Assessment of alternative uses for lignocellulosic waste products

NUI MAYNOOTH

Ollscoil na hÉireann Má Nuad

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Declaration of Authorship

This thesis has not been submitted in whole or in part, to this or any other university, for any degree and is, except where otherwise stated the original work of the author.

Signed: _____

Derek Carr

Date: _____

Dedication

For my Parents

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Abstract

Lignocellulosic materials are a vast potential resource that can be harnessed to produce a range of value added products which can have a number of applications. These applications typically include enzyme, antioxidant and bioethanol production in addition to use as an animal feed.

Production of hydrolytic enzymes employing solid state fermentation (SSF) of relatively cheap agricultural wastes such as corncob, can potentially reduce the cost of production compared to traditional methods. Two fungi (Aspergillus niger 102.12 and Aspergillus oryzae 553.65) were selected following an initial screening, with the optimal conditions for enzyme production subsequently determined. Supplementation with yeast extract at 5 % (w/v) appeared to have the most significant impact on the enzyme production from both strains, with xylanase and cellulase activity being increased by as much as 31 and 44-fold, respectively.

Lignocellulosic material can also be utilised as a substrate for bioethanol, although structural modification of the material to remove the lignin first needs to be achieved. Biological pretreatment with two selected strains of white rot fungi revealed that fermentation with Pleurotus ostreatus Oyrm 1 appeared to be the optimal strain for biological pretreatment, with as much as 75 % (w/v) lignin degradation observed. These pretreated materials were subsequently used to replace a portion of corn mash during ethanol production. The addition of corncobs which were pretreated with both white rot fungi and Aspergillus appeared to increase the rate of ethanol production during batch fermentation, whilst an increase in the final ethanol yield was observed in a number of fermentations.

Biological pretreatment with both Trametes versicolor 3086 (TV 3086) and Pleurotus ostreatus Oyrm 1 was also successful at upgrading the potential value of forage feed. In a number of fermentations the crude protein content of the material was significantly ($p<0.05$) improved during pretreatment, with increases of up to 205 % noted following fermentation. The digestibility of the forage can also be increased using biological pretreatment with increases in the digestibility of as much as 2.2-fold detected following fermentation.

The potential of producing antioxidants through SSF of lignocellulosic wastes by selected strains of white rot fungi was also assessed. Pretreatment of the residues improved the antioxidant potential of these residues further, with the phenolic content of the liquid extracts from fermentation being notably increased.

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACP	Adjusted Crude Protein
ADF	Acid Detergent Fibre
AFEX	Ammonia Fibre expansion
ATP	Adenosine Triphosphate
A_w	Water activity
BG	β-glucosidase
BHT	Butylated Hydroxytoluene
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolase
CBP	Consolidated Bioprocessing
CE	Carbohydrate Esterase
CMC	Carboxymethyl Cellulase
CMCu g^{-1}	Cellulase Units per gram
CMCu ml^{-1}	Cellulase Units per millilitre
CP	Crude Protein
CSH	Cottonseed Hulls
CTAB	Cetyl Trimethylammonium Bromide
DDM	Digestible Dry Matter
DMI	Dry Matter Intake
DE	Digestible Energy
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic Acid
DM	Dry Matter
DPPH	2,2-diphenyl-1-picrylhydrazyl
EE	Ether Extract
EDTA	Ethylenediaminetetraacetic Acid
EG	Exoglucanase
EMP	Embden Meyerhof Pathway
ESC	Ethanol Soluble Carbohydrates
GH	Glycoside Hydrolase

Gly	Glycine
GRAS	Generally Regarded As safe
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HUT g^{-1}	Protease Units per gram
HUT ml^{-1}	Protease Units per millilitre
IVTD	*In Vitro* True Digestibility
LCC	Lignin-Carbohydrate Complexes
LDL	Low-Density Lipoprotein
LiP	Lignin Peroxidase
MIBK	Methyl Isobutyl Ketone
MnP	Manganese Dependent Peroxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NDF	Neutral Detergent Fibre
NFC	Non-Fibre Carbohydrate
PDA	Potato Dextrose Agar
QSS	Quasi-Steady State
RA	Relative Activity
RDP	Rumen Degradable Protein
RH	Relative Humidity
RI	Refractive Index
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
SHF	Simultaneous Hydrolysis and Fermentation
SimSF	Simultaneous Saccharification and Fermentation
SmF	Submerged Fermentation
SP	Soluble Protein
Spp.	Species
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Solid State Fermentation
TCA	Trichloroacetic Acid
TDN	Total Digestible Nitrogen

TE	Trolox Equivalents
TEAC	Trolox Equivalent Antioxidant Capacity
UV	Ultra Violet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
Xut g^{-1}	Xylanase Units per gram
Xut ml^{-1}	Xylanase Units per millilitre
YM	Yeast Malt
YPD	Yeast Peptone Dextrose
Vis	Visible

1. Introduction

The potential of lignocellulose and its applicability have been widely recognised over the last number of years (Zaldivar *et al.*, 2001; Malherbe *et al.*, 2002; Perez *et al.*, 2002; Alvira *et al.*, 2010). Despite the drawbacks associated with the structure of this material, such as the complex nature of the lignin fraction, a significant bulk of research has been undertaken to deal with these concerns and unlock the true potential of lignocellulose. In the following section the issues associated with lignocellulosic degradation and possible solutions to these obstacles will be outlined. In addition, the value of lignocellulose as a substrate and its potential application in areas such as antioxidant production, enzyme synthesis, ethanol fermentation and the potential of fermented agricultural by-products to be used as forage feed will also be discussed.

1.1 Lignocellulose and lignocellulosic biomass

Lignocellulosic polymers comprise over 60 % of plant biomass and represent a vast potential resource for foodstuffs, animal feed, biofuels, fertilizers and other industrial processes (Tengerdy *et al.*, 2003). For example, lignocellulose is the major raw material for the paper industry. The potential of this material is clear when one considers that 10^{11} tons of plant biomass (containing lignocellulose) is degraded every year, a figure which equates to 640 billion barrels of oil (Nagy *et al.*, 2007). However, to fully utilise the potential of lignocellulosic materials, the lignocellulose needs to be converted using either chemical or biological processes (Tengerdy *et al.*, 2003). Lignocellulose consists of three different polymers that are strongly bound by non-covalent forces and covalent cross-linkages (Perez *et al.*, 2002). By virtue of these linkages, the accessibility of the carbohydrates within the material is severely inhibited and as a result, significant amounts of carbon/potential energy remain unused (Akin *et al.*, 1996).

Only a limited amount of the lignocellulosic material that is produced as by-products of the farming and forestry sectors is used, with the rest being either incinerated or considered waste (Sánchez, 2009). In the US pulp and paper industry, on average 60 kg of primary and 20 kg of secondary sludge are generated per tonne of pulp produced. This equates to between 2-13 million tonnes of primary and secondary sludge annually (Duff *et al.*, 1996). Consequently, significant pollution of the

environment can occur as a direct result of this incorrect disposal of such waste materials.

With the advent of biofuels over the last decade, competition has arisen between food and fuel companies for grain crops, specifically corn maize and as a result the price of this commodity has increased over two-fold. However, even if the production capacity of bioethanol plants was increased, there would still not be enough arable land in the world to provide fuel for every car. As a consequence, there has been significant research into second generation biofuels as a method of producing fuel ethanol (Ingram *et al.*, 1995; Gáspár *et al.*, 2007; Alvira *et al.*, 2010). Second generation biofuels are classed as ethanol which is produced from pretreated lignocellulosic material. These materials can include a number of common agricultural waste products such as straw, corn silage, woody residues, cotton seed hulls and rice husks, which would otherwise go to waste.

1.1.1 Structure and composition of lignocellulose

Lignocellulose consists of three polymers; cellulose, hemicellulose and lignin, which form structures that are linked through covalent and non-covalent bonds, resulting in a rigid structure that is extremely difficult to degrade. Cellulose is the most abundant polymer in lignocellulose, followed by hemicellulose and lignin, respectively (Baldrian *et al.*, 2008). Cellulose's compact, in part crystalline structure forms microfibers which are densely packed between layers of lignin that protect it from hydrolysis by either chemical or biological methods (Leonowicz *et al.*, 1999). The composition and proportions of these polymers vary greatly between plants. Table 1.1 details the composition of a number of well known lignocellulosic materials.

Table 1.1. Composition of some commonly used lignocellulosic materials.
Modified from Graminha *et al.* (2008).

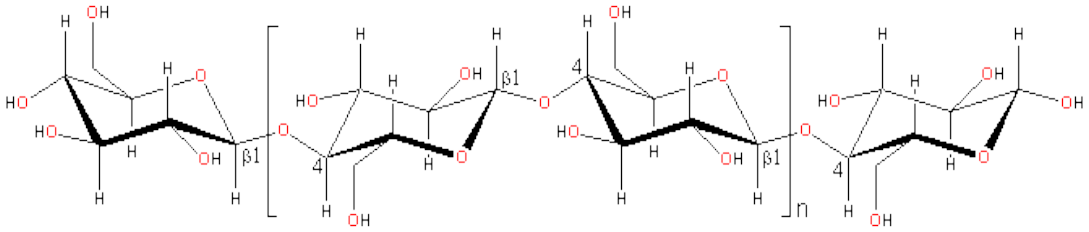
Material	Cellulose*	Hemicellulose*	Lignin*
Corn Silage	380-400	280	70-210
Corn	--	--	--
Straw	335	249	78
Stalk	336	237	87
Leaf	245	273	54
Cob	377	396	73
Bran	338	393	49
Coconut fibre	360-430	15-25	410-450
Wheat straw	270-400	210-320	98-200
Wheat bran	300	500	150
Rice bran	350	250	170
Rice straw	280-470	190-280	43-240
Sorghum stalks	270	250	110
Barley bran	230	320	214
Barley straw	310-450	254-380	110-190
Oat bran	493	250	180
Oat straw	300	220	85
Grape seed	71	311	435
Grape vine	299	353	229
Coniferous plants	400	300	200-300

*g kg^{-1} dry matter

1.1.1.1 Cellulose

Cellulose is an organic polysaccharide consisting of a linear chain of β-1,4 linked D-glucose units, with each polymer containing a hydroxyl residue at the C2, C3 and the C6 atoms (Figure 1.1). These form long chains linked together via hydrogen bonds and Van der Waals interactions (Sánchez, 2009). Hydroxyl residues bind with the oxygen atoms on the other neighbouring chains to form microfibrils. The length of the cellulose chain can vary from between 2,000 to 12,000 glucose units, depending on the source; however, the majority of cellulose molecules are between 8,000 and 12,000 units in length. Each glucose unit is rotated 180° relative to its two neighbouring glucose units, thus the smallest repetitive unit in the glucose polymer is the disaccharide cellobiose (Figure 1.1) (Zhang *et al.*, 2004). Cellulose exists in a highly crystalline form with a number of different allomorphs of cellulose known, namely cellulose I, II, III and IV. Cellulose from wood and higher plants usually consists of long thin fibres of the cellulose I allomorph surrounded by hemicellulose and lignin (Howell *et al.*, 2011). The crystalline regions of cellulose in plant cells are usually separated by amorphous regions where the degree of crystallinity is dependent of the

source of the cellulose. Cellulose I is a natural variant which consists of a mixture of 1α (triclinic) and 1β (monoclinic) crystalline forms (Atalla *et al.*, 1984). Cellulose II is formed as a result of regeneration of cellulose fibres. In contrast to cellulose I, cellulose II is arranged in an antiparallel arrangement in a two chain unit cell. Cellulose III is formed through the treatment of cellulose I or II with liquid ammonia, whereas cellulose IV is formed through the heat treatment of cellulose III allomorphs (Zugenmaier, 2001).



Cellobiose

Figure 1.1 Schematic representation of the cellulose backbone.

Adapted from Brown Jr *et al.* (1996)

1.1.1.2 Hemicellulose

Hemicellulose is a highly complex heterogeneous polysaccharide consisting of D-xylose, D-glucose, D-arabinose, D-galactose, D-mannose, D-4-*O*-methyl-glucuronic, D-galacturonic and D-glucuronic acid and other ester linked acetyl, coumaryl and feruloyl moieties (Perez *et al.*, 2002). Although hemicellulose is structurally more complex than cellulose and requires substantially more enzymes for complete hydrolysis, it does not form packed crystalline structures like cellulose and therefore is generally more susceptible to enzymatic hydrolysis (Saha, 2003). Hemicelluloses are typically found in the secondary wall and are linked with cellulose; however, they can also be found in the primary wall of the cell. The hemicellulose fraction of plants varies from plant to plant but generally the principle hemicellulose in hardwoods and grasses is glucuronoxylan, while the main component in softwoods is glucomannan (Jeffries, 1994). The sugars are usually linked together with β-1,4 glycosidic bonds, although occasionally they are linked with β-1,3 glycosidic bonds (Perez *et al.*, 2002). Xylans are the most abundant hemicellulose and display a large structural variety, conferred by the degree and nature of the linked side groups (Mazeau *et al.*, 2005).

The most significant difference between cellulose and hemicellulose is that the latter consists of short lateral chains of different sugars, whereas cellulose consists of oligomers which are easily hydrolysable (Sánchez, 2009). Hemicellulose usually

consists of a long linear backbone of one sugar type and branched side chains which are composed of acetate and sugars (Duff *et al.*, 1996). In plants, the linear backbone contains β-(1,4)-D-xylopyranan with side chains consisting mainly of single α-L-arabinofuranosyl and α-D-glucopyranosyl uronic acid units with 4-*O*-methyl ether, while rhamnose, xylose and galactose have also been located next to the acetyl groups and phenolic acids (Mazeau *et al.*, 2005). The hemicellulosic constituents vary from one plant species to another (Perez *et al.*, 2002); for example, Birchwood hemicellulose contains 89.3 % xylose, 1 % arabinose, 1.4 % glucose and 8.3 % anhydrouronic acid (Kormelink *et al.*, 1993), whereas in corn fibre (Figure 1.2), the hemicellulosic fraction consists of between 48-54 % xylose, 33-35 % arabinose, 5-11 % galactose and 3-6 % glucuronic acid (Doner *et al.*, 1997). Furthermore, the hemicellulose content can also vary between different portions of the same plant, for example between the stem and the leaves (Perez *et al.*, 2002).

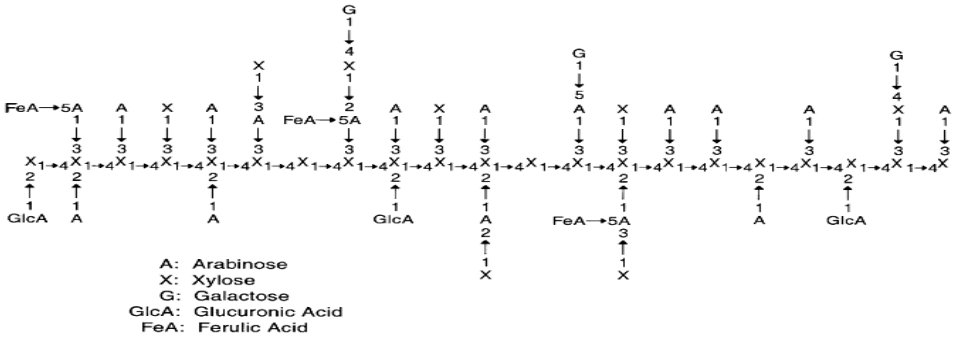


Figure 1.2 Schematic representation of the structure of corn fibre heteroxylan. Retrieved from Saulnier *et al.* (1995).

1.1.1.3 Lignin

Lignin is a complex three dimensional, aromatic heteropolymer found predominantly in the xylem of plants (Donaldson, 2001). It is synthesised by the polymerization of its three alcohol constituents (displayed in Figure 1.3); *p*-coumaryl alcohol (*p*-hyroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol) and sinapyl alcohol (syringyl alcohol) to form the three-dimensional lignin polymer (Buranov *et al.*, 2008). Over ten different interphenylpropane linkages occur in lignin polymerization including β-*O*-4, α-*O*-4, β-5, β-1, 5-5, 4-*O*-5 and β-β; however, the most prominent linkage is the β-*O*-4 type linkage which is found between over 50 % of units (Lee, 1997). Biosynthesis is peroxidase mediated and consists mainly of radical coupling (Buranov *et al.*, 2008). During polymerization, secondary reactions can lead to cross-linking between lignin and hemicellulose fractions. Generally, plants are composed of

different concentrations of each constituent, for example the main lignin constituent of hardwoods is guaiacol and syringyl, while the main component in softwood is coniferyl alcohol (Jeffries, 1994; Perez *et al.*, 2002). In plants the main functions of lignin is to provide structural support to the cell wall, to form an impermeable physical barrier, to prevent microbial attack or oxidative stress and to improve sap conduction through lignified vascular elements (Boudet, 2000). Lignification is one of the final stages of xylem cell differentiation, where lignin is accumulated in the carbohydrate matrix, filling in the interlamellar cavities while forming bonds with non-cellulosic carbohydrates. The process usually begins in the middle lamella and S1 regions, subsequently spreading across the secondary wall to the lumen (Donaldson, 1991). The middle lamella is lignified once the construction of the secondary wall is initiated, while secondary wall lignification occurs when the construction of the wall is complete (Donaldson, 2001).

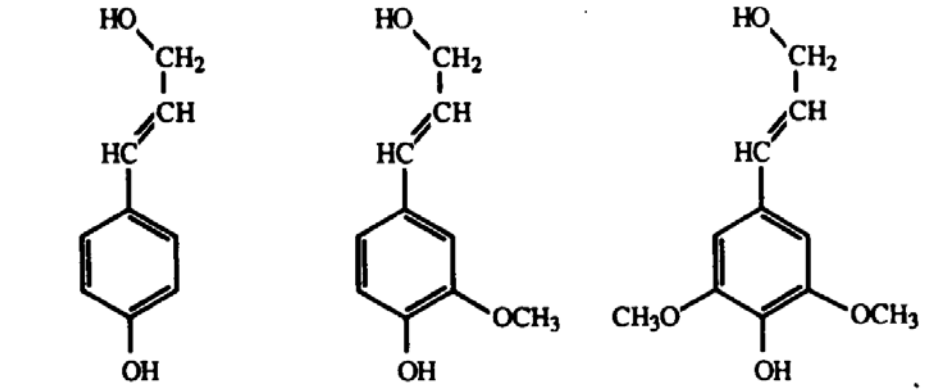


Figure 1.3 Constituent molecules of lignin polymers.

Retrieved from Jeffries (1994).

1.1.1.4 Lignin-carbohydrate and cellulose-hemicellulose interactions

Cell walls are a critical component in plants as they add structural stability as well as transport nutrients around the plant. Lignin-carbohydrate and hemicellulose-cellulose interactions are thought to have an influence on the rigidity of the plant, whilst also influencing the composition and structure. Softwood lignin carbon complexes (LCCs) are unique in that their carbohydrate portions contain glactomannan, arabino-4-*O*-methylglucuronoxylan and arabinoglactan linked to lignin (Azuma *et al.*, 1981). Comparatively, carbohydrates from hardwoods and grasses generally consist of 4-*O*-methylglucuronoxylan and arabino-4-*O*-methylglucuronoxylan (Azuma *et al.*, 1988).

These complexes are linked through hydrogen bonds, although other linkages such as α-ether and α-ester linkages are also thought to exists between LCCs (Zhang *et al.*, 2011).

The interactions between hemicellulose and cellulose via hydrogen bonds and Van der Waals forces in plants dictate the biosynthetic processes and structural properties of lignocellulosic material (Kabel *et al.*, 2007). Previous research has indicated that hemicellulose plays a passive role in the cell wall, filling the voids between the cellulose microfibrils binding lignin oligomers to add to the rigidity of the plant. However, Atalla *et al.* (1993) suggested that hemicellulose also acts as a regulator in the structure of cells walls by influencing the aggregation of cellulose microfibrils. It has been further proposed that hemicellulose forms an acid coating, binding to the cellulose microfibrils thus preventing flocculation (Dammstrom *et al.*, 2009).

Lignin is thought not to have a direct influence on the aggregation of the hemicellulose and cellulose; however, it is suggested that it plays an important role in the formation of strong carbohydrate-lignin frameworks in the secondary cell wall. It has been proposed that lignin does not exist as a pure fraction because all lignin found is linked with both cellulose and hemicellulose (Zhang *et al.*, 2011). In pulp processing, enzymes are used to solubilize the hemicellulose fraction remaining after bleaching and extraction; however, despite the addition of xylanases, mannanases and endo-glucanases, the hemicellulose fraction was still not fully removed. Thus, it has been suggested that the hemicellulose fraction is partially inaccessible to hemicellulases due largely to lignin-carbohydrate complexes (Gübitz *et al.*, 1998). Hemicellulose also appears to affect the structure of lignin during cell wall construction, where the cell wall structure is altered by genetic modifications on the hemicellulose biosynthetic pathway (Uhlin *et al.*, 1995).

Xyloglucans are thought to make up a significant fraction of hemicellulose in softwoods. They are organised into three macromolecular domains with the major part being closely associated with the surface of the cellulose microfibrils, while the other fractions form cross-links within and to other cellulose microfibrils (Chambat *et al.*, 2005). In softwoods, glucomannans are thought to also interact with cellulose, while in hardwoods a fraction of xylan is associated with the cellulose in a similar way (Dammstrom *et al.*, 2009). Glucomannans are generally thought to bind to cellulose

with a stronger affinity than xylans and are very difficult to separate (Ãkerholm *et al.*, 2001).

1.1.2 Lignocellulosic degradation

The binding of lignin with the hemicellulosic components of the plant cell wall, through both covalent and physical forces, reduces the accessibility of the cellulosic and hemicellulosic carbohydrates to carbohydrolase enzymes, thus limiting the potential of the lignocellulosic material. Recently, researchers have investigated several methods of delignification which have the potential to release tightly bound carbohydrates and to remove the lignin fraction (Alvira *et al.*, 2010). Pretreatment techniques for conversion of lignocellulose can be categorised into four groups; specifically physical, physico-chemical, chemical and biological.

During the pretreatment process, the crystallinity of cellulose should be reduced to increase the amorphous region as this is a more suitable form of cellulose for hydrolysis. The most abundant fraction of hemicellulose should also be hydrolysed, while the lignin fraction should be removed or degraded (Sánchez *et al.*, 2008). Table 1.2 outlines the advantages and disadvantages of a range of pretreatment techniques.

Table 1.2 Advantages and disadvantages of lignocellulose pretreatment techniques. Adapted from Alvira *et al.* (2010).

Pretreatment method	Advantages	Disadvantages
Biological	Degradation of lignin and hemicellulose	Low rate of hydrolysis
	Low energy cost	
Milling	Reduction in cellulose crystallinity	High power and energy consumption
Steam explosion	Causes lignin transformation and hemicellulose solubilisation	Generation of toxic compounds
	Cost effective	Partial hemicellulose degradation
	Higher yield of glucose and hemicellulose in the two step process	
Ammonia fibre expansion (AFEX)	Increases accessible surface area	Not efficient for materials with high lignin content
	Low formation of inhibitors	High cost of ammonia
CO_2 explosion	Increases accessible surface area	Does not affect lignin-hemicellulose interactions
	Cost effective	High pressure requirements
	Does not generate toxic compounds	
Wet oxidation	Efficient lignin removal	High cost of oxygen and alkali catalysts
	Low formation of inhibitors	
	Minimises energy demand	
Ozonolysis	Reduces lignin content	High cost of the large amount of ozone needed
	No generation of toxic compounds	Pollution concerns
Organosolv	Causes lignin and hemicellulose hydrolysis	High cost
		Solvents need to be drained and recycled
Concentrated acid	High glucose yields	High cost of acid and the need for it to be recovered
	Ambient temperatures	Reactor corrosion issues
		Formation of inhibitors
Diluted acid	Less corrosion than concentrated acid	Generation of degradation products
	Less formation of inhibitors	Low sugar concentrations in exit stream

1.1.2.1 Factors to consider for effective pretreatment of lignocellulose

There are a significant number of factors that must be considered to ensure a low cost efficient pretreatment process (Yang *et al.*, 2008; Zheng *et al.*, 2009; Alvira *et al.*, 2010). These include:

- *High yields for multiple crops, site ages and harvesting time:* It has been shown that different pretreatment techniques are better suited to specific biomass. Ammonia fibre expansion (AFEX) for example, is extremely effective against agricultural waste but less so with softwoods.
- *Highly digestible pretreated solids:* The material to be pretreated should have a high degree of digestibility to ensure efficient processing (Yang *et al.*, 2008).
- *No significant sugar degradation:* High yields of up to 100 % of fermentable cellulose are desirable, with minimal loss due to heating, as this can impact on the efficiency of the fermentation.
- *Minimal production of toxic compounds:* Harsh pretreatments like acid and alkali pretreatment can lead to the formation of toxic compounds such as furfural, carboxylic acids and 5-hydroxymethylfurfurals, which can affect fermentation in downstream processing (Palmqvist *et al.*, 2000a; Alvira *et al.*, 2010).
- *Biomass size reduction not required:* Milling and grinding are expensive and energy intensive processes. Therefore an ideal material would require little processing prior to pretreatment.
- *Low capital cost:* Reactors for pretreatment are expensive depending on the pretreatment process; for example, a reactor for acid hydrolysis needs to be resistant to corrosion from concentrated acids which can add to the capital cost of the pretreatment (Alvira *et al.*, 2010).
- *Effectiveness at low moisture content:* The use of material with high dry matter content would reduce the energy costs (Alvira *et al.*, 2010).
- *Lignin recovery:* Lignin recovery would be beneficial to simplify downstream processing while also aiding in the reduction of the cost of hydrolysis (Yang *et al.*, 2008).
- *Minimum heat and power requirements:* Minimising heat and power requirements reduce the cost of pretreatment (Zheng *et al.*, 2009).

1.1.2.2 Physical pretreatment

Physical degradation focuses on three main techniques; mechanical comminution, pyrolysis and extrusion. Mechanical comminution (i.e. chipping, grinding or milling) is a process whereby the lignocellulosic materials are reduced in size to minimise the

crystallinity of the material. During chipping, the materials are reduced to between 10-30 mm, whereas grinding and milling reduces the size to 0.2-2 mm (Alvira *et al.*, 2010). Milling and grinding can be effective in increasing the surface area of the material, thus increasing cellulose exposure towards enzymatic hydrolysis. The cost involved in this form of physical degradation is proportional to the final practical size.

In pyrolysis, materials are heated to temperatures above 300 ^{o}C rapidly decomposing its cellulose and reducing its crystallinity. This process can also be carried out at lower temperatures; however, the decomposition is much slower as less volatile compounds are formed (Sun *et al.*, 2002). Extrusion is a novel process in which the lignocellulosic material is heated, mixed and sheared resulting in both physical and chemical modification (Alvira *et al.*, 2010). Physical degradation remains an unattractive technique due to the amount of energy used in the process and capital costs involved (Sánchez, 2009).

1.1.2.3 Physico-chemical pretreatment

Steam explosion (autohydrolysis) is by far the most studied physico-chemical pretreatment technique. It involves the hydrolysis of chipped biomass with high pressure saturated steam for a specific time, after which the pressure is quickly reduced, thereby initiating explosive decompression within the material. The temperatures used range between 160-260 °C, with higher temperatures requiring shorter contact times. In general, lower temperatures and longer residence times are employed due to the formation of inhibitors at higher temperatures (Duff *et al.*, 1996). This process causes extreme hemicellulosic degradation and lignin transformation thus, increasing the availability of the cellulose fraction (Sun *et al.*, 2002). Up to 90 % efficiency of enzymatic hydrolysis of poplar wood can be achieved using this pretreatment method whereas only 15 % efficiency can be obtained without pretreatment (Grous *et al.*, 1986). The addition of H_2SO_4 or CO_2 to the process can improve subsequent enzymatic hydrolysis considerably as the digestibility of the substrate is increased due to a higher degree of lignin degradation (Sun *et al.*, 2002). Autohydrolysis is one of the most cost effective treatment processes for hardwoods and agricultural wastes; however, it is less effective with softwoods as these materials are more rigid and usually contain a higher lignin content (Shahbazi *et al.*, 2005). Despite the effectiveness of this pretreatment process, destruction of a portion of the xylan fraction will most likely occur resulting in the generation of compounds such as

furfural that may be inhibitory to microorganisms downstream. Other physico-chemical pretreatments include ammonium fibre expansion (AFEX) and CO_2 explosion. These methods are fundamentally similar to steam explosion; however, their efficiencies are generally lower (Sun *et al.*, 2002).

AFEX pretreatment involves the treatment of lignocellulosic biomass with concentrated aqueous ammonia at relatively moderate temperatures ranging from 80-150 °C, under pressure. After a treatment period of 5-30 minutes, the pressure is explosively released causing the depolymerisation of the cellulose and the partial solubilisation of the hemicellulose fraction (Bals *et al.*, 2010). This is an effective method for pretreatment of hardwoods and grasses; however, like autohydrolysis, it is not as effective in the pretreatment of materials with high lignin contents such as softwood. No inhibitors are produced using this method, while high levels of depolymerisation can be achieved (Sánchez *et al.*, 2008). However, the hemicellulosic fraction is not completely solubilised which can affect the economic viability of the pretreatment process (Sun *et al.*, 2002).

Carbon dioxide explosion involves the use of carbon dioxide as a super critical fluid where gas is compressed at temperatures above its critical point to a liquid density (Alvira *et al.*, 2010). The biomass is contacted with carbon dioxide at temperature ranging from 35-165 °C for a specific period of time after which, the pressure is released causing decomposition in the biomass and rupturing the cellulose structure. This method has been reported to work better with rigid wet materials, with the process temperature dependent on the amount of moisture in the material (Kim *et al.*, 2001; Srinivasan *et al.*, 2010). Similar to AFEX pretreatment, this method does not cause the formation of inhibitory compounds; however, the yields obtained are lower the other physico-chemical methods described (Sun *et al.*, 2002).

1.1.2.4 Chemical pretreatment

Chemical pretreatment involves the depolymerisation of lignocellulosic materials with chemical agents such as acid, inorganic solvents, alkalis and peroxidases. The most successful and thoroughly studied techniques involve the use of dilute and concentrated inorganic acids such as H_2SO_4 and HCl.

Dilute acid hydrolysis is widely used due to the high reaction rates that can be achieved with hemicellulose which significantly improves the availability of the cellulose fraction for hydrolysis (Sánchez *et al.*, 2008). The reaction is carried out

between 121-220 ^{o}C under pressure; however, lower temperatures (~121^{o}C) are optimal to reduce the formation of inhibitors such as hydroxymethyl furfural and to minimise sugar degradation (Saha *et al.*, 2005). The reaction time is dependent on the temperature used, where higher temperatures require shorter reaction times (Alvira *et al.*, 2010). Although the cost of dilute acid pretreatment is higher than other techniques such as steam explosion, the recoveries of hemicellulose are generally much higher (Sánchez *et al.*, 2008). Dilute acid hydrolysis is primarily used as a method for hemicellulose pretreatment with subsequent enzymatic hydrolysis used for cellulose pretreatment, due to the formation of inhibitory compounds at the higher temperatures which are required for cellulose pretreatment with this process (Gírio *et al.*, 2010). This method is strikingly similar to steam explosion using an acid catalyst; however, the biomass is suspended in the acid solution while the particles used are also smaller (Duff *et al.*, 1996).

Concentrated acid hydrolysis involves the conversion of lignocellulose material with concentrated acids at lower temperatures of between 20-30 ^{o}C, which can result in lower operational costs in comparison to other techniques (Gírio *et al.*, 2010). Unlike using acids such as H_2SO_4 and HCl at lower concentrations, these acids are powerful agents in cellulosic hydrolysis when used at high concentrations (Sun *et al.*, 2002), while the hemicellulose fraction can also be solubilized. The concentration of the acid usually depends on the length of pretreatment of the biomass. Teixeira (1999) for example, achieved up to 93.1 % of cellulose conversion during a 120 hour pretreatment with 21 % acid, while a similar conversion was realised with 60 % acid over a 24 hour incubation period. Although it is regarded as an effective process, the use of concentrated acids as a pretreatment technique can result in high capital costs due to acid corrosion and subsequent toxicity to microorganisms in downstream processes (ethanol fermentation) as a result of the formation of inhibitory compounds (Alvira *et al.*, 2010). In addition, the acids must be recovered after the process to make it economically viable (Sun *et al.*, 2002).

Organosolv uses hot organic or aqueous solvents such as ethanol and acetone, under pressure to hydrolyse the internal lignin and hemicellulose bonds. These solvents can also be mixed with acid to increase the hemicellulose recovery, although at higher temperatures, acid catalysts are not required as the acid released from the biomass can act as a catalyst (Aziz *et al.*, 1989). Relatively pure lignin is recovered with this technique (Zhao *et al.*, 2009), while high recovery rates for cellulose and

hemicellulose are also achieved (Sánchez *et al.*, 2008). Despite this, the process can prove costly due to the expense involved in purchasing solvents; although, this cost can be reduced through solvent recycling. The toxicity of the solvent towards microorganisms in downstream processing is also a concern as the efficiency of the fermentation can thus be affected (Aziz *et al.*, 1989; Sun *et al.*, 2002).

Alkali pretreatment involves the saponification of ester bonds between xylan hemicelluloses, lignin and other hemicelluloses with alkali solutions such as sodium hydroxide, potassium hydroxide and ammonium hydroxide (Sun *et al.*, 2002). They are used to remove the lignin and hemicellulose, whilst the cellulose is left exposed for enzymatic hydrolysis as a result of swelling, which increases the surface area of the substrate biomass. This method is generally thought to be more effective against agricultural residues than woody biomass (Kumar *et al.*, 2009). Pretreatment usually occurs at lower temperatures; however, this method needs long hydrolysis periods and high alkali concentrations (Binod *et al.*, 2010). In comparison to other chemical techniques, this method is one of the most effective methods for breaking the ester bonds between lignin, hemicellulose and cellulose (Gáspár *et al.*, 2007). Low concentrations of inhibitors are formed using this method while good lignin removal and hemicellulose hydrolysis can also be achieved.

1.1.2.6 Biological pretreatment

Biological pretreatment is a complex process involving multiple enzymes which are mediated by a number of small molecules that work synergistically. Biological pretreatment is typically carried out by wood rotting Basidiomycetes, incorporating brown, white and soft rot fungi. Brown rot fungi primarily attack cellulose, while white and soft rot fungi attack cellulose, hemicellulose and lignin. Typical fungi used for biological pretreatment include *Pleurotus ostreatus*, *Trametes versicolor*, *Lentinula edodes* and *Phanerochaete chrysosporium*. These fungi penetrate the wood which results in the formation of more easily metabolizable carbohydrates (Leonowicz *et al.*, 1999).

Biodegradation of lignin is a secondary metabolic process which occurs in the presence of low levels of nutrients and a suitable carbon source. The main enzymes involved in lignin degradation are extracellular laccases and peroxidases such as lignin and manganese peroxidase. Lignin peroxidase (LiP), in the presence of H_2O_2, degrades lignin through oxidative cleavage of the carbon-carbon backbone, oxidation

and hydroxylation of benzylic methylene groups and oxidation of phenol alcohols. Meanwhile, manganese dependent peroxidases (MnP) oxidise Mn^{2+} to Mn^{3+} which acts as a redox couple oxidising phenolic substrates to phenoxy radicals (Lee, 1997). Laccase is a multicopper polyphenol oxidase which is able to catalyze the oxidation of a large number of organic and inorganic molecules such as diphenol, polyphenols and aromatic amines via the transfer of electrons (Halaburgi *et al.*, 2011). These enzymes work in synergy to hydrolyse the lignin polymer leaving the hemicellulose and cellulose fraction more exposed to enzymatic hydrolysis.

Biological pretreatment has low energy requirements, high lignin specificity with some fungal strains, mild environmental conditions and little or no formation of inhibitors in comparison to chemical and physico-chemical techniques (Sánchez *et al.*, 2008). However, this technique requires long reaction times due to the low rates of hydrolysis by these fungi, thus limiting its application in industrial processes (Sun *et al.*, 2002). Biological pretreatment is usually carried out through submerged or solid state fermentation (Section 1.4).

1.2 Lignocellulosic enzymes and their roles in lignocellulosic degradation

Lignocellulosic degradation involves a collection of enzymes collaborating together to hydrolyse the biomass substrate. Due to the complexity of the lignocellulosic material, a selection of three or more different enzymes groups are typically required depending, on the intended final product or the degree of hydrolysis required. These three groups of enzymes include lignin and cellulose degrading enzymes, in addition to hemicellulases.

1.2.1 Lignin degrading enzymes

There are a number of enzymes which function in delivering effective hydrolysis of the lignin polymer; specifically manganese peroxidase, lignin peroxidase, laccase, glycol oxidase, sugar oxidase, alcohol oxidase and quinone oxidoreductase (Sarikaya *et al.*, 1997).

Lignin peroxidase is a glycoprotein with a molecular mass of between 38-48 kDa which contains a heme as a prosthetic group in its active centre. Due to the size of this protein, it cannot enter the plant cell, hence lignin degradation takes place at the exposed regions of the lumen (Perez *et al.*, 2002). Lignin peroxidase is oxidised by

H_2O_2 or organic peroxides, whereby two electrons are derived from the substrate molecules to give the intermediate, compound 1 (Lundell *et al.*, 2010). The oxidised LiP in turn oxidises the aromatic nuclei in lignin by one electron, resulting in the generation of cation radicals. These radicals react with nucleophiles and oxygen resulting in the cleavage of C-C and C-O linkages, depolymerising the lignin polymer and opening the aromatic ring. These compounds subsequently enter the hyphae where they are oxidised. Next, the compound 1 form of LiP produces veratryl alcohol radical cations, which remove one electron from the methoxylated aromatic ring of the lignin, thus initiating cleavage. Finally, the radical reacts with oxygen resulting in active oxygen species which oxidises the lignin resulting in decomposition (Kirk *et al.*, 1987; Sarikaya *et al.*, 1997).

Manganese dependent peroxidases (MnP) are molecularly very similar to LiPs i.e., they are also glycosylated proteins containing a heme group but have a marginally higher molecular mass (45-60kDa) (Perez *et al.*, 2002). The catalytic cycle is also similar, although in contrast to other peroxidases, MnP uses Mn^{2+} which is abundant in lignocellulose.

Briefly, the catalytic cycle of MnP is initiated by the binding of H_2O_2 to a native ferric enzyme with the formation of an iron-peroxide complex. As a result of the donation of two electrons from the heme, MnP complex I is formed. Subsequently, the dioxygen bond is cleaved leading to the expulsion of one water molecule. Next the MnP complex I is reduced to compound II, where a monochealated Mn^{2+} ion acts as an electron donor and is oxidised to Mn^{3+}. Similarly, compound II is reduced and another Mn^{3+} is formed, resulting in the expulsion of an additional water molecule and the formation of the native enzyme (Hofrichter, 2002). Mn^{3+} is a very strong oxidant which can oxidise phenolic compounds in lignocellulose. It typically does not attack non-phenolic units, although some Mn^{3+} molecules have been known to oxidise non-phenolic compounds in the presence of Mn^{2+}. MnP also produces phenoxy-radicals which undergo a variety of reactions leading to depolymerisation of the lignin (Perez *et al.*, 2002). MnP is extremely sensitive to high concentrations of hydrogen peroxide, which can result in the inactivation of the enzyme through the formation of compound III (Wariishi *et al.*, 1988).

Laccases are a group of glycoproteins which can be found both intra and extracellularly in Basidiomycetes. Over 100 laccases have been classified from lignin degrading fungi to date and can range in size from 60-70kDa (Baldrian, 2006).

Laccases are copper-containing oxidases that catalyse the electron oxidation of primarily phenolic and lower-redox potential compounds, although they can oxidise non-phenolic compounds in the presence of mediators, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid**)** (ABTS). The phenolic nucleus is oxidised by the removal of one electron generating a phenoxy free radical which leads to the cleavage of the polymer through a host of non-enzymatic reactions. This in turn leads to alkyl-phenyl cleavage, demethoxylation and C_{α}-oxidation (Perez *et al.*, 2002), which can be crucial in the degradation of phenolic units in lignin, as well as phenolics released during lignin decomposition (Sarikaya *et al.*, 1997).

Glycol oxidase, sugar oxidase and alcohol oxidase are a diverse group of enzymes which are also associated with lignin degradation. They are involved in the production of H_2O_2, which is vital in ligninase activity through the oxidation of ligninase enzymes thus activating them (Sarikaya *et al.*, 1997).

1.2.2 Cellulase enzymes

It is estimated that around 100 billion tons of lignocellulosic biomass is produced through photosynthesis every year. Approximately 50 % of this biomass is cellulose (Ryu *et al.*, 1980), indicating that cellulases have a crucial role to play in the hydrolysis of lignocellulosic biomass. Cellulose hydrolysis is carried out by 3 groups of cellulases (Figure. 1.4), specifically endoglucanases (carboxymethylcellulases), exogluconases (cellobiohydrolases) and β-glucosidases (cellobiases) (Singhania *et al.*, 2010).

Carboxymethylcellulases randomly hydrolyse the amorphous, swollen region of the cellulose chains resulting in a decreased chain length and a slow increase in the number of reducing groups. They are generally inactive towards crystalline cellulose and cellobiose with the final products of hydrolysis consisting of a mixture of shorter chain cellulose fibres that can subsequently be broken down by cellobiohydrolases (Ryu *et al.*, 1980). Cellobiohydrolases (CBH), namely CBHI and CBHII are exo-acting enzymes that cleave from both ends of the cellulose molecule. CBHI acts on the reducing ends of the cellulose unit removing both glucose and cellobiose molecules resulting in a large increase in reducing groups, whereas CBHII acts from the non-reducing end of the carbohydrate. CBHs are the only enzymes that can effectively degrade the crystalline fraction of cellulose as endoglucanases can only act on the amorphous region (Perez *et al.*, 2002). Cellobiohydrolases are the major component in

fungal cellulases representing between 40-70 % of the overall cellulolytic activities produced.

β-glucosidase hydrolyses the β-1,4 glucosidic linkages in cellobiose with glucose units being produced. They are important enzymes in the complete hydrolysis of cellulose as cellobiohydrolases are inhibited by high concentrations of cellobiose; therefore exogenous β-glucosidases are required to reduce cellobiose to glucose if it is not already present (Sánchez *et al.*, 2008). A multitude of different cellulases can exist in a single enzyme preparation depending on its origin. A typical cocktail from *Trichoderma reesei* for example, contains at least six different endoglucanases, two cellobiohydrolases (CBH I and CBH II) and two β-glucosidases (Sánchez *et al.*, 2008). Cellulases are classified into families depending on their amino acid sequence and/or similarities in their catalytic domain. The active site for substrate hydrolysis is contained within the catalytic domain, while the cellulose binding domain (CBD) facilitates binding to the insoluble substrate (Linder *et al.*, 1997). Cellulases usually contain three domains; the catalytic domain, a cellulose binding domain and a glycosylated flexible linker which connects the other two domains together (Hall *et al.*, 2011). To date, a total of 12 families of catalytic domains and 14 distinct families of CBD's have been identified based on amino acid sequencing (Duff *et al.*, 1996; Levy *et al.*, 2002).

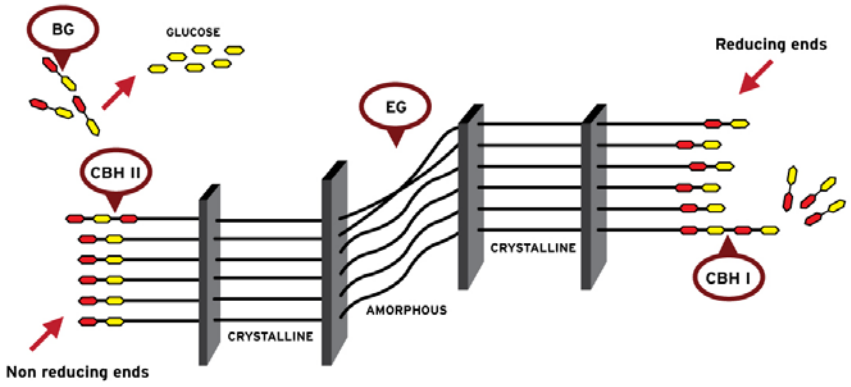


Figure 1.4 Enzymatic degradation of cellulose to glucose.

Adapted from Perez *et al.* (2002). Cellobiohydrolase I (CBH I) acts on the reducing ends, Cellobiohydrolase II (CBH II) acts on the non-reducing ends, Endoglucanases (EG) hydrolyse the internal bonds normally in the amorphous region and β-glucosidase (BG) cleaves the cellobiose dimers to glucose.

1.2.3 Hemicellulolytic enzymes

The enzymes required for efficient hydrolysis of hemicellulose include endoarabinanase, endoxylanase, α-L-arabinosidase and exoxylanase, with the complete set of enzymes required listed in Table 1.3 (Coughlan *et al.*, 1993). Due to the heterogeneity of hemicellulose, significantly more enzymes are required for total hydrolysis and decomposition in comparison to other polysaccharides such as cellulose (Malherbe *et al.*, 2002). The enzymes involved in hemicellulose degradation are invariably categorised into two catalytic modules; glycoside hydrolases (GH) which hydrolyses the glycosidic bonds and carbohydrate esterases (CE) which hydrolyses the ester linkages of ferulic acid or acetate side groups (Shallom *et al.*, 2003).

Xylan is the principle carbohydrate found in hemicellulose and consequently the mode of action of xylanases and their related accessory enzymes are of great interest in understanding the hydrolysis of hemicellulosic substrates. Xylanases such as endo 1,4-β-xylanase hydrolyse the β-1,4 bonds within the xylan backbone, yielding randomly sized and branched xylooligomers. Accessory enzymes such as arabinofuranosidases and α-L-arabinases hydrolyse the arbinofuranosyl containing hemicelluloses to remove L-arabinose, while α-D-glucuronidases cleave the α-1, 2-glycosidic bonds of the 4-*O*-methyl-D-glucuronic acid side chains of xylans. Meanwhile, hemicellulolytic esterases such as xylan esterase, ferulic and ρ-coumaric esterases cleave the *O*- acetyl groups on the xylose moieties as well as the ester bonds between the arabinose substitution and ferulic acid (Shallom *et al.*, 2003; Gírio *et al.*, 2010). The xylooligomers produced by xylanase are subsequently hydrolysed by β-1,4-xylosidases which degrade the xylooligomers into single xylose units (Sánchez, 2009). Similarly, β-mannosidases act on the β-1,4 manno-oligmers produced from β-mannan to produce single mannan units.

Any of a number of hemicellulosic enzymes are required to work together to achieve efficient hydrolysis of a specific chain of hemicellulose. For instance, *O*-acetyl-4-*O*-methylglucuronoxylan, which is one of the most abundant hemicellulose complexes, requires four enzymes (endo-1,4-β xylanase, acetyl esterase, α-D-glucuronidase and β-xylosidase) for complete hydrolysis (Perez *et al.*, 2002).

Table 1.3 List of hemicellulosic enzymes and their function in hemicellulose degradation.
Adapted from Coughlan *et al.* (1993).

Enzyme	Function
Endo-acting	
Endoarabinanase	Cleavage of the α-1.5-linked backbone of arabinan
Endogalactanase	Random cleavage of α-1,4 and α-1,3 linked backbone of arabinogalactans
Endomannanase	Cleavage of the β-1,4-linked backbone of galactoglucomannans
Endoxylanases	Randomly cleaves the β-1,4-linked backbone of xylans as well as the β-1,4-linked backbone of arabinoxylans and glucuronoxylans at specific intervals
Exo-acting	
α-L-Arabinosidase	Releases 1,3- or 1,2-α-L-arabinosyl substituents from arabinoxylans while also releasing α-1,2 or 1,3-linked L-arabinose from artificial arabinosides, arabinoxylans, arabinosyl-substituted pectins and xylo-oligomers
β-L-Arabinosidase	Releases β-linked L-arabinopyranosyl side chains of arabinogalactans
α-1,2-L-Fucosidase	Releases L-fucose α-1,2-linked to D-galactosyl substituents of xyloglucans
α-D-Galactosidase	Releases α-1,6 linked D-galactopyranosyl side chains of galactoglucomannans
β-D-Galactosidase	Releases β-1,6 linked D-galactopyranosyl side chains of arabinogalactans and galactoglucomannans
β-D-Glucosidase	Hydrolysis of 1,4-β-D-glucopyranose at non-reducing end of the oligosaccharides released from galactoglucomannans by endomannanases
α-D-Glucuronidase	Releases α-1,2-linked D-glucopyranosuronic acid or 4-*O*-methylglucopyranosuronic acid side chains of substituted oligomers released from glucuronoxylans
β-D-Mannosidase	Hydrolysis of β-1,4 linked mannosides including mannobiose, and releases mannose from non-reducing end of manno-oligosaccharides Releases mannose from non-reducing end of β-1,4 linked galactomannans, mannans and manno-oligosaccharides Releases mannobiose from the non-reducing end of β-1,4-linked galactomannans, mannans and manno-oligosaccharides
β-D-Xylosidase	Releases xylose from artificial xylosides and from the non-reducing end of β-1,4-linked xylans and xylo-oligosaccharides
Exoxylanase	Release of xylose from the non-reducing end of β-1,4-linked xylans and xylooligomers
Acetylgalactan esterase	Release of acetyl groups from arabinogalactans
Acetylmannan esterase	Release of acetyl groups from galactoglucomannans
Acetylxylan esterase	Release of acetyl groups from xylans
Courmaric acid esterase	Release of arabinose-linked courmaric acid from cereal arabinoxylans
Ferulic acid esterase	Release of arabinose-linked ferulic acid from cereal arabinoxylans and pectins

1.2.4 Determination of enzyme activity

The presence of an enzyme is commonly distinguished by the occurrence of the chemical reaction that is catalyzed, where the amount of enzyme can be determined

through measurement of the rate of reaction (NC-IUB, 1979). The standard International Unit of enzyme activity is the Katal, although the non-SI unit of enzyme activity, the Enzyme unit, is more commonly used (IUPAC-IUB, 1973). One unit of enzyme activity is defined as the amount of enzyme required to catalyse the transformation of 1 micromole of the substrate per minute under standard assay conditions (IUB, 1965). The Katal is used to express the catalytic activity of the enzyme which measures the increase in the rate of the reaction in the assay system (NC-IUB, 1979).

The measurement of enzyme activity is carried out using one of the four commonly used assays. These include: initial rate, progress curve, transient kinetic and relaxation experiments. The most commonly used of these procedures are initial rate experiments. They involve the mixing of an enzyme with an excess of substrate which results in the build up of an enzyme-substrate intermediate. The reaction subsequently reaches a quasi-steady state (QSS) where the enzyme substrate complex (C) remains relatively constant while the reaction changes slowly. Next the reaction rate is measured over a short period of time generally by the accumulation of product (Schnell *et al.*, 2006). An example of such assays are the colorimetric assay based on the release of dye from a paper substrate (Poincelot *et al.*, 1972) or the measurement of the formation of reducing sugar from the hydrolysis of carboxymethylcellulose (Miller *et al.*, 1960). Progress curve experiments were commonly used in the early years for the detection of enzymes; however, they are used less so now. They require the monitoring of the reaction rate of the enzyme as a function of time as it approaches equilibrium to determine the concentration (Schnell *et al.*, 2006). Transient kinetic experiments monitor the reaction behaviour during the initial fast transient period prior to the reaction reaching the QSS interval (Nolting, 1999). Finally, relaxation experiments monitor the return to equilibrium of the reaction after a change in reaction conditions such as pH or temperature. A drawback of this method however, is that a fully reversible reaction is required (Fersht, 1999).

When assessing the activity of a specific enzyme the conditions of the reaction such as storage, pI, substrate type, substrate concentration, pH and temperature must be closely monitored (Headon, 1993). Changes in any of these parameters can have a significant effect on the determination of enzyme activity and need to be standardised to obtain consistent data which is comparable between assays. For example, Hristov *et*

al. (1996) showed that three commercial enzyme preparations had differing activities depending on the type of substrate used when assaying via a reducing sugar assay.

Another issue that arises when determining enzyme activity is the lack of a standard assay for the determination of each enzyme, which would make it easier to compare and contrast results with other works. For instance, a number of assay methods are used for the determination of manganese dependent peroxidase. These include the monitoring of oxidation of phenol red (Orth *et al.*, 1993), the determination of changes in the oxidative state of Mn^{2+} to Mn^{3+} (Ganesh Kumar *et al.*, 2006), the monitoring of the oxidation of ABTS (Gold *et al.*, 1988) and the determination of MnP using 2,6 dimethoxyphenol (Grgic *et al.*, 2003).

1.3 Filamentous fungi and their role in lignocellulosic degradation

Lignocellulosic degradation by filamentous fungi is predominantly carried out by a specialised group of basidiomycetous fungi that can be categorised into three groups based on their habitat and lifestyle. These three groups include wood decaying fungi, litter decomposing fungi and mycorrhizal fungi. Wood decaying fungi can further be classed into three different types, specifically brown, white and soft rot. Brown rot fungi attack cellulose and hemicellulose while leaving the lignin fraction relatively untouched (Eriksson *et al.*, 1990). Soft rot fungi cause wood to lose its mechanical strength and become wet and spongy through degradation of the cellulose and hemicellulose fractions. The only groups of fungi that have evolved to efficiently mineralise lignin are white rot fungi and related litter decomposing fungi (Kirk *et al.*, 1998). Other filamentous fungi such as *Aspergillus spp.*, *Trichoderma spp.* and *Penicillium spp.* are also of interest, due to their capacity to degrade cellulosic and hemicellulosic fractions in forestry and agricultural wastes.

1.3.1 White rot fungi

White rot fungi degrade lignocellulose during secondary metabolism under low nutrient conditions through a cocktail of enzymes including lignin peroxidases, manganese peroxidases, laccases, cellulases and hemicellulases (Hatakka, 2005). There is however, a hypothesis that wood rotting fungi can also degrade cell wall polysaccharides through non-enzymatic decomposition (Baldrian *et al.*, 2008). White rotting fungi can usually be classified into three distinct groups based on their ligninolytic enzyme patterns. The first group of fungi produce the enzymes LiP, MnP

and laccase, while the second group contain the enzymes LiP and laccase. Finally, the third group of fungi produce MnP and laccase (Hatakka, 1994).

White rot fungi can vary considerably in their ability to degrade lignocellulosic material. A large number of white rot fungi colonize the cell lumina, resulting in zones of hydrolysis in the cell wall, which subsequently coalesce resulting in larger hydrolysis zones. These voids are then filled with mycelia in a process known as non-selective degradation. The fungal strain *Trametes versicolor,* a well known white rot fungus, is understood to degrade lignin non-selectively. Other white rot fungi preferentially degrade the lignin without significant loss to the cellulose and hemicellulose fractions (Blanchette, 1995). However, some of these fungi are also capable of producing both types of fungal biodeterioration, such as *Ganoderma applanatum*. White rot fungi which selectively degrade lignin are of most interest in biotechnological applications as cellulose and hemicellulose are the desired products of the process. It must be noted that individual fungi can vary significantly in their ability to degrade specific substrates under the same conditions (Eriksson *et al.*, 1990). For example, both *Ceriporiopsis subvermispora* and *Pleurotus eryngii* partially remove lignin from the middle lamella in straw, while *Phlebia radiata* removes the lignin from the secondary cell wall (Burlat *et al.*, 1998).

A screening study carried out to find a suitable fungus for biopulping revealed a selection of fungi that displayed specificity towards lignin degradation (Hatakka, 2005). These fungi included *Pleurotus ostreatus*, *Pleurotus eryngii*, *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora.* White rot fungi are more commonly found on hardwoods rather than softwoods, as syringyl units found in angiosperms are preferentially degraded by white rot fungi, whereas the guaiacyl units found in gymnosperms are more resistant to degradation (Hatakka, 2005). Softwoods consist mainly of guaiacyl type lignin whereas hardwoods and grasses contain guaiacyl-syringyl type lignin (Singh *et al.*, 2008).

1.3.2 Brown rot fungi

Brown rot and white rot fungi are taxonomically related and can be found in the same division (Hatakka, 2005). Brown rot fungi however, tend to degrade the hemicellulose and cellulose fractions, although they have been known to degrade the lignin fraction to a certain extent (Eriksson *et al.*, 1990). As brown rot fungi only utilise cellulose and hemicellulose, the wood stays predominantly brown (due in part to the lignin) while

keeping its general shape. Examples of brown rot fungi include *Serpula lacrymans*, *Lenzites trabea*, *Fomitopsis lilacano*, *Meruliporia incrassate*, *Gloeophyllum trabeum* and *Coniphora putenana* (Blanchette, 1995). The initiator of lignocellulosic breakdown by these fungi are thought to be as a result of small molecular weight proteins that diffuse out from the hyphae penetrating into wood cells and initiating hydrolysis (Shimada *et al.*, 1997). Fungal hyphae penetrate from one cell to another through the existing pores in the wood cell walls resulting in depolymerisation. Diffusion starts in the cell lumen where the fungal hyphae are found in close connection to the S3 layer, while decay is thought to start in the S_2 layer (Eriksson *et al.*, 1990). The cell wall of wood generally consists of 7 layers; the middle lamella, the primary wall, the outer layer of the secondary wall (S1), the middle layer of the secondary wall (S2), the inner layer of the secondary cell wall (S3), the helical thickening layer (W) and finally the warty layer (Butterfield *et al.*, 1980). Unlike white rot fungi, brown rot rapidly depolymerise the cellulose and hemicellulose and in doing so, form degradation products, as not all the carbohydrate and nutrients are utilised during metabolism (Cowling, 1961). In spite of this, wood decay in brown rot fungi is quite similar to white rot in that it involves the formation of radicals, requires a low pH and decay is enhanced by high oxygen tension (Hatakka, 2005).

1.3.3 Soft rot fungi

Soft rot fungi cause the wood to lose its mechanical strength and become wet as a result of extensive degradation. They function by attacking the cellulose and hemicellulose fraction; however, they have been known to have the ability to degrade lignin polymers. Fungi classified as soft rot are taxonomically classified in the phylum Ascomycota and Deuteromycota. Two forms of soft rot degradation exist currently; type 1 is characterised by long horizontal cavities that are formed in the secondary cell wall, whereas type 2 is distinguishable by complete erosion of the secondary cell wall (Blanchette, 2000). Examples of soft rot fungi include *Cadophora* spp., *Chaetomium funicola*, *Daldinia concentric*, *Chrysonilia sitophila* and *Kretzschmaria deusta.*

Of the three categories of wood rotting fungi in existence, white rot fungi have been the most extensively studied due to their ability to depolymerise the lignin polymer while leaving the other fractions relatively intact.

1.3.4 Additional lignocellulolytic fungi

Other fungal species of interest due to their capacity to produce high titres of heterogeneous enzymes are ascomycetous fungi, such as *Aspergillus* spp. and *Trichoderma* spp. Aspergilli can be found in a wide variety of ecosystems as they have an extremely flexible metabolism which enables them to consume a wide range of carbon and nitrogen sources (Flipphi *et al.*, 2009). Within the genus of Aspergilli the two families *A. niger* and *A. oryzae* have been the most extensively studied, as well as being the most commonly utilised strains in industrial processes (Hu *et al.*, 2011). Both of these strains produce a broad range of enzymes that are involved in hemicellulose and cellulose degradation. These include cellobiohydrolase, endoglucanase, β-glucosidases, xylanases, xyloglucanases, glactomannanases and pectinases (de Vries *et al.*, 2001). Research has also indicated that Aspergilli are involved in the degradation of a wide variety of lignin related aromatic compounds (Cardoso Duarte *et al.*, 1994). For instance, Milstein *et al.* (1983) determined that *A. japonicas* was adept at non-oxidative and oxidative decarboxylation, oxidation of aromatic alcohols, aldehydes, associated side chains and cleavage of aromatic rings.

Trichoderma, specifically *Trichoderma reesei*, is one of the most studied ascomycetous fungi and is used throughout the world for production of commercial cellulolytic and hemicellulolytic enzymes (Sánchez, 2009). *T. reesei* produces very high titres of extra-cellular enzymes, which consist mainly of cellobiohydrolases and endoglucanases, but produces very little cellobiase activities (Kim *et al.*, 1997). As a result, cellobiose is accumulated, which in turn can lead to end product inhibition of enzymes such as cellobiohydrolase (Fang *et al.*, 2010). It has been shown that cellulase production in *T. reesei* is extremely dependent on fermentation conditions, substrate type and environment. For example, a high pH can enhance β-glucosidase production by up to 50 % (Juhasz *et al.*, 2005).

1.4 Solid state fermentation

A brief look through history tells us that after 1940, with the adaption of submerged fermentation (SmF), solid state fermentation (SSF) was largely ignored by researchers in the Western world. However, the discovery of mycotoxin production through SSF and the advent of protein enriched cattle feed from fermentation of low cost agricultural wastes, renewed researchers interest in solid state fermentation (Pandey, 2003). Furthermore, the development of soil pollution, toxins in the food chain

(treatable through bioremediation) and the production of valuable secondary metabolites through SSF have also helped to renew this interest (Durand, 2003).

Solid state fermentation is defined as the cultivation of a microorganism on a solid substrate in the absence or near absence of water, although the fermentation must possess enough moisture to support the growth and metabolism of the microorganism. In contrast submerged fermentation involves a variety of stirred and non-stirred vessels, where microorganisms are cultivated in a liquid medium (Viniegra-Gonzalez, 1997). Although the mixing and diffusion of water and nutrients throughout the biomass is lower in SSF, solubility and diffusion of oxygen is greater. In addition, heat transfer is greater is SmF because of its liquid form (Viniegra-Gonzalez, 1997).

Although submerged fermentation is the most popular technique used in the production of enzymes and other secondary metabolites, there are a number of advantages that SSF has over submerged fermentations. These include:

- The use of low-cost agricultural waste residues in SSF as a substrate and support matrix, which reduces substrate cost, while also solving the issue of disposal of such residues (Singhania *et al.*, 2009).
- The ability to mimic the natural cycle of the fungi, which makes it easier to control their morphological cycle (Viniegra-Gonzalez, 1997).
- The lower energy and waste water costs in SSF in comparison to SmF, which results in lower cost fermentations. For instance, it has been estimated that the cost of cellulase production by SSF is around \$0.2 kg^{-1}, while the same product would cost \$20 kg^{-1} if produced in a submerged fermentation system (Tengerdy, 1996).
- Higher enzyme yields with SSF, while the enzymes produced tend to have greater stability. Acuña-Argüelles *et al.* (1995) demonstrated exo-pectinase yields of up to 50 times higher in SSF by *A. niger* compared to SmF. It has still not been determined why there are higher titres of enzymes produced in SSF, although it is thought to be due in part to the fungi being closer to their natural state.
- Lesser need for sterility in SSF due to the low water activity in the substrate.
- Less susceptibility to catabolic repression in SSF, which can limit enzyme production and activity (Hölker *et al.*, 2004). Solís-Pereira *et al.* (1993) for example, reported that although more exo-pectinase per gram of biomass was

produced in SmF, the enzyme activity from SSF exhibited maximal values because of lower protease levels.

Although there are significant advantages to SSF, a number of drawbacks also exist. These are mainly involving process control, a factor that is hindering the large scale adoption of SSF. The drawbacks include: a build-up of gradient temperature, pH control, moisture and substrate concentrations, which are difficult to control due to of the lack of moisture in the system (Hölker *et al.*, 2004).

1.4.1 Factors affecting solid state fermentation processes

There are several factors that affect the use of SSF for the large scale production of secondary metabolites. These factors include: substrate, moisture level/water activity, control of temperature, relative humidity, size and type of inoculum, period of cultivation, maintenance of uniformity in the SSF system and reactor design (Pandey *et al.*, 1999). For efficient solid state fermentation it is desirable that, all physiochemical parameters be optimised.

1.4.1.1 Substrate

The choice of substrate is a significant factor that can affect the efficiency of the fermentation. Substrates are required to provide a macromolecular matrix for the fungi to grow on, as well as supply adequate nutrients for growth (Raimbault, 1998). Without sufficient concentrations of the correct nutrient, fungi will not colonise the chosen substrate. Zadrazil *et al.* (1997) showed that *Pleurotus spp., Dichomitus squalens, Abortiporus bienni* and other wood rotting fungi grew well on substrates such as rape straw, beech sawdust and reed straw; however, they did not grow on rice husks. In addition, the variation in the nitrogen content influenced the rate of decomposition of the substrate (Henriksen *et al.*, 1999).

An ideal substrate should provide all the nutrients required for growth and production of the desired product, although nutrient supplementation of the substrate can be employed (Pandey *et al.*, 1999). The substrate must also act as a solid support for fungal mycelial expansion. This support can either be the substrate itself or an inert material such as polyurethane foam (Pandey, 2003). Natural substrates have the advantage of being relatively cheap; however, the substrate is degraded during fermentation, thus leading to changes in the structure and rigidity, ultimately affecting the heat and mass transfer throughout the system (Krishna, 2005).

Substrate size can also affect fungal growth and product formation. Substrates with small particles provide a larger surface area for microbial attack which is optimal for efficient growth. On the other hand, if the particles are too small it can result in agglomeration, hindering the transfer of air around the substrate and resulting in poor growth. Conversely, coarse material provides a smaller surface area for nutrient release but allows for better aeration (Pandey *et al.*, 1999). Therefore the optimum substrate should be large enough to allow efficient aeration, whilst being small enough to provide a sufficient surface area. To achieve a suitable size the substrate may be pretreated via grinding, chopping or chipping as discussed in Section 1.1.2.2.

1.4.1.2 Temperature

The temperature of the fermentation can affect the growth of the microorganism as well as the formation of spores and fermentation products. Although fungi can grow over a wide temperature range, the thermal optimum required for specific product formation may differ (Bhargav *et al.*, 2008).

Temperature has been shown to have a significant effect on the growth and function of the microorganism in solid state fermentation. Zadrazil *et al*. (1997), revealed that increasing the temperature from 22 °C to 30 °C significantly impacted on the rate of decomposition of the substrates as well as the sequence of decomposition. Additionally, Sohail *et al.* (2009) showed that increasing the incubation temperature during cellulase production had a detrimental effect on endo-glucanase and β-glucosidase activity.

Heat is generated during growth due to the metabolic activities of the fungi; however, the solid substrates used in SSF typically have low thermal conductivities, leading to the accumulation of heat within the system, causing denaturation of the product of interest. In the early stages of fermentation, oxygen transfer remains uniform throughout; however, as the fermentation proceeds, oxygen transfer becomes limited resulting in heat formation within the system (Pandey, 2003).

As a consequence, the main body of research in SSF process integration has revolved around heat removal from the system through aeration and other techniques (Bhargav *et al.*, 2008). Temperature gradients can vary by up to 20 °C within the fermentation, therefore efficient monitoring of the substrate is required to ensure good growth and secondary and/or primary metabolite formation. Low moisture content and poor thermal conductivity make it difficult to control the fermentation temperature in

SSF. Nevertheless, minimizing the depth of the reactor bed is an effective method to reduce heat build-up and allows for easier transfer of oxygen and CO_2 removal. Mixing of the substrate using novel reactors such as a rotating drum reactor, can also improve heat transfer; however, this method cannot be used with fungi that are affected by high sheer stress. Furthermore, sparging the system with air can effectively reduce the heat in the system, although large quantities of air are required, far in excess of the amount needed for respiration (Gervais *et al.*, 2003). The use of larger particles can also reduce the heat accumulation, as these larger particles have increased space between them resulting in easier aeration although as discussed, the surface area of the particle is reduced, which can affect fungal growth (Bhargav *et al.*, 2008).

High temperatures within the system can affect the germination and growth, as well as the formation of metabolites (Bhargav *et al.*, 2008). Mitchell *et al.* (2000b) modelling experiments of a packed bed reactor for SSF revealed that the rate of cooling at the top of the fermentation was greater than at the bottom. In addition they also noted that the highest fungal biomass concentration existed above the bottom of the reactor where the temperature was lower than optimum, whilst the lowest biomass concentration was at the top of the reactor which had a higher than optimal temperature.

1.4.1.3 Moisture content and Water activity (A_w)

Water has a number of roles in solid state fermentation; it functions at a cellular level providing nutrients to the cells, removing wastes and metabolites and works to impart stability to the cell at a molecular and cellular level. At a molecular level, water acts to stabilise the biopolymer, proteins and nucleotides; while at a cellular level it stabilises the lamellar structure of the plasmic membrane thus maintaining the membrane permeability (Quinn, 1985). Insufficient water content can result in a slowdown of the cellular metabolism due to a lack of nutrients and gas, or through high concentrations of secondary metabolites which are normally removed by the liquid. The metabolic pathway of fungi can also be affected by a lack of water, as this can impact on the maintenance of the functional properties of the enzymes in the pathway. Water mass transfer is also strongly dependent on other physiochemical parameters such as aeration and temperature (Gervais *et al.*, 2003).

The term water activity (A_w) was developed to define the association that water has with other non-aqueous constituents and solids. Water activity is defined as the ratio of vapour pressure of an aqueous solution to that of pure water at the same temperature (Bhargav *et al.*, 2008). Scott (1953) introduced the term water activity (A_w) in 1953 and it is still widely used today as a criterion for the physiological functioning of a microorganism. In SSF, low moisture within the system can limit growth and metabolism of the microorganism as these two functions almost always occur in the liquid phase. Gervais and Molin (2003) investigated the affect that water activity has on the production of metabolites, hyphal growth, orientation and fungal physiology. They determined that A_w impacted significantly on the radial extension rate of *T. viride* and *P. roqueforti,* with the optimal A_w for *T. viride* being 0.99 and 0.97 for *P. roqueforti*. They also observed no radial extension below 0.90.

Fungal sporulation was also induced by low water activity in response to unfavourable growth conditions. Optimal fungal sporulation was observed at 0.98 for *T. viride* and at 0.96 for *P. Roqueforti,* with sporulation decreasing above and below these values. The A_w of sporulation for both *T. viride* and *P. roqueforti* was lower than what is required for optimal fungal growth. Water activity was also shown to be the main parameter in spore formation from *Metarhizium anisopliae* (Dorta *et al.*, 1998), although other factors such as oxygen concentration also had an influence. Germination was also affected by A_w as hydration of the medium is an important factor in germination (Snow, 1949), although it is also influenced by other factors such as carbon and nitrogen availability (Martín *et al.*, 1970). Furthermore, secondary metabolite formation can be dependent on water activity in many cases. It was demonstrated that production of polygalacturonase, D-xylanase and β-glucosidase from *Trichoderma viride* were all significantly affected by water activity of the system. Maximal production of polygalacturonase and D-xylanase occurred at 0.99, while maximum β-glucosidase production was observed at between 0.96 and 0.98 (Grajek *et al.*, 1987).

1.4.1.4 Influence of pH

Control of pH is important in both SmF and SSF, aiding in providing optimal growth conditions for fungal metabolism and secondary metabolite formation. In SmF it is relatively simple to control the pH; however; in SSF it can be very difficult due to the inability to uniformly mix the growth matrix, as well as the difficulty in adequately

determining the pH of solids (Krishna, 2005). In SSF the pH of the culture can be reduced as a result of fungal metabolism in which organic acids, such as citric, acetic and lactic acids are secreted. Conversely, the pH can be increased due to urea hydrolysis, which can result in alkalinisation and organic acid assimilation (Raimbault, 1998).

Filamentous fungi usually have a broad pH range in which they can grow; however, for optimal growth and metabolite formation a pH of 3.8-6.0 is typically optimum (Gowthaman *et al.*, 2001). Attempts have been made over the years to control the pH of the SSF fermentation. This can be accomplished by considering the buffering capacity of the components while formulating the substrate, or by using chemicals that have no effect on the biological activity of the fungi (Krishna, 2005). Raimbault *et al.* (1980) for example, adjusted the pH of an *Aspergillus niger* fermentation using ammonia salt and urea to give a final pH of 4.5. Similarly, Durand *et al.* (1988a) also managed to control the fermentation by spraying it with urea.

1.4.1.5 Aeration

Aeration has a vital role in SSF; providing oxygen to the fungi for metabolic growth as well as removing heat, moisture, volatile metabolites and carbon dioxide from the system (Krishna, 2005). The requirement for air within the system is generally regulated by the rate of growth of the microorganism, the requirement for removal of CO_2 and the need to reduce the temperature. The operation parameters of various reactor configurations can influence the rate of oxygen transfer through the system. These parameters include; the bed depth, porosity and size of the substrate, moisture content and impeller or drum rotation speed (Krishna, 2005). In tray fermentations, the bed depth must be kept to a minimum to allow for better aeration and to reduce the formation of heat gradients. Mitchell *et al.* (1990) studied the effect of oxygen on the growth of *Rhizopus oligosporus* in SSF. They determined that the transfer of oxygen was dependent on the interfacial gas-liquid surface area and the thickness of the wet fungal layer. Moo-Young *et al.* (1983), determined that the most important oxygen transfer mechanism is the interparticle mass transfer and that the dew point of the air used to control the system must be found to effectively control the relative humidity of the system.

1.4.1.6 Reactor configuration

Historically, reactor design in solid state fermentation was not given much attention, due to the poor thermal conductivities and mass transfer issues in the solid state reactors compared to those in SmF. However, in recent years reactor design has increasingly gained a lot of interest (Singhania *et al.*, 2009). A number of factors are thought to influence the reactor design including; the varieties of substrates that are involved, the size, mechanical resistance, water holding capacity and the porosity of the substrate. Submerged fermentations are generally easier to scale-up than SSF systems because of better heat and mass transfer in the system, resulting from better mixing and aeration in the vessel. Other factors are also known to impact on the reactor design, such as fungal morphology, the transfer of water through the system, the fungi's resistance to sheer forces and the need for sterility (Durand, 2003).

Several SSF reactor configurations have been investigated that can overcome the problems associated with heat removal and other limiting factors, although very few have been scaled up to large scale processes (Singhania *et al.*, 2009). Commonly used reactor configurations include trays, packed bed, horizontal drum and fluidised bed reactors, with each reactor configuration having their own advantages and disadvantages (Rodriguez Couto *et al.*, 2005).

Drum reactors use a perforated drum or rotating paddles to mix the fermentation whilst tumbling the substrate, thus allowing for continuous contact with the vessel wall which may be surrounded by a cooling water jacket. These configurations; however, are only useful for fungi that have high tolerance to shear stress (Durand, 2003). Tray fermenters are one of the most popular techniques for the cultivation of fungi using solid state fermentation as they have low capital costs due to the simplicity of the system. These reactors consist of flat trays which are filled to a height of about 1.5-2 cm with inoculated substrate. They are kept on trolleys in a temperature regulated chamber with passive or forced aeration (Rodriguez Couto *et al.*, 2005). The bed depth in the tray is kept to a minimum and the trays are generally perforated to reduce heat accumulation while allowing easier aeration. Tray reactors generally suffer from slow diffusion of air resulting in temperature variations throughout the tray, with the tray centre having the highest temperature.

A packed bed reactor consists of a cylindrical column which is packed to large heights with the substrate. Air is forced up through the bottom of the reactor to cool

the system and supply oxygen to the fungi (Krishna, 2005). Durand and his team at INRA in France developed a reactor similar to a packed bed model, which consists of, a relative humidity probe, heating cover for the vessel and a cooling coil within the system which work together to regulate the water content during the process (Durand *et al.*, 1988b).

A fluidised bed reactor involves passing a solid or liquid through the substrate bed at high velocities to cause the system to behave like a fluid in a process known as fluidisation. This system offers advantages such as uniform conditions throughout the system and an increased surface area (Bhargav *et al.*, 2008). A number of other reactor designs exist based on the aforementioned reaction types which use the principle of heat reduction through either forced aeration, cooling coils or conduction plates (Mitchell *et al.*, 2000a; Suryanarayan, 2003).

1.5 Biologically pretreated lignocellulosic waste products and the metabolites from this pretreatment

Lignocellulosic waste products can have a number applications in industrial and agricultural processes, although at present most of these materials are being disposed of or used as low quality animal feed. These applications can include enzyme, antioxidant and ethanol production, whilst the material can also be used as an upgraded animal feed.

As discussed in Section 1.1.2.6 biological pretreatment of these waste materials through solid state fermentation using white rot fungi can reduce the lignin content and leave the more hydrolysable cellulose and hemicellulose fractions available for fermentation. These pretreated materials can then be fermented to ethanol through a two stage process, where the material is hydrolysed with hollocellulosic enzymes and subsequently fermented to ethanol using a selection of different microorganisms.

A number of enzymes can also be produced through solid state fermentation of these lignocellulosic wastes with fungi. The principle enzymes produced from solid state fermentation of lignocellulosic wastes are hydrolytic enzymes, namely cellulases, proteases, hemicellulases, ligninases. These enzymes have found a wide variety of uses in industrial processes such as the production of food, clothing, beverages and paper amongst others. Currently, the majority of enzymes are produced through submerged fermentation in defined media; however, solid state fermentation of

lignocellulosic wastes offers a low cost alternative with the potential to achieve higher enzyme titres (Bhargav *et al.*, 2008).

Pretreated lignocellulosic material also has the potential to be used as an upgraded forage feed for livestock. As pretreatment removes the lignin fraction, the cellulose and hemicellulose fractions become more available for utilisation by the animal due to the increased digestibility of the feed. Exogenous hydrolytic enzymes produced from solid state fermentation of lignocellulosic substrates can also be used in the animal diet to improve the feed digestibility through direct addition of these enzymes to the forage (Bedford, 1993).

At present the vast majority of antioxidants used are chemically produced. Solid state fermentation of waste material with white rot fungi offers the potential to produce natural enzymatic antioxidants at a low cost without the inherent dangers that can be associated with those that are chemically produced (Cheung *et al.*, 2003). White rot fungi have long been known to have the potential to produce antioxidants and it has been thought that the medicinal effects of the mushrooms used in Chinese medicine are believed to be related to the antioxidant properties they exhibit. Fungi have become an attractive source of antioxidants as the can be rapidly produced, whilst the medium can be manipulated to produce excess quantities, which is not typically the case with antioxidant produced from other natural sources (Ferreira *et al.*, 2009).

1.5.1 Production of enzymes from lignocellulosic waste material by SSF

Enzymes are used in a large number of industrial processes, such as in detergents to remove stains, and have found extensive application in the brewing, paper making and fruit juice industries (Rodriguez Couto *et al.*, 2005). Currently, almost all commercial enzyme production is carried out using fungi and bacteria, with the majority being produced using genetically modified organisms in submerged fermentation (Krishna, 2005). Over the last decade however, there has been a trend towards enzyme production using SSF, largely due to the high cost of production using SmF in comparison to SSF. Castilho and Polato (2000) carried out a cost analysis between SSF and SmF for the production of lipase and concluded that SmF required a 78 % higher capital investment in comparison to SSF, while the cost to produce the product in SSF was also lower. Other advantages of SSF include; less production of waste, lower need for sterility due to lack of water in the system and higher enzyme titres (Bhargav *et al.*, 2008). It is not known why higher enzyme titres are achieved;

however, it is perhaps due to SSF working as a fed batch type system where oxidation is high and carbohydrate supply is low (Viniegra-Gonzalez *et al.*, 2003). Alternatively, it may be that the fungus is able to germinate in its natural state. Diaz-Godinez *et al.* (2001), showed that higher pectinase titres were achieved in solid state fermentation because of higher biomass yields, while there was lower protein hydrolysis within the fermentation from contaminating proteases. In SSF processes the crude enzyme extract can also be used directly, reducing the processing cost to recover the enzyme as the product will only need to be dried (Pandey *et al.*, 1999).

Currently, the vast majority of research published on the production of enzymes in solid state fermentation are laboratory scale processes (Durand, 2003). A wide variety of lignocellulosic waste materials have been used in solid state fermentation including; sugar cane baggase, wheat straw, oat straw, banana waste, corn stover and corncobs. Wheat bran however, is the most extensively used lignocellulosic substrate and has been used for the production of various enzymes, although other potentially suitable substrates exist in vast quantities (Pandey *et al.*, 1999).

Presently, about 60 million tonnes of corncobs are being produced in China and the US every year, with it being mainly used as a low cost animal feed (Bai *et al.*, 2008). The potential of corncob as a substrate for lignocellulolytic production is realised when one considers the composition of the material. Corncob consists of ~35 % hemicellulose and ~45 % cellulose (Saha, 2003) making it an ideal substrate for enzyme production. Table 1.4 illustrates a number of enzymes that can be produced using solid state fermentation techniques, the microorganisms that can be used to produce these enzymes and the various substrates which have been used in production.

Table 1.4 Substrates, enzymes and microorganisms associated with SSF.

Retrieved from Pandey *et al.* (1999).

Substrates	Enzymes	Microorganisms
Bagasse, rice husk, rice straw, wheat bran, wheat straw, sweet sorghum, silage, sugar beet pulp, sawdust, cassava waste, soy-hull, paddy straw, etc.	Cellulolytic enzymes, laccase, xylanase, polygalacturonase, ligninase	*Aspergillus* spp., *Trichoderma* spp., *Penicillium* spp., *Pleurotus* spp., *Neurospora* spp., *Sporotrichum pulverulentum*, *Cerrena* spp., *Botritis* spp., *Gliocladium* spp., *Phanerochaete* spp.
Rice straw, corn hull, corncobs, wheat bran, wheat straw, bagasse, rice straw, cotton stalks, soy-hull, kraft pulp, sugar beet pulp, rice husk, apple pomace, coffee processing waste, barley straw, oat straw	Xylanases, b-xylosidase, α-arabinofuranosidase, acetoesterase, catechol-oxidase	*Aspergillus* spp., *Trichoderma* spp., *Penicillium* spp., *Phlebia radiata*, *Melanocarpus albomyces*, *Pycnoporus sanguineus*, *Thermomyces lanuginosa*, *Thermoascus aurantiacus*, *Taloromyces emersonii*, *Thermomonospora* spp.
Bagasse, wheat bran, wheat straw, saw-dust, cotton stalk, cellulose powder, wood chips	Laccase, Li-peroxidase, Mn-peroxidase, aryl-alcohol oxidase, catalase, phenol oxidase	*Penicillium* spp., *Pleurotus* spp., *Phlebia radiata*, *Trametes versicolor*, *Polyporus* spp., *Panus tigrinus*, *Trichoderma versicolor*
Wheat bran, sunflower flour, coffee husk, soybean meal, rice bran, corn bran, rice hull, sweet potato residue	Protease (acidic, neutral and alkaline)	*Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., *Bacillus* spp., *Trichoderma* spp.
Wheat bran, coconut cake, rice bran	Lipase	*Candida* spp., *Aspergillus* spp., *Rhizopus* spp., *Neurospora sitophila*, *Penicillium candidum*, *Mucor* spp.
Wheat bran, soybean cake residue	α-galactosidase, b-galactosidase	*Aspergillus niger*, *Aspergillus oryzae*, *Rhizomuco*r spp., *Kluyveromyces lactis*
Wheat bran, rice bran, sugarcane bagasse, corn flour, saw-dust, soybean meal, sweet potato, sugar beet pulp	α-amylase, b-amylase, glucoamylase	*Aspergillus* spp., *Rhizopus* spp., *Mucor* spp., *Bacillus* spp., *Saccharomyces* spp.
Wheat bran, rice husk, saw dust, coconut oil cake	Glutaminase	*Vibrio costicola*
Wheat bran, chicory roots	Inulinase	*Staphylococcus* spp., *Kluyveromyces marxianus*
Canola meal	Phytase	*Aspergillus ficuum*, *Aspergillus carbonarius*
Wheat bran + tannic acid	Tannase	*Rhizopus oryzae*
Wheat straw	Feruloyl ρ-coumaroyl esterase	*Penicillium pinophilum*

Currently there are three different areas in which enzymes are used in industry. These include technical enzymes for detergent and biopulping, food enzymes for the production of confectionary and animal feed enzymes like phytase.

The enzyme industry is thought to be worth about $2 billion annually with proteases and amylase being the two most important enzymes, making up approximately 45 % of total enzyme sales (Krishna, 2005). Production of enzymes in SSF is a relatively simple process; however, a number of factors have to be considered for the efficient production of the enzyme of interest. These factors include the selection of a suitable substrate that will induce enzyme production, whilst other factors that influence the yield of enzyme from SSF such as temperature, humidity, type and size of the inoculum, water content and water activity should also be considered (Krishna, 2005). Hydrolytic enzymes such as cellulase, hemicellulase and amylase are usually produced from fungi as these enzymes are required in their natural habitat for growth (Pandey *et al.*, 1999).

1.5.1.1 Amylase production

Amylase production in industry is mainly carried out via the submerged fermentation of *Aspergillus spp*. and *Rhizopus spp.* The two main classes of amylase enzymes produced are glucoamylase and α-amylase. Glucoamylase hydrolyses single glucose units from the non-reducing end of amylose, while α-amylase hydrolyses the 1,4-α-D-glucosidic linkages between glucose units in starch and glycogen randomly, yielding maltose and glucose units (Krishna, 2005). These enzymes are used mainly in the brewing and food industry for starch saccharification (O' Rourke, 1996), whilst they can also be used in detergents to breakdown starch stains (Eriksen, 1996). Wheat bran has been reported as the best substrate for α-amylase production in SSF (Bhargav *et al.*, 2008), although other materials such as banana waste, corn flour and sweet potato waste have been shown to be potential substrates (Pandey *et al.*, 1999). There are less instances of glucoamylase production from SSF, although it has been shown to be viable, with Ellaiah *et al.* (2002) producing glucoamylase on a number of lignocellulosic substrates such as wheat bran, rice bran and wheat straw.

1.5.1.2 Cellulase production

Cellulases are, as outlined in Section 1.2.2, a group of enzymes responsible for the hydrolysis of cellulosic materials into glucose. These enzymes have gained a lot of interest due to the advent of second generation biofuels which involves the conversion of cellulose into ethanol (Bhargav *et al.*, 2008). Presently, the production of cellulase enzymes is typically carried out using SmF, with the fungi *T. reesei, T. longibrachiatum* and *A. niger* being the most extensively utilised microorganisms (Singhania *et al.*, 2010). A number of lignocellulosic waste materials have been investigated for the production of cellulolytic enzymes in both solid state and submerged fermentation. Xia *et al.* (1999) produced cellulases through the solid state fermentation of corncob residue and wheat bran by *T. reesei*, whilst cellulase production from other lignocellulosic materials such as rice straw (Kang *et al.*, 2004), wheat straw (Deswal *et al.*, 2011), sorghum bagasse (Dogaris *et al.*, 2009) and soyhull (Jha *et al.*, 1995) has also been reported. Currently, the cost of cellulase production is expensive in comparison to other secondary metabolites, hence the focus on SSF to reduce this cost. For instance, the cost of cellulase production in SmF is currently three times the cost of penicillin production, whilst it has a lower commercial value (Tengerdy, 1996). Tengerdy also compared the cost of cellulase production in SSF and SmF and determined that cellulase production in solid state fermentation was 100 times cheaper than with submerged fermentation. Currently, cellulase have found uses in a number of industrial processes such as in the detergent industry to remove the broken fibres that cause prills (Bhat, 2000) and in the textile industry, during bio-stoning, to produce the stone wash effect in jeans (Galante *et al.*, 1998b).

1.5.1.3 Xylanase production

Xylanase production can be carried out by fungi, yeast and bacteria, although in most commercial processes filamentous fungi are used as they produce much higher titres than other microorganisms (Rodriguez Couto *et al.*, 2005). Xylanases hydrolyses the β-1, 4-linkages in xylan which aids in the conversion of hemicellulose to pentose sugars. Xylan is the second most plentiful polysaccharide in the world after cellulose, making xylanase enzymes of great interest. Xylanase is mainly produced by *Trichoderma* spp. and *Aspergillus* spp. (Li *et al.*, 2005); however, recent research has uncovered strains of *Thermomyces lanuginosus* which are of great interest due to their ability to produce high concentrations of cellulase free thermostable xylanase (Singh *et*

al., 2003; Gaffney *et al.*, 2009), which are more suited to high temperature processes such as simultaneous saccharification and co-fermentation. Xylanase, like most other hydrolytic enzymes are mainly produced by SmF; however, research into production by SSF has resulted in the discovery of more thermostable xylanases than those produced from SmF (Krishna, 2005). Xylanase can have a number of applications in industry; such as during pulp and paper production, to attack the surface xylan and xylan-lignin complexes to make the remaining lignin more susceptible to bleaching (Tolan, 1996).

1.5.1.4 Protease production

Proteases are one of the most commercially significant enzymes produced by filamentous fungi. They account for a large portion of the enzyme market and have numerous applications in industries, like in leather and food processing and in the detergent industry (Rodriguez Couto *et al.*, 2005). The main fungi involved in the production of proteases includes, but are not limited, to *A. oryzae, A niger, A. flavus* and *Rhizopus oligosporus*. Various attempts have been made to produce alkaline, acidic and neutral proteases via SSF, as a number of different proteases are required depending on the application. Furthermore, a diverse range of substrates have been investigated for production of these proteases including rice bran, spent brewers grain, coconut oil cake, wheat bran and soy meal, although wheat bran is once again considered the substrate of choice (Bhargav *et al.*, 2008). Protease production is usually carried out in SmF; however, higher titres are generally achieved through SSF. For instance, George *et al.* (1997), produced as much protease in 1 g of SSF as there was in 100 mL of SmF culture. Proteases are typically used in leather processing to dehair the skin (Godfrey, 1996), in detergents to remove protein based stains (Eriksen, 1996), whilst they are also used in brewing to remove protein haze from the final product (O' Rourke, 1996).

1.5.1.5 Ligninase production

Ligninases are a group of enzymes that hydrolyse lignin through a number of oxidative reactions. The three most significant enzymes in lignin hydrolysis include manganese dependent peroxidase (MnP), lignin peroxidase (LiP) and laccase, as discussed in Section 1.2.1. The main applications of these enzymes are in the bioethanol and paper industries (Rodriguez Couto *et al.*, 2005). In bioethanol production, these enzymes

which are typically produced from white rot fungi, can be used as a biological pretreatment step to delignify lignocellulosic waste material to increase the digestibility of the substrate, whilst they are used in the paper industry to degrade the wood so as to make it more susceptible to mechanical pulping. Fujian *et al.* (2001), investigated the production of MnP and LiP produced from steam exploded wheat straw in both submerged and solid state fermentation and determined that the maximal activity of 2600 U g^{-1} was obtained through SSF.

1.5.2 Enzymes in lignocellulosic degradation and their potential for application in animal nutrition

There is vast potential for the use of lignocellulosic enzymes as a pretreatment process in animal feed diets. Currently, exogenous enzymes are used to improve the digestibility of the feed, remove anti-nutritional factors and to supplement endogenous enzymes in pigs and poultry where they may be deficient (Bedford, 1993; Galante *et al.*, 1998a).

Phytase is used in the diets of pigs and poultry to help utilise the phosphorus that is present in cereals as phytic acid (Selle *et al.*, 2009). Previously, BSE-related bone meal was used as a phosphorus source; however, it has since been banned in many countries. As a consequence, the addition of phytase to the monogastric diets is now one of the only ways to efficiently use phytic acid in the diet (Kirk *et al.*, 2002).

Over the last two decades, xylanase and β-glucanase have also been used in monogastric feeds, as these animals are unable to fully utilise plant-based feeds due to the presence of cellulose and hemicellulose (Kirk *et al.*, 2002). For example, β-glucanase can be added to broiler diets (which can contain over 10 % barley) as they can suffer from reduced performance due to high β-glucan content in the barley. This β-glucan increases the viscosity in the gastro-intestinal tract, resulting in a reduction in the digestibility of starch and protein (Campbell *et al.*, 1992). The addition of β-glucanases improved digestion while also aiding weight gain (Cowan, 1996).

Supplementation of enzymes in the ruminant diet has gained some interest recently due to positive responses such as, increased milk production in cows (Kung Jr *et al.*, 2000; Yang *et al.*, 2000), weight gain in beef cattle (Burroughs *et al.*, 1960) and improved feed efficiency with high forage diets (Beauchemin *et al.*, 1997). Burroughs *et al.* (1960) determined that supplementation of beef cattle's mixed feed diet with an enzyme cocktail containing cellulase, amylase and protease increased the weight of the

cattle by between 6.8-24 %. Furthermore, Beauchemin (1995) reported live weight gains of up to 35 % in cattle through the addition of xylanase and cellulase to a hay diet.

Pretreatment of agricultural waste products through direct fermentation of the material with fungi or by the addition of exogenous enzymes produced from these fungi (e.g. ligninase, cellulase and xylanase) can improve the feed digestibility creating a feed of higher quality. Several studies have investigated using basidiomycetous white rot fungi to selectively degrade the lignin, whilst leaving the other components (cellulose & hemicellulose) relatively untouched for use in animal feed (Jung *et al.*, 1992; Karunanandaa *et al.*, 1995). Results however, have indicated that it is difficult to control the degradation patterns of most fungi, resulting in partial loss of the cellulose and hemicellulose fractions (Graminha *et al.*, 2008).

The direct addition of enzymes to the diet has also been investigated (Yang *et al.*, 2001), although many of these studies have been carried out *in vitro* which will not always directly mimic what is happening *in vivo* (Lewis *et al.*, 1996; Krause *et al.*, 1998; Graminha *et al.*, 2008). They have however, indicated that adding lignocellulosic enzymes to the feed increases the digestibility of the substrate, although results from forage diets were more inconsistent (Beauchemin *et al.*, 2003), with the addition of these enzymes to high grain diets delivering more consistent results. For example, Beauchemin *et al.* achieved between 6-11 % improved feed efficiency in a 95 % barley grain diet depending on the enzyme dosage rate (Beauchemin *et al.*, 1997; Beauchemin *et al.*, 1999). Another consideration to note when developing an enzyme product for the addition to different animal feeds is that commercial ruminant diets tend to have a number of different types of forages in varying volumes. As a consequence, to obtain maximum nutritive value a number of different enzymes from different sources would have to be used (Beauchemin *et al.*, 2003).

Colombatto (2003) examined the use of adding enzyme from thermophillic fungi to ruminant diets. Mesophilic fungi tend to produce enzymes with optimal temperatures of between 45-60 °C, which can be lower than the temperature found in the later stages of silage processing (Graminha *et al.*, 2008). They determined that enzymes from thermophillic fungi often had increased thermostability in comparison to mesophilic enzymes (Colombatto *et al.*, 2003). The mode of action of enzymes in animal feed is not fully understood, although it is thought that the addition of enzymes to the feed simply improves the digestibility by the hydrolysis of the material in the

rumen (Morgavi *et al.*, 2000). Although, ultimately it could be as complex as removal of structural barriers, allowing interaction with ruminal microorganisms (Colombatto *et al.*, 2003).

Direct solid state fermentation of the agricultural waste can also help improve the protein content of the material. Typically lignocellulosic wastes have poor protein content, which can lead to the need to supplement the feed with exogenous nitrogen and protein. However, feeding ruminants pretreated lignocellulosic forages can reduce this need for protein supplementation, thus reducing the feed cost. Shojaosadati *et al.* (1999) demonstrated that pretreatment of waste material can increase the protein content by 10-15 %, while also increasing the digestibility of the material. The degradability of the protein from the fermented agricultural wastes can also be higher in comparison to untreated material (Iconomou *et al.*, 1998). Proteins in ruminant are required for growth of the animal and milk production (Oltjen, 1969). On average mature beef cows require 70 g kg^{-1} of crude protein in the diet, while high-producing dairy cows require 190 g kg^{-1} (NRC, 1984 ; NRC, 1989). In ruminants, protein digestion is complex due to loss of nitrogen from the rumen, through urine for example. Up to 75 % of crude protein is typically degraded in the rumen, while the remaining 25 % is passed to the intestines where the absorption is more efficient (Merchen *et al.*, 1994). Generally, the average protein content found in warm season grasses is 100 g kg^{-1} dry matter, whereas in cold season grasses the crude protein content increases to 130 g kg^{-1}. Legumes such as alfalfa usually have a even higher crude protein of about 170 g kg^{-1} (Minson, 1990), with the stems usually containing nearly twice as much as the leaves (Buxton, 1996).

1.5.2.1 Methods for determining forage feed quality

The detergent system is the method commonly used for determining feed quality for ruminant diets (Van Soest, 1994). Forage quality can vary significantly depending on factors such as herbage maturity, plant environment, season of the year and geographical location (Buxton, 1996). As a result, the forage quality has to be assessed to determine the quality of forage in the diet. The plant cell wall from the forage provides the fibrous portion of the diet which is required for normal ruminant function. It is composed of between 40 and 80 % lignin and structural carbohydrates, which can limit the digestibility of the material. Typically, ruminants can only utilise about half of this material; however, degradation can release the contents from within

the cell walls, which can be nearly completely digested (Buxton, 1996). Neutral detergent fibre (NDF) is used to estimate the concentration of cell wall carbohydrates in the forage and is negatively correlated to dry matter intake (Golding *et al.*, 1985), while acid detergent fibre (ADF) is used to assess the concentration of cellulose and lignin in the material (Van Soest, 1967). The ADF content is inversely proportional to the digestibility of the substrate, where the higher the ADF content the lower the digestibly of the forage. The NDF concentration in grasses is generally higher than that of legumes, which is caused mostly by the difference in the structural carbohydrates between grass and legume leaves (Buxton *et al.*, 1989). The optimal NDF concentration in the diet of high producing dairy cows is between 270-290 g kg^{-1}. In mature beef cows, plant cell wall concentrations of up to 750 g kg^{-1} will not hinder animal production; however, finishing ruminants would require a much lower NDF content of as low as 150 g kg^{-1} (Mertens, 1994).

Another method which has been used to assess the digestibility of forage is the method first introduced by Tilley *et al.* (1963), for determining the *in vitro* dry matter digestibility. This method involves the incubation of the forage of interest in rumen fluid, under anaerobic conditions for a defined period of time. It aims to mimic the rumen of the animal so as to determine the true digestibility of the forage. Over the years this method has undergone a number of modifications, where the current method uses a Daisy II incubator (Ankom Technologies, New York, U.S.A.) to incubate and mix the digestion samples for a more accurate determination (Holden, 1999).

1.5.3 Bioethanol production

Ethanol or ethyl alcohol is a solvent which has been produced through fermentation of mono- and disaccharides for centuries. Ethanol is typically produced from a variety of cereal grains and fruits where it is produced in a low oxygen environment. During this process yeast becomes stressed due to a lack of oxygen and ethanol is produced as an antimicrobial defence mechanism. Various microorganisms can produce ethanol including the bacteria *Zymomonas mobilis* and yeast strains such as *Saccharomyces cerevisiae* and *Pichia stipitis*, amongst others.

Ethanol as a fuel has gained increasing attention over the last number of years due to rising cost of oil, which exceeded $100 dollars a barrel for the first time in 2008 (Sánchez *et al.*, 2008). This volatility in the market, the concern over the impact of fossil fuels on the environment and the increasing demand from emerging markets

such as China and India, has lead to significant interest in the production of bioethanol (Hahn-Hägerdal *et al.*, 2006). Plans by governments to substitute oil with renewable fuels through the offering of grants and subsidies have also lead to increased production of bioethanol and a reduction in cost, which has lead to a more competitively priced fuel (Balat, 2011). Currently, the main feed stock that is used for the production of bioethanol is corn in the United States and sugar cane in Brazil, with these two countries being the largest producers of ethanol in the world. At present, 73 % of total ethanol production throughout the world is used as fuel, with the remaining 27 % being used for beverage production and industrial processes (Sánchez *et al.*, 2008). Although ethanol is currently being produced through starch conversion of sugar cane and various cereal crops, the increasing demand for fuel bioethanol has resulted in establishing ethanol production in direct competition with the food supply, resulting in the increased cost of the substrate (Binod *et al.*, 2010). Consequently, a switch to more sustainable substrates such as agricultural wastes, forestry lumber and paper wastes would be a more desirable alternative to corn and sugar cane.

As mentioned previously in Section 1.1, the potential of these waste products is extremely high considering that 10^{11} tons of lignocellulosic wastes are degraded every year, equating to roughly 640 billion barrels of oil (Nagy *et al.*, 2007). However, one drawback that can be associated with lignocellulosic ethanol production is the variation in fermentation yields due to the variation of substrate composition from season to season with some lignocellulosic wastes (Binod *et al.*, 2010).

For the efficient production of lignocellulosic bioethanol, the material must be efficiently pretreated to remove the lignin fraction and solubilize the hemicellulose and cellulose fractions. A number of these pretreatment methods have been described previously in Section 1.1.2. Biological pretreatment through SSF offers a low cost alternative, which is relatively free from inhibitors that can be produced from chemical and physico-chemical pretreatments. This method can achieve extensive lignin degradation leaving cellulosic fractions available for enzymatic hydrolysis. Unlike sucrose and starch-based fermentations, production of lignocellulosic bioethanol involves a mixed sugar fermentation consisting of glucose and pentose sugars which can be difficult to ferment efficiently. This liquor can also contain inhibiting compounds such as phenolics and furan derivatives (a result of the pretreatment process) making fermentation strain selection vital (Larsson *et al.*, 2000). Other aspects that influence the efficacy of lignocellulosic bioethanol production is the cost

effective use of the lignin fraction, advanced process integration to minimise energy demand, improvement in ethanol yield and the production of cheaper enzymes (Hahn-Hägerdal *et al.*, 2006; Gnansounou, 2010).

1.5.3.1 Fermentation of lignocellulosic hydrolysate

Fermentation of lignocellulosic hydrolysates produced from pretreatment is usually carried out using simultaneous saccharification and fermentation (SimSF) or separate enzymatic hydrolysis and fermentation (SHF). SimSF however, is generally considered the more favourable approach as it can increase the ethanol yield through reducing the end product inhibition of cellulases, which are inhibited by cellobiose and to a lesser extent by glucose (Sun *et al.*, 2002). Öhgren *et al.* (2007) compared SHF and SimSF for the production of ethanol from steam-pretreated corn stover and obtained a 13 % higher overall ethanol yield with SimSF in comparison to SHF. Similarly, Niklitschek *et al.* (2010) investigated the fermentation of native forest residues and also achieved higher ethanol yields with SimSF. Other advantages of SimSF include the lower enzyme dosage required because of less product inhibition, lower costs due to a reduction in the number of reactors required, lower temperatures and a lower requirement for sterility as the substrate is being utilised as it is formed (Sun *et al.*, 2002). However, there are also a number of drawbacks to this process namely; the temperature of the process not being optimal for enzyme hydrolysis or growth of the organism. As a result, higher enzyme loadings may be required which can increase the cost of the fermentation (Sarkar *et al.*, 2012), whilst the enzymes can be inhibited due to the ethanol concentration (Sun *et al.*, 2002). Conversely, in SHF the optimal temperatures for both saccharification and fermentation can be utilised as it is a two stage process. Despite this, SHF suffers from decreased yields due to product inhibition, higher capital costs because of the need for two fermenters and higher operating cost owing to longer fermentation times and higher process temperatures (Sarkar *et al.*, 2012).

Other ethanol production processes which have garnered interest are consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF). CBP involves biomass conversion, cellulase production and fermentation in the one reactor (Binod *et al.*, 2010). For this process to work, an organism that can ferment cellulose to ethanol is needed. These microbes are generally not found in nature and have to be genetically engineered, although some do exist

(Olson *et al.*, 2012). The main microorganisms that are used in this process are bacterial strains such as *Clostridium phytofermentans* (Tolonen *et al.*, 2009) and *Clostridium japonicas* (Gardner *et al.*, 2010). In addition, researchers have also utilised genetically modified fungal strains to produce ethanol from cellulose such as *Trichoderma reesei* (Xu *et al.*, 2009) and *Trametes hirsuta* (Okamoto *et al.*, 2011). The main advantages of this technique is the cost of hydrolysis is lower as the expense of producing enzyme separately is eradicated, while the capital costs are also less, as only one reactor is needed. However, currently yields are low in comparison to the other techniques described previously (Xu *et al.*, 2009; Olson *et al.*, 2012). For instance, yields of 9.1, 4.3 and 3.0 g L^{-1} were obtained during fermentation of 20 g L^{-1} of starch, wheat bran and rice straw, respectively by *Trametes hirsuta*, where the highest ethanol yield of 89 % of the theoretical yield was obtained through the fermentation of starch (Okamoto *et al.*, 2011).

Simultaneous saccharification and co-fermentation (SSCF) is similar to SimSF in that saccharification and fermentation are carried out in the same vessel; however, in SSCF pentose sugars from the hydrolysate are also fermented. Yeast strains such as *Saccharomyces cerevisiae* TMB3400 (Öhgren *et al.*, 2006; Olofsson *et al.*, 2010) and *Saccharomyces cerevisiae* 424A (Jin *et al.*, 2012), which are genetically modified are generally used in SSCF as they can ferment pentose sugars as well as mono- and disaccharides. SSCF is typically considered to be superior to SHF, although it suffers from similar problems to SimSF, such as differences in optimum fermentation and saccharification temperatures in addition to inhibition of enzymes by ethanol, while poor xylose fermentation by the yeast is also a concern (Jin *et al.*, 2005). Öhgren *et al.* (2006), achieved up to 64 % conversion of the theoretical yield of glucose/xylose using this method, where 98 % of the theoretical yield of the glucose fraction was achieved.

1.5.3.2 Microorganism selection for ethanol fermentation

Traditionally, ethanol fermentation is carried out using yeast strains such as *Saccharomyces cerevisiae;* however, a variety of other yeasts and bacteria can also be used. There are many considerations to be taken into account when choosing a strain to ferment pretreated lignocellulosic material. Table 1.5 displays the characteristic of the most commonly used microorganisms in the production of lignocellulosic ethanol.

Table 1.5 Characteristics of a selection of microorganisms used in ethanol production from lignocellulosic biomass.

Adapted from Gírio *et al.* (2010)

Characteristics	*E. coli*	*Z. mobilis*	*S. cerevisiae*	*P. stipitis*
Glucose utilisation	+	+	+	+
Other hexose fermentation (e.g. galactose and mannose)	+	-	+	+
Pentose fermentation (xylose and arabinose)	+	-	-	+
Direct hemicellulose fermentation	-	-	-	W
Anaerobic fermentation	+	+	+	-
Mixed-product formation	+	W	W	W
High ethanol productivity	-	+	+	W
Ethanol tolerance	W	W	+	W
Tolerance to inhibiting products from lignocellulose	W	W	+	W
Osmotolerance	-	-	+	W
Acid pH range	-	-	+	W
GRAS microorganism	-	+	+	+

+: Positive, -: negative, W: weak.

S. cerevisiae is commonly used in cellulosic, starch, glucose and maltose based fermentations as these strains can ferment mono- and disaccharides efficiently, are generally regarded as safe (GRAS), are able to produce high quantities of ethanol, can tolerate inhibiting products from pretreatment/saccharification and have a high resistance to osmotic pressure (Gírio *et al.*, 2010). Furthermore, *S. cerevisiae* fermentation rate is not significantly reduced by ethanol concentrations under 10 % (v/v) (Casey *et al.*, 1986). The main drawback of using Saccharomyces for the fermentation of lignocellulosic hydrolysate; however, is its inability to ferment pentose sugars. Lignocellulosic materials can contain up to 50 % of pentose sugar, although generally they contain between 5-20 % (Graminha *et al.*, 2008). As a result, researchers have investigated the use of different strains and species including genetically modified organisms that can ferment pentose sugars in the hydrolysates. Xylose is the most abundant pentose sugar found in lignocellulosic material, therefore genetic engineering has centred on engineering pathways for fermentation of this sugar in microorganisms (Dien *et al.*, 2003).

Currently, the hemicellulosic fraction is generally being wasted in pilot plant fermentations, making bioethanol production from lignocellulosic hydrolysates an expensive process. Additionally, it is difficult for it to compete in the commercial gasoline market without subsidies or the conversion of the hemicellulosic fraction to

ethanol (Gírio *et al.*, 2010). Hinman *et al.* (1989), carried out a cost analysis and determined that utilisation of the hemicellulosic fraction of the fermentation could reduce the cost of ethanol by up to 25 %.

Xylose utilisation requires a pathway distinctly different to conventional glucose pathways. Anaerobic conditions are required in most yeast for xylose fermentation where cellular respiration is promoted resulting in poor ethanol yields (Lee, 1997). Microorganisms that have been used to replace *Saccharomyces* in ethanolic fermentations are *Pichia stipitis* and *Candida utilis* as they are able to ferment xylose to ethanol. However, ethanol yields from both these strains are up to five times lower than *S. cerevisiae* (Sarkar *et al.*, 2012), while they are also less tolerant to pH changes and lignocellulosic derived inhibitors (Hahn-Hagerdal *et al.*, 1994).

Escherichia coli are a species which posses several advantages for it to be considered for use in ethanol production including the capacity to ferment a wide range of substrates such as glucose, maltose and xylose. However, it suffers from low tolerance to ethanol and inhibitors and is only able to grow over a narrow pH range (Dien *et al.*, 2003). *Z. mobilis* is also a bacterium that can produce ethanol, yet similar to Saccharomyces it cannot ferment pentose sugars. This bacterium has higher specific ethanol production than *S. cerevisiae* as it produces less cell mass, although it can suffer from ethanol inhibition at concentrations as low as 2 % (w/v) (Gírio *et al.*, 2010).

As a result of the disadvantages outlined above, most research efforts have concentrated on strain development through genetic modification to obtain suitable strains for ethanol production (Dien *et al.*, 2003; Jeffries, 2006).

1.5.3.3 Bioreactor configuration for ethanol production

The production of ethanol is normally carried out using one of three different configurations; namely batch, fed-batch and continuous processes. The choice of fermentation is dependent on a number of factors such as the desired product, microbial growth and fermentation properties of the microorganism as well as process economics (Olsson *et al.*, 1996).

Batch fermentation was the traditional method for fermentation in which the process started with high concentrations of substrates and low concentrations of product, ending with high product formation and low substrate concentrations.

However, batch fermentations generally have low fermentation productivity due in part to product inhibition (Shama, 1988). Consequently, batch fermentation tends not to be used for ethanol production on an industrial scale.

In continuous fermentations, small concentrations of substrate are added to the reactor while the equivalent amount of converted nutrient solution is removed simultaneously. Various reactor configurations exist in continuous systems such as plug flow or continuous stirred tank reactors, which can be either in series or single (Shama, 1988). In a continuous process, the microorganism is cultivated in a low substrate environment with high ethanol concentrations, therefore the risk of contamination is relatively low. Continuous fermentations give higher productivities than batch fermentations; however, yields are usually lower as all the substrate in not consumed (Olsson *et al.*, 1996), whilst the yeast can lose their ability to synthesis ethanol over long periods of time (Sánchez *et al.*, 2008).

In fed-batch systems the substrate is fed throughout the fermentation, leading to ethanol being accumulated in the reactor. The microorganism is kept in a lower substrate environment thus leading to higher yields. For instance, yields of 0.41 g g^{-1} were obtained with fed-batch fermentation of xylose with *P. tannophilus* in comparison to 0.29 g g^{-1} with a batch process (Woods *et al.*, 1985). Furthermore, fermentation and conversion rates are also greater in fed-batch fermentations in comparison to batch. Lui *et al.* (2010) reduced the fermentation rate by 100 hours in comparison to a batch reactor for the production of ethanol from corncob residues, while the enzyme dosage decreased almost two-fold with an increase in conversion rate from 70.9 % to 85.2 %. Fed-batch is typically the most popular production method in ethanol fermentation as there are possibilities to achieve much higher productivities than with other methods (Sánchez *et al.*, 2008).

Inhibitors are a major concern in the fermentation process and can influence the type of reactor used in ethanol production. The amount of inhibitors depends on the method of pretreatment and the type of biomass that is used as the substrate. Inhibitors can come from lignin degradation in the form of aromatic rings and polyaromatic rings (Olsson *et al.*, 1996) or as furfural and 5-hydroxymethyl furfural which are released during prehydrolysis as a result of sugar degradation (Clark *et al.*, 1984). Additionally, lactic acid, formic acid and ethanol produced during fermentation can also act as inhibitory compounds (Maiorella *et al.*, 1983). There are numerous methods for the detoxification and removal of inhibitors from lignocellulosic

hydrolysates including activated charcoal, extraction with organic solvents, ion-exchange, overliming, steam stripping and molecular sieves. One of the most popular methods is overliming which involves adding a hydroxide such as $Ca(OH)_2$ until the liquor reaches pH 10. The resulting precipitate is removed and the pH is reduced to pH 6.5 using sulphuric acid (Olsson *et al.*, 1996). This method is unlikely to be used in a production scale facility however, as precipitation of calcium salts could foul distillation units and evaporators (Hahn-Hägerdal *et al.*, 2006).

The addition of substrate, at low rates as with continuous and fed-batch fermentations, aids in reduction of inhibitors as they are kept at low concentrations (Palmqvist *et al.*, 2000a), thus reducing the need for detoxification prior to fermentation. For instance, Taherzadeh *et al.* (1999b) determined that when comparing fermentations of spruce and birch wood hydrolysates to ethanol, the yeast strain was strongly inhibited in batch fermentations. As a consequence, batch fermentation was not possible, whereas in fed-batch fermentation the glucose and mannose fraction were completely fermented.

High cell density is another method that can help reduce the effect of inhibitors while also increasing productivity (Olsson *et al.*, 1996). High cell density is usually achieved through immobilisation of the microorganism on polymeric matrices or by cell recycling. Furthermore, high cell density can also increase the tolerance to high substrate concentration and products (Yao *et al.*, 2011b).

1.5.4 Natural Antioxidants

Antioxidants are defined as any substance that, when present at low concentrations, delays, prevents or removes oxidative damage to the target molecule (Gutteridge *et al.*, 2010). Oxidation is the term used to describe a chemical reaction where electrons or hydrogen is transferred to the oxidising agent from another substance which can produce free radicals and start chain reactions, resulting in cell death. Free radicals are produced during the natural metabolism of aerobic cells in the form of reactive oxygen and nitrogen species (ROS/RNS). However, overproduction of free radicals or loss of antioxidant defences can result in the free radicals oxidising lipids, DNA and proteins causing cell damage (Valko *et al.*, 2007). Antioxidants act by terminating free radical intermediates and other oxidation reactions, reducing the damage to the cells and cellular functions (Sies, 1997). Proteins in particular are exposed to oxidative damage

as a result of their structural flexibility and reactive amino acid residues (Stadtman *et al.*, 2003).

Fungi have been shown to possess health related benefits such as antioxidant antitumor, antimicrobial, antiallergic and hypoglycaemic properties (Palacios *et al.*, 2011). The main fungi known to produce natural antioxidants are wild mushrooms such as *Pleurotus ostreatus, Agaricus blazei, Lentinula edodes* and *Volvariella volvacae*. These fungi offer a low cost natural alternative to some chemical antioxidants like butylated hydroxytoluene (BHT), which has been shown to be a suspected tumour promoter (Witschi, 1984).

1.5.4.1 Free radicals

Free radicals are defined as any atom or molecule possessing unpaired electrons in their outer orbit (Halliwell *et al.*, 2003). Free radicals derived from molecular oxygen, also known as reactive oxygen species (ROS), are known to be extremely reactive and represent the most important class of radicals in living organisms (Miller *et al.*, 1990). The most common free radicals in oxidative stress are superoxide anion, alkoxyl, peroxyl and hydroxyl radicals as well as sulphur and nitrogen centred radicals. Mitochondria are the main source of ROS; however, they are also the first target as the ROS has access to membrane lipids which are prone to attack by free radicals (Ferreira *et al.*, 2009).

Superoxide radicals which are formed by the addition of one electron to molecular oxygen is produced by a number of biological reactions. It can be generated either from membrane-associated NADPH oxidase or through leakage from mitochondrial electron transport (Burnaugh *et al.*, 2007). They do not directly initiate lipid peroxidation or damage DNA; however, they serve as a precursor to reactive species such as hydroxyl radicals, where superoxide is dismutated to hydrogen peroxide by superoxide dismutase which, can in turn be converted to hydroxyl radical by Fe^{2+} (Jayakumar *et al.*, 2011). Hydroxyl radicals, although having a very short lifetime, are the most reactive of the ROS and can induce severe damage to surrounding biomolecules (Gutteridge, 1984). They are responsible for damage to DNA, purines and pyrimidines, as well as to the structure of deoxyribose (Halliwell *et al.*, 1990). Peroxyl radicals are formed as a result of lipid peroxidation, where ROS attack lipids and extracts the hydrogen atom from the polyunsaturated lipid chain. These in turn can react with other lipids to form lipid hydroperoxides, which are

subsequently broken down to form lipid radicals, thus initiating a chain reaction (Valko *et al.*, 2007; Ferreira *et al.*, 2009). Reactive nitrogen species are also responsible for oxidation although not to the same extent as ROS. The most common RNS is nitric oxide, which is formed via the metabolisation of arginine to citrulle by nitric oxide synthases (Ghafourifar *et al.*, 2005). Nitric oxide reacts with superoxide anion to form peroxynitrite which can cause damage to DNA, proteins and lipids (Fang *et al.*, 2003). Nitric oxide has been shown to play a significant role as a signalling molecule in a variety of physiological processes such as neurotransmission, blood pressure regulation, defence mechanisms and immune regulation (Bergendi *et al.*, 1999).

1.5.4.2 Antioxidants from natural sources

Typically, organisms are sufficiently protected against oxidation by free radicals. However, when the mechanism of antioxidant protection becomes unbalanced due to ageing amongst other factors, degeneration of proper physiological functioning can occur. This can result in diseases such as cancer, cardiovascular disease, immune system decline, membrane protein damage and DNA mutation (Mau *et al.*, 2001; Halliwell *et al.*, 2003). Oxidation is an essential method of producing energy for biological functions in many living organisms; however, the uncontrolled production of free radicals derived from oxygen in the human body can cause oxidation of biomolecules, which can lead to cell and tissue damage (Elmastas *et al.*, 2007). There is evidence to suggest that oxidative stress plays a role in numerous disorders and diseases, which has led to significant attention from researchers in antioxidants (Papas, 1999).

Natural antioxidants can be found in many organic materials such as vegetables, fruits, cereals and spices, whilst synthetic antioxidants also exist. The two most popular synthetic antioxidants are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Moure *et al.*, 2001). These synthetic antioxidants have been used as stabilisers in food and for preservation to reduce rancidity. However, over the last 10 years contradictory research has indicated that these antioxidants are both carcinogenic as well as anticarcinogenic, while they have also been shown to cause liver damage (Sherwin *et al.*, 1990) and promote tumour formation (Botterweck *et al.*, 2000). As a result, the use of these chemicals as antioxidants has been restricted (Xiu-Qin *et al.*, 2009), with focus shifting to natural additives as a source of antioxidants

(Jayakumar *et al.*, 2011). Nutraceutical and cosmetic companies have lead the way, as natural antioxidants can be produced cheaply and sold at a high commercial value.

Antioxidants generally consist of proteins, enzymes and other small molecules. They can be classified into a number of categories depending on their mode of action, namely preventing, scavenging and repair antioxidants (Niki *et al.*, 1995). The roles of preventative antioxidants are to suppress the formation of reactive oxygen and nitrogen species (ROS/RNS) and act as the first line of defence. For example, these antioxidants can reduce hydrogen peroxide and lipid hydroperoxides to water and lipid hydroxides, respectively. Scavenging antioxidants remove the active species before they can attack essential molecules. Examples of common scavenging antioxidants are superoxide dismutase and β-carotene. These scavenger molecules function by donating hydrogen atoms or electrons as well as transferring protons to the oxidant to form stable compounds. Finally, repair antioxidants are a class of enzymes that act through repair mechanisms clearing waste in the cells, while adaption mechanisms deliver the enzymes to the right location at the correct concentrations (Niki, 2010). When studying antioxidants, one must be aware of the reductive side of the equation as antioxidants have to go somewhere after they have carried out their function. For example, antioxidants can get oxidised while acting as an antioxidant and become damaged (Gutteridge *et al.*, 2010).

Common antioxidants found in foods include: superoxide dismutase, vitamin E (α-tocopherol), vitamin C (ascorbic acid), β-carotene, phenolic compounds and flavonoids. Phenolics are aromatic hydroxylated compounds possessing one or more aromatic rings with one or more hydroxyl groups (Ribeiro *et al.*, 2007). Phenolics can be sub-categorised as flavonoids, phenolic acids, lignins, tannins, stilbenes and oxidised polyphenols (Ferreira *et al.*, 2009). These molecules can function by scavenging radicals, chelating metal ions or modulating enzyme activity, while they also affect the signal transduction activity of transcription factor and gene expression (Srinivasan *et al.*, 2005). The effectiveness of the phenolic compounds is dependent on the involvement of the phenolic hydrogen in the reaction, the stability of the antioxidant radical that is formed during the reaction and the chemical substitutions present on the structure (Hall, 2001). Meanwhile, substitutions on the structure are thought to be the most significant with regard to the ability of the antioxidant to control the radical reaction (Ferreira *et al.*, 2009). Flavonoids are a group of phenolic antioxidants that can be found in many fruits, vegetables, plants and nuts. They

usually consist of two aromatic rings which are linked together by a three carbon chain bridge (C-ring) (Iwashina, 2000). Flavonoids have been demonstrated as good scavengers of a variety of oxidising molecules (Wright *et al.*, 2001).

Carotenoids are potent radical scavengers. They are usually 40 carbons in length and can consist of hydrocarbons with oxygenated functional groups that act in two ways; by quenching the highly reactive free radical oxygen singlet and by interfering with radical-initiated reactions, particularly with regard to those which result in lipid peroxidation (Krinsky, 1998). The reactivity of carotenoids depends on the chain length of the conjugated double bonds, as well as characteristics of the end groups (Ferreira *et al.*, 2009). Carotenoids are abundant in nature and can be commonly found in mushrooms and vegetables.

Vitamin C functions by reacting directly with oxy- or peroxy radicals, preventing damage to the cell (Beyer, 1994), while it can also act as a chain-breaking antioxidant in lipid peroxidation (Doba *et al.*, 1985). Additionally, ascorbic acid is known to have a role in the inhibition of mutagenesis and carcinogenesis, regulation of aging, inhibition of exercise injury and protection of the brain. Free radicals tend to act in chain reactions, for example one compound with an unpaired electron will react with another which results in the other compound having an unpaired electron. Chain-breaking antioxidants like ascorbic acid function by interacting with free radicals to break the chain and subsequently interact with a water soluble compound to regenerate if possible (Sies, 1997). Not all chain-breaking antioxidants can regenerate; although, it is a desirable feature. Ascorbic acid is also known to interact with vitamin E to produce synergistic antioxidant reactions. Vitamin E scavenges free radicals and as a result is converted to a vitamin E radical which can attack polyunsaturated lipids. However, vitamin C can reduce vitamin E radical to form vitamin E before the radical can attack the lipids (Niki, 2010).

Vitamin E is a group of chemical compounds, consisting of four tocopherols and four tocotrienols (α, β, γ, δ), which possess a similar chemical structures, namely a chromanol head and an isoprene side chain (Heleno *et al.*, 2010). Vitamin E's antioxidant activity is associated with the redox properties of the chroman ring, which can react with peroxyl radicals such as those generated in polyunsaturated fatty acids and in membrane phospholipids (Sies *et al.*, 1995). It functions as a radical scavenger and a chain breaker and is thought to have an active role in the prevention of cancer by facilitating immunocompetence, membrane and DNA repair and reduction of DNA

damage via oxidation (Kimmick *et al.*, 1997; Blokhina *et al.*, 2003). It is widely believed that α-tocopherol is a lipid-phase antioxidant, where it functions by reducing peroxyl radicals from polyunsaturated fatty acids in phospholipids in membranes or lipoproteins. Tocopherols interact with the lipid peroxyl radicals to form tocopheroxyl radicals which interrupt the radical chain reaction (Sies *et al.*, 1995).

The efficiency of an antioxidant to scavenge radicals can be affected by the fluidity of the local environment and the mobility of the antioxidant. For instance, vitamin E has a long side chain which is essential for incorporation and retainment of the radicals; however, this side chain also reduces its mobility between the membrane and the lipoprotein thus reducing its capacity (Niki, 2010).

1.5.4.3 Detection of Antioxidants

Detection of antioxidants and determination of the antioxidant capacity is evaluated using a number of methods. There is no one standardised method as antioxidants efficacy is measured by their potential to scavenge radicals, sequester metal ions and decompose peroxides, with each antioxidant having a different capacity for each. For example, α-tocopherol has good radical scavenging activity but it is a poor antioxidant against lipid peroxidation. In contrast, carotenes are weak radical scavenging antioxidants but good inhibitors of oxidation by singlet oxygen (Niki *et al.*, 2000). The majority of chemical tests available are based on the ability of the antioxidant to scavenge radicals, with few methods focusing on the chelation ability and the reducing power (Mau *et al.*, 2001; Moure *et al.*, 2001). Different diagnostic methods are used to determine antioxidant activity including the measurement of total oxidative DNA damage, level of antioxidant enzymes, low molecular weight molecules and vitamins and oxidative damage to lipids or protein damage (Aruoma, 1997).

Additionally, some antioxidants can have quite a different capacity *in vitro* in comparison to *in vivo* as antioxidants may not be retained, absorbed or distributed properly, while some others can undergo biotransformation due to enzymatic conjugation with sulphate, methyl and glucuronide groups (Terao, 2009). For instance, the potent antioxidant 2,2,5,7,8-pentamethyl-6-chromanol is effective against lipid peroxidation of membranes and oxidative modification of LDL but its efficacy *in vivo* is quite limited as it is rapidly excreted (Niki *et al.*, 2003). Currently, it is very difficult to determine whether antioxidants are effective as there is no reliable method for detection *in vivo* (Niki, 2010).

1.5.4.4 Antioxidants in fungi

Fungal fruiting bodies and mycelia are known for their chemical and nutritional properties as well as their flavour and texture (Elmastas *et al.*, 2007). They are considered a therapeutic food and are useful in the prevention of diseases such as hypertension, hypercholesterolemia and cancer (Manzi *et al.*, 2001). Other observed medicinal properties of fungi include reduction of blood cholesterol, prevention or alleviation of heart disease, antitumour, antiviral and antithrombotic effects, all of which have been linked to their antioxidant properties (Jayakumar *et al.*, 2011). The most common mushrooms that produce antioxidants include *Agaricus bisporus*, *Pluerotus ostreatus* (oyster mushroom), *Volvariella volvacae* (paddy straw mushroom) and *Lentinula edodes* (shiitake) (Yang *et al.*, 2002). A wide variety of fungi have been reported to possess antioxidant potential which is mainly associated with the phenolic compounds present. A review of associated literature reveals that the main antioxidant compounds found in fungi include phenolic compounds such as flavonoids and phenolic acids; however, other antioxidants such as tocopherols, carotenoids and ascorbic acid are also produced, but in lower concentrations (Ferreira *et al.*, 2009). Over 8000 naturally occurring phenolic compounds are known to exist in nature. These phenolic compounds consist of at least one aromatic ring with one or more hydroxyl groups attached. Natural phenolic compounds accumulate as end products of the shikimate and acetate pathways (Apak *et al.*, 2007). The most commonly found phenolic compounds in fungi are ρ-hydroxybenzoic, protocatechuic, gallic, gentisic, caffeic, cinnamic and ρ-coumaric acids. Phenolic acids can be divided into two sub-groups, namely the hydroxybenzoic acids and the hydroxycinnamic acids, which are derived from benzoic and cinnamic acid, respectively (Ferreira *et al.*, 2009). Hydroxybenzoic acid derivatives, which are typically found in the bound form are part of complex components such as lignins and hydrolysable tannins. They can also be found as part of sugars and organic acids in plants (Liu, 2004). Hydroxycinnamic acid derivatives are generally present in the bound form, where they are found linked to cell-wall structural components such as lignin, cellulose and associated organic acids (Ferreira *et al.*, 2009).

Fungi are an attractive organism for the production of antioxidants as they can be produced on low cost lignocellulosic wastes or in liquid medium. Furthermore,

both fermentation strategies can be optimised to produce higher quantities of the antioxidant of interest (Ferreira *et al.*, 2009).

1.6 Applications of lignocellulosic waste

Due to the abundance of lignocellulosic waste produced every year in numerous industrial and agricultural processes, methods need to be developed to utilise these materials which are environmentally friendly and economically sustainable. In this thesis, a number of potential applications for lignocellulosic materials will be investigated to determine the viability of these processes. In particular this thesis will focus on the production of hydrolytic enzymes from solid state fermentation of these materials, which offers a viable alternative to the production of enzymes through submerged fermentation. Additionally, the biological pretreatment of lignocellulosic waste materials will be investigated as a method of removing the lignin from the lignocellulose, thus making the cellulose fraction available for enzymatic hydrolysis and subsequent ethanol production. The potential of improving the nutritional value of these agricultural materials as forage feeds through the pretreatment with white rot fungi will also be investigated, with special attention paid to the improvement in the digestibility of the forages as well as the potential to increase the protein content. Finally, the potential for producing antioxidants from these lignocellulosic materials through SSF will also be examined, in which the antioxidant properties of the liquid extracts from SSF will be assessed.

1.7 Project objectives:

a) To screen a selection of fungi for the production of cellulosic and hemicellulosic enzymes.

b) To optimise cellulase and xylanase production from the selected strains through substrate manipulation and altering culture conditions.

c) To determine the stability of cellulase and xylanase enzymes produced from strains of Aspergillus fungi.

d) To investigate the degradation profile of lignocellulosic wastes by white rot fungi.

e) To assess the efficacy of using pretreated lignocellulosic wastes as an upgraded animal feed.

f) Screening of selected yeast strains to achieve maximal ethanol production from corn mash.

g) Substitute biologically pretreated lignocellulosic biomass into corn mash fermentations to assess the impact on ethanol production.

h) To investigate the production of ethanol from biologically pretreated lignocellulosic wastes.

i) Determination of antioxidant production by white rot fungi on lignocellulosic wastes.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, solvents and other reagents

All chemicals used were of a molecular biology grade or higher where appropriate.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid**),** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)$^{+}$, acetic acid, acetone, acetonitrile, agar, α-amylase, α-tocopherol, ammonium dihydrogen phosphate, ammonium phosphate, ammonium tartrate, ammonium sulphate, β-carotene, butylated hydroxytoluene (BHT), carboxymethyl cellulose (CMC), cellobiose, cellulase from *Trichoderma viride*, cetyltrimethylammonium bromide (CTAB), chloramphenicol, chloroform, citric acid, copper sulphate anhydrous, corn starch, diethyl ether, dinitrosalicyclic acid (DNS), di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH), ethylenediaminetetraacetic acid (EDTA), ethanol, folin-ciocalteau reagent, ferric chloride, galactose, gallic acid, glucose, glycine, glycerol, guaiacol, haemoglobin, hydrochloric acid, hydrogen peroxide, iodine, laccase, lactic acid, lignin peroxidase, linoleic acid, magnesium chloride, magnesium sulphate heptahydrate, malt extract, maltose, maltotriose, manganese peroxidase, manganese oxide, manganese, mannose, methanol, methyl isobutyl ketone (MIBK), methyl red indicator, mineral oil, peptone, phenol, potassium chloride, potassium ferricyanide, potassium hydrogen phthalate, potassium iodide, potassium sodium tartrate, potassium sulphate, potato dextrose agar (PDA), skimmed milk, sodium acetate, sodium borate decahydrate, sodium hydrogen carbonate, sodium hydroxide, sodium lauryl sulphate, sodium phosphate di-sodium salt, sodium phosphate mono-sodium salt, sodium sulphate, sucrose, sulphuric acid, tributyl citrate, trichloroactetic acid, triethylene glycol, trypitic soya broth, trisodium citrate dihydrate, trolox, TWEEN 20, urea, xylan from birchwood, xylose and veratryl alcohol were all supplied by Sigma-Aldrich, Tallaght, Ireland.

Strains of fungi for initial screening were supplied by Alltech Bioscience Centre Dunboyne, Co. Meath, Ireland.

Phanerochaete chrysosporium 1547, 1556, 6909, *Pleurotus eryngii* 9619, *Trametes versicolor* 3086, *Phlebia radiata* 5111, *Pleurotus ostreatus* 1833, *Pleurotus salmoneostramineus* 5338, *Pleurotus pulmonarius* 9558 and *Candida utilis* 70167 were supplied by DSMZ GmbH, Inhoffenstraße 7 B, 38124 Braunschweig, Germany.

Pleurotus ostreatus 32783, *P. chrysosporium* 24725 and 32629, *Hericium erinaceus* 52487 and *Lentinula edodes* 66784 were supplied by American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108, U.S.A.

Saccharomyces cerevisiae 72, 358, 88 and 1026 were supplied by the National Collection of Yeast Cultures, Norwich, United Kingdom.

Pichia jardinii 621 and *Pichia thermotolerans* 7012 were supplied by Central Bureau for Fungal Cultures, Utrecht, Netherlands.

Saccharomyces cerevisiae 9846, 7044 and 10676 were obtained from Alltech, Sao Pedro, Brazil.

The yeast strains, Lab 1, Lab 2 and *Pleurotus ostreatus* Oyrm 1 were obtained from Alltech Inc., Nicolasville, Kentucky, U.S.A.

Oat straw was sourced from Alltech Bioscience Centre, Dunboyne, Co. Meath, Ireland.

Becton Dickinson (BD) Difco yeast extract, yeast malt (YM) broth, skim milk and yeast peptone dextrose (YPD) broth were supplied by Unitech, Citywest, Dublin, Ireland.

Ground corn was supplied by Cooley Distilleries Riverstown, Co. Louth, Ireland.

Corn stems, leaves and rumen fluid was supplied by Teagasc Research Centre, Grange, Dunsany, Co. Meath.

Lodex 5 maltodextrin starch was supplied by Cargill, Haubourdin, France.

Switchgrass, corncobs, cottonseed hulls and coconut fibre were obtained from Alltech Inc., Nicholasville, Kentucky, U.S.A.

Spezyme® α-amylase and Distillase L 400® glucoamylase solutions were obtained from Alltech Inc., Nicholasville, Kentucky U.S.A.

2.2 Methods

2.2.1 Growth and maintenance of filamentous fungi

2.2.1.1 Storage of fungal strains

Fresh spores of fungi were prepared by spreading a spore suspension on plates of potato dextrose agar. The plates were subsequently incubated at 30 °C for 3-6 days depending on the strain. Following this, they were harvested by washing with 10 mL of 0.05 % (w/v) TWEEN 20 and stored at 4 °C for up to 1 year. For long term storage, 1 mL of this spore suspension was mixed with 9 mL of 20 % (w/v) skimmed milk. Aliquots (1 mL) of this mixture were then transferred into glass vials (Wheaton, New Jersey, U.S.A.) and lyophilized using a Virtis freezemobile 25XL freeze-drier (Virtis, Ipswich, Suffolk, U.K.) (Smith *et al.*, 1994).

2.2.1.2 Liquid culture and growth conditions

Fungal strains were cultivated in a liquid seed medium at 30 °C and 200 rpm for 3 days in a shaking incubator (Weiss-Gallenkamp, Loughborough, U.K.). Each seed medium consisted of 60 g L^{-1} corn starch, 5 g L^{-1} glucose, 18 g L^{-1} peptone, 1.5 g L^{-1} magnesium sulphate heptahydrate, 0.5 g L^{-1} potassium chloride and 1 g L^{-1} potassium phosphate monobasic. The medium was sterilised at 121 °C for 20 minutes and allowed to cool prior to inoculation with the appropriate fungal strain.

2.2.1.3 Solid state fermentation and growth conditions for enzyme production

Corncobs (20 g) were weighed to a 250 mL Erlenmeyer flask, sealed with Bioshield® sterilisation wrap and sterilised at 105 °C for 30 minutes. The flasks were inoculated with 12 mL of a dilute liquid seed culture and subsequently incubated for 3-6 days (strain dependent) in a humidity chamber (Sheldon Manufacturing Inc., Oregon, U.S.A.) at 30 °C and 80 % relative humidity (RH).

2.2.1.4 Harvesting of flasks and preparation of enzymatic preparations

Fungal koji obtained, as outlined in Section 2.2.1.3 was transferred to a Ziploc bag and lyophilised. Following this, 5 g of dry koji was weighed to a sterlin and extracted with 30 mL of H_2O on a rotary shaker (IKA KS260 Basic) at 300 rpm for 1 hour. The extract was filtered through muslin cloth and subsequently centrifuged (MPW 350R, MPW Medical Instruments, Warsaw, Poland) at 6000 rpm for 5

minutes. The supernatant was collected, filtered through a 0.45 µm chromafil filter unit (Lab Unlimited, Dublin) and analysed for extracellular enzymatic activity (Section 2.2.2). Samples were stored at 4 °C overnight, although for longer term storage, samples were kept at -20 °C.

2.2.2 Analysis of fungal extracts for enzyme activity

2.2.2.1 Determination of xylanase activity

Xylanase activity of the crude enzyme preparation (Section 2.2.1.4) was determined using a modified version of the assay described by Bailey and Poutanen (1989). A 1.8 mL aliquot of 2 % (w/v) xylan from birchwood in 50 mM sodium citrate pH 5.3 was hydrolysed by 0.2 mL of xylanase extract at 50 °C. After exactly 5 minutes, 3 mL of 1 % (w/v) dinitrosalicyclic acid (DNS) solution was added to each tube to terminate the reaction. The samples were subsequently removed and boiled for 5 minutes, then placed in an ice bath to cool. The absorbance of each sample was read at λ_{540nm} on a UV visible-spectrophotometer (Simadzu UV 1601PC). Samples were measured against a blank, in which 3 mL of DNS was added to 0.2 mL of enzyme sample and mixed prior to the addition of 1.8 mL of substrate. A standard curve was constructed using known concentrations of xylose from which unknown xylanase activities were calculated. One xylanase unit was determined as the amount of enzyme required to liberate 1 µmole of xylose per minute under assay conditions.

2.2.2.2 Determination of carboxymethylcellulase activity

Carboxymethylcellulase (CMCase) activity was determined using a modified version of the assay described by Miller *et al.* (1960). Carboxymethylcellulose (CMC) substrate was prepared in 0.05 M sodium acetate at pH 4.8. Aliquots (300 µL) were hydrolysed by 300 µL of enzyme solution, which was diluted appropriately in 50 mM sodium acetate buffer. Samples were incubated at 50 °C for 5 minutes, following which 900 µL of 1 % (w/v) DNS was added to each tube to terminate the reaction. These sample tubes were incubated at 100 °C for 5 minutes, cooled in an ice bath with the absorbance subsequently being determined using a UV visible spectrophotometer at λ_{540nm}. Samples were measured against a blank, where DNS was added to the enzyme mixture prior to the addition of the substrate. A standard curve was constructed using glucose to determine the sample's carboxymethylcellulase activity. One CMC unit of activity liberates one µmole of

reducing sugar (expressed as glucose equivalents) in one minute under the assay conditions outlined.

2.2.2.3 Determination of proteolytic activity

Proteolytic activity was determined using method previously described by the Assembly of Life Sciences (1981). Aliquots (5 mL) of 2 % (w/v) haemoglobin in 0.5 M glycine-HCl buffer pH 2.5 were hydrolysed by 1 mL of dilute enzyme solution for 30 minutes at 40 °C. Samples were mixed intermittently throughout the incubation. After 30 minutes elapsed, 5 mL of 14 % (w/v) TCA was added to each tube to stop the reaction. The sample tubes were removed from the water bath and incubated at room temperature for 1 hour, mixing frequently throughout to allow colour development. Following incubation, the samples were filtered through Whatman #42 filter paper and the absorbance was determined at λ_{275nm} on a UV - visible spectrophotometer using quartz cuvettes. Samples were compared to blanks, where TCA was added to the enzyme solution prior to the addition of haemoglobin to denature the enzyme. Subsequently the samples were appropriately diluted to obtain a range of between 0.2 and 0.5 absorbance units for each sample. One HUT unit is the amount of enzyme that produces a hydrolysate with the same absorbance at λ_{275nm} as a solution containing 1.10 µg ml^{-1} tyrosine in 5 mN HCl. Absorbance of tyrosine is a known characteristic, therefore there was no need to develop a calibration curve.

2.2.3 Characterisation of crude enzymatic extracts

2.2.3.1 The effects of pH and temperature on cellulase and xylanase stability

The effect of pH and temperature on crude cellulase and xylanase extracts from *A. niger* 102.12 and *A. oryzae* 553.65 was determined. Enzyme extracts were incubated at temperatures between 24-60 °C over a defined pH range. The buffers used to acquire each specific pH were: 50 mM sodium citrate (pH 3.1 to 6.1) and 50 mM sodium phosphate pH (6 to 8). Samples (2 mL) were taken intermittently for 50 hours and assayed for xylanase and cellulase activity as outlined in Sections 2.2.2.1 and 2.2.2.2.

2.2.3.2 Analysis of pH optimum of cellulase and xylanase enzymes under assay conditions

Optimum pH was determined by quantifying xylanase and cellulase activity at pH values between pH 2-8. The buffers used to facilitate the required pH range were: 50 mM Gly-HCl (pH 2 to 3), 50 mM citrate buffer (pH 3 to 6) and 50 mM phosphate buffer (pH 5.7 to 8). Enzyme activity was quantified as detailed in Sections 2.2.2.1 and 2.2.2.2. CMC and xylan substrates used in each enzyme assay were prepared using the buffer with appropriate pH.

2.2.3.3 Analysis of the effects of temperature on cellulase and xylanase activity under assay conditions

The effect of temperature on the detection of cellulase and xylanase activity from *A. niger* 102.12 and *A. oryzae* 553.65 was determined by assaying enzyme activity at different temperatures using the assays procedures described previously in Sections 2.2.2.1 and 2.2.2.2. The temperatures tested ranged from 24 - 90 °C to determine the optimum assay temperature.

2.2.4 Evaluation of fermentation conditions on enzyme activity

2.2.4.1 Carbon and nitrogen supplementation

Liquid seed media (Section 2.2.1) was inoculated with the strains *A. niger* 102.12 and *A. oryzae* 553.65 and incubated for 3 days at 30 ^{o}C and 200 rpm. Each culture was diluted appropriately with solutions of respective carbon and nitrogen sources prior to inoculation. Corncobs (20 g) were subsequently inoculated with 14 mL of supplemented culture and incubated at 30 °C and 80 % RH for 5 days. The supplemented cultures were inoculated at a final concentration of between 0.5, 1 and 5 % (v/v). The carbon sources used were glucose, xylose, maltose and sucrose, while the nitrogen sources were peptone, urea, ammonium dihydrogen phosphate and ammonium sulphate. Samples were analysed for cellulase and xylanase activity using the assay procedures outlined in Sections 2.2.2.1 and 2.2.2.2.

2.2.4.2 Initial moisture content

To determine the effect of moisture on enzyme activity, flasks were inoculated with a range of different inoculum volumes of dilute seed medium (10-20 mL) per 20 g of substrate. Each inoculum contained yeast extract at a 5 % (w/v) final concentration

in the fermentation flask. The flasks were incubated and harvested as described in Sections 2.2.1.3 and 2.2.1.4 and subsequently analysed for cellulase and xylanase activity using the methods outlined in Sections 2.2.2.1 and 2.2.2.2.

2.2.4.3 Incubation time

Erlenmeyer flasks of corncobs were inoculated with the fungal strains *A. niger* 102.12 and *A. oryzae* 553.65 and propagated as detailed in Section 2.2.1.2. Each inoculum contained yeast extract at a final concentration of 5 % (w/v). Subsequently, triplicate flasks were incubated at 30 ^{o}C and 80 % RH for between 2-7 days, with flasks harvested every 24 hours. The fermentation extracts were subsequently prepared and analysed for extracellular cellulase and xylanase activity (Sections 2.2.2.1 and 2.2.2.2).

2.2.4.4 Effect of fermentation temperature on cellulase and xylanase production

Liquid seed culture was prepared with the fungal strains *A. niger* 102.12 and *A. oryzae* 553.65 (Section 2.2.1.2). Following this, the cultures were suitably diluted with dH_2O and yeast extract to obtain a final concentration of 5 % yeast extract in each sterile corncob fermentation. Each set of triplicate flasks were incubated at temperatures of between 25 and 40 °C for five days and subsequently harvested. Afterwards, the cell free extracts from each flask were analysed for cellulase and xylanase activity (Sections 2.2.2.1 and 2.2.2.2).

2.2.5 Solid state fermentation of lignocellulosic materials with a selection of white rot fungi

2.2.5.1 Fungal maintenance and storage

A loop of mycelium from the selected strain of white rot fungi was transferred from stock slants of fungal strains to yeast malt (YM) agar plates. Plates were subsequently incubated at 30 ^{o}C for 5 - 7 days following which, a loop of the sub-cultured mycelia was transferred to freshly prepared sterile YM slants and incubated for a further 5 to 7 days. These slants were suitable for storage for up to 3 months. For longer term storage, sterile high purity mineral oil was decanted to each slant to a depth of 1 cm above the uppermost level of the medium (Buell *et al.*, 1947). These slants were suitable for storage for up to one year.

2.2.5.2 Propagation and solid state fermentation of lignocellulosic material

In total, 10 lignocellulosic materials were fermented including corncobs, oat straw, switchgrass, corn leaves, upper and lower stem from corn stalks, cottonseed hulls, pine wood shavings, a cottonseed hulls and coconut fibre mixture, coconut fibre and ash chippings.

YM medium (200 mL) was inoculated with a plug of mycelia from a slant of either *Trametes versicolor* 3086 or *Pleurotus ostreatus* Oyrm 1 and incubated for 5 days at 30 ^{o}C and 200 rpm. The flasks were subsequently harvested and diluted with an appropriate volume of sterile H_2O. Corncob (200 g) and cottonseed hulls (30 g) at a ratio of 7 mL culture to 10 g of material were inoculated with this culture, sealed with sterilisation wraps (Kimberly-Clark, Roswell, U.S.A) and incubated at room temperature (~24 °C) for up to 240 days.

Switchgrass and oat straw were soaked in cool dH_2O for 45 minutes to wet the material. Excess water was removed and the moisture content of the material was determined using a moisture analyser (Sartorius MA 150). Aliquots (500 g) of this material were separated into sterile polypropylene bags (Unicorn Bags, Garland, Texas), which contain a membrane that permit gas exchange and were subsequently autoclaved at 121 °C for 20 minutes. Mycelia from plates prepared previously (Section 2.2.4.1) were harvested and used to inoculate the sterile bags of lignocellulosic material. These bags were sealed and incubated at 24 °C for 210 days.

Moist lower and upper corn stems and corn leaves (250 g) were transferred to Unicorn fermentation bags and sterilised at 105 ^{o}C for 30 minutes. Samples were taken from each bag and the moisture content was determined. These bags were inoculated with plugs of mycelia from a plate of appropriate fungal material, sealed and incubated at 24 °C for 240 days.

Blocks of dried coconut fibre (~500 g) were moistened with 3 litres of dH_2O and allowed to steep for 45 minutes, with the excess water being removed by draining. An aliquot (1 kg) of material was transferred to separate fermentation bags, sterilised at 105 °C for 1 hour and inoculated with plugs of mycelia from agar plates. These bags were subsequently sealed and incubated at 24 °C for up to 210 days.

Three blocks of dried coconut fibre (500 g) were mixed with 9 litres of dH_2O and 2.1 kg of cottonseed hulls and mixed to obtain a uniform substrate. The material was allowed to steep for 45 minutes, the excess water was removed and the material (500 g) was weighed to bags for sterilisation and inoculation as described previously.

Pine wood shavings (500 g) were mixed with 1 litre of dH_2O water. The material was left to steep for 45 minutes and drained to remove excess water. An aliquot (500 g) was weighed to each bag and inoculated as previously described.

Each bag was incubated at 24 ^oC for a period of 210-240 days. Samples were taken each month for compositional analysis, ethanol fermentation and antioxidant determination.

2.2.5.3 Secondary solid state fermentation of fermented lignocellulosic material

Corncobs from Section 2.2.4.2 which were pretreated for 120 days were freeze-dried and ground to pass through a 2 mm sieve. Ground material (20 g) was weighed to each flask and inoculated with a liquid culture (Section 2.2.1.2). A 1:5 dilution of each culture was prepared with a yeast extract solution to give a 5 % (w/v) final concentration in SSF flasks. Flasks were incubated at 30 ^oC and 80 % RH for between 3-5 days (depending on the Aspergillus strain) and harvested as per Section 2.2.1.4.

2.2.6 Yeast propagation, ethanol fermentation and sample analysis

2.2.6.1 Yeast propagation, maintenance and storage

Yeast cultures were cultivated on YPD agar which consisted of 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} dextrose and incubated for 1-2 days at 30 ^oC. Single colonies were picked from these plates and transferred to YPD slants containing chloramphenicol and incubated at 30 ^oC for 1 to 2 days. The slants can be stored at 4 °C for up 2 months. For longer term storage YPD broth was inoculated with a single colony of yeast and incubated at 30 ^oC at 200 rpm for 16 hours. Aliquots of this culture (200 µL) were re-suspended in 40 % (v/v) glycerol (800 µL) and stored at -75 ^oC (Sherman, 1986).

2.2.6.2 Corn mash preparation

A 20 % (w/v) corn mash medium was prepared by adding coarsely ground corn to an appropriate volume of water, allowing for the initial moisture content of the corn.

The mash was heated to 85 ^{o}C and 0.06 % (w/v) of α-amylase (Spezyme®) was added per weight of grain. The mash was stirred for 20 minutes at this temperature and autoclaved at 121 ^{o}C for 20 minutes to simulate a jet cooker. Each Belco jar was removed, allowed to cool to 85 ^{o}C and 0.04 % (w/v) of α-amylase was added to the mixture. This mixture was stirred for one hour and allowed cool before 1 g L^{-1} of urea was added. A portion (250 mL) was transferred to an Erlenmeyer flask and sterilised at 121 °C for 20 minutes. Glucoamylase (Distillase L 400®), 0.06 % (w/v) was added to each flask prior to inoculation.

2.2.6.3 Ethanol fermentation

Flasks with 200 mL of YPD were inoculated with single colonies of yeast and incubated for between 16-18 hours at 30 ^{o}C and 200 rpm. The contents of each flask were transferred to sterile tubes and centrifuged for 5 minutes at 6000 rpm. The supernatant was decanted and the yeast pellet was resuspended in sterile dH_2O to make a slurry containing 2 x 10^{10} cells mL^{-1}, as determined using a haemocytometer and an optical microscope (Olympus CX41). Each flask was inoculated with 1 mL of yeast slurry and incubated at 30 ^{o}C for 48 hours or until the fermentation was complete. Samples were taken intermittently throughout the fermentation and analysed for lactic acid, maltose, glucose, maltodextrin, acetic acid, glycerol and ethanol using high performance liquid chromatography (HPLC).

2.2.6.4 High performance liquid chromatography

Samples were diluted to an appropriate level and filtered using 0.2 µM filters (Chromafil ultra RC). The samples were analysed using a Dionex DX500 chromatography system with a Waters 2410 refractive index detector. Samples were run for 30 minutes at 0.6 mL min^{-1}, using 5 mM sulphuric acid as the mobile phase. The column used for the separation was a Bio-rad Aminex HPX-87H with accompanying guard columns, which were maintained at 60 ^{o}C in a column heater throughout the analysis. Column guards were maintained at room temperature during the analysis. Samples were quantified against reference standards of known concentrations of glucose, maltose, maltodextrin, maltotriose, lactic acid, glycerol, acetic acid and ethanol. The efficiency of each fermentation was determined using the formula:

Efficiency = (Ethanol concentration % (v/v) / Potential Alcohol) x 100/1

2.2.6.5 Corn mash substitution

Corn mash was prepared as detailed in Section 2.2.6.2. Freeze-dried, fermented lignocellulosic material prepared as outlined in Sections 2.2.5.2 and 2.2.5.3, was used to replace 2-6 % (w/w) of the corn mash (depending on the lignocellulosic material). Each flask contained a final volume of 250 mL with the flasks being sterilised and glucoamylase added prior to inoculation. Fermentations were carried out using the methods detailed in Section 2.2.6.3, with control fermentations of 100 % (w/v) corn mash prepared as a reference for each fermentation.

2.2.6.6 Ethanol production from lignocellulosic material

A quantity of fermented lignocellulosic substrate was weighed to a conical flask and an aliquot of deionised water was added to give a final volume of 150 mL. This substrate was subsequently sterilised at 121 ^{o}C for 20 minutes. Following this, a sterilised crude enzyme extract prepared using the method described in Section 2.2.1.4 was diluted with three parts water and an appropriate amount of this cocktail (depending on the amount of lignocellulosic material) was added to each flask to give a final volume of 250 mL. Urea (1 g L^{-1}), glucose (1 g L^{-1}), glucoamylase (50 µL) (Distillase L 400®) and α-amylase (50 µL) (Speczyme ®) were also added to the mixture. Each flask was inoculated with 1 mL of a 2 x 10^{10} cells ml^{-1} yeast culture and incubated at 30 ^{o}C for 72 hours. Samples were taken intermittently and analysed via HPLC as described in Section 2.2.6.4.

2.2.7 Antioxidant determination

2.2.7.1 Propagation and extraction of SSF material

Lignocellulosic materials for antioxidant analysis were fermented as per Section 2.2.5.2. Biologically pretreated lignocellulosic material was freeze-dried and an aliquot (5 g) was weighed to a sterlin and extracted for 1 hour in 45 mL dH_2O at 200 rpm. Extracts were subsequently centrifuged and filtered using a 0.45 µM filter to remove contaminants and cellular debris from each sample. Each sample extract was a representative value of the water soluble antioxidant potential of the treated substrate material, where the fungal biomass was not removed.

2.2.7.2 Determination of antioxidant activity using the β-carotene bleaching assay

Antioxidant activity was determined using a method described previously by Lopes-Lutz (2008). An aliquot (1 mL) of crystallised β-carotene (0.2 mg mL^{-1} in chloroform) was transferred to a round bottom flask with purified linoleic acid (20 µL) and 200 mg of Tween 20 being added to the mixture. The chloroform was subsequently removed using a Heidolph laborota 4000 rotary evaporator and 50 mL oxygenated dH_2O (shaken at 400 rpm for 30 minutes) was added to the flask and shaken vigorously. The β-carotene / linoleic acid emulsion (2.5 mL) was mixed with 200 µL of extract and analysed on a UV spectrophotometer at λ_{470nm}. Samples were incubated for 3 hours at 50 ^{o}C and subsequently the absorbance was determined again at λ_{470nm}. Antioxidant activity was determined using the equation:

$$AA\ \% = 100x[1-(A_0-A_t)/(A_{00}-A_{0t})]$$

where, A_0 is the absorbance at the beginning of the incubation with the extract, A_t is the absorbance after incubation with the extract, A_{00} is the absorbance at the beginning of the incubation without the extract and A_{0t} is the absorbance after 3 hours with the extract.

Samples were read against blanks containing the emulsion without β-carotene. Positive controls of BHT and α-tocopherol, as well as a negative control of dH_2O were included with each batch of samples.

2.2.7.3 Determination of antioxidant activity using a reducing power assay

Antioxidant reducing power was determined using an assay previously described by Yang (2002). Fungal extract (1 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1 % (w/v) potassium ferricyanide. The reaction mixture was incubated for 20 minutes at 50 ^{o}C and 2.5 mL of 10 % (w/v) TCA was subsequently added to terminate the reaction. Next, the mixture was centrifuged for 10 minutes at 3000 rpm using a Heraeus multifuge (DJB labcare Ltd., Buckinghamshire, U.K.). Afterwards, a 2.5 mL aliquot of the supernatant was mixed with 2.5 mL of dH_2O and 0.5 mL of 0.1 % (w/v) ferric chloride. Samples were transferred to cuvettes and the absorbance of the samples was determined at λ_{700nm} against a sample blank. Positive controls of BHT and α-tocopherol as well as a negative control of dH_2O were included with each batch of samples.

2.2.7.4 Determination of total phenol content from SSF material

The total phenol content of each sample was determined using a method previously described by Slinkard *et al.* (1977). Sample extracts (100 µL) were mixed with 100 µL of 10 % (v/v) Folin-Ciocalteau's reagent. After 2 minutes at room temperature, 200 µL of 10 % (w/v) sodium carbonate solution (10 % w/v) was added to the reaction. The samples were incubated in the dark for 1 hour and the absorbance was read at λ_{765nm} using a plate reader (Biotek Synergy HT). A standard curve was prepared using appropriate concentrations of 20 - 250 µg mL^{-1} gallic acid. Total phenol content was expressed as gallic acid equivalents, which reflects the phenol content as the amount of gallic acid units in 1 g of extract.

2.2.7.5 Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity was determined using a method described previously by Lopes-Lutz *et al.* (2008). Briefly, a 50 µL aliquot of 0.2 mM DPPH in water was added to 200 µL of appropriately diluted extract. The samples were shaken well and allowed to stand in the dark for 30 minutes at room temperature. A plate reader was used to monitor the decrease in absorbance during the 30 minute incubation period at λ_{517nm}. Positive controls of BHT and α-tocopherol as well as a negative control of dH_2O were included on each plate. Percentage radical scavenging activity was determined as:

$$[(\lambda_{517nm} \text{ of control} - \lambda_{517nm} \text{ of extract}) / \lambda_{517nm} \text{ of control} \times 100]$$

The EC 50 value is the effective concentration at which DPPH radicals were scavenged by 50 % and was obtained by interpolation from linear regression analysis using Minitab 16 (Coventry, U.K.).

2.2.7.6 ABTS radical cation decolourisation assay

The ABTS radical cation scavenging activity was determined using a method described by Katalinic *et al.* (2006). A stock solution (7mM) of $ABTS^+$ was mixed with 2.45 mM potassium persulfate (1:1 v/v) and allowed to stand in the dark at room temperature to stabilize. Next the $ABTS^+$ stock solution was diluted in 95 % ethanol to an absorbance in the range of between 0.7 and 0.75 at λ_{734nm} to produce the $ABTS^+$ reagent. The assay was conducted by mixing 180 µL of $ABTS^+$ reagent, 20 µL of sample and measuring the optical density of the sample at λ_{734nm} at time zero. A positive control of trolox was included in each plate, whilst a negative

control which consisted of dH_2O was also incorporated. The radical scavenging activity of each sample was determined using the following equation:

$$E= [(A_c-A_t)/A_c] \times 100$$

where A_t is the absorbance of the samples and A_c is the absorbance of the negative control.

The antioxidant activity of each sample was expressed as the trolox equivalent antioxidant capacity, where a standard curve of trolox ranging from 10-50 µg ml^{-1} was prepared.

2.2.8 Compositional analysis of lignocellulosic material

2.2.8.1 Construction of a standard curve for the determination of acid soluble lignin concentration by UV-Vis spectroscopy

A standard curve was constructed according to a method by Hyman *et al*. (2008). A 400 mL aliquot of hydrolysate liquor was added to a separation funnel with 100 mL of methyl isobutyl ketone (MIBK) and gently inverted to avoid forming an emulsion. The mixture was allowed to separate for 30 minutes and fractions were collected in a separation beaker. This process was repeated at least two more times to ensure efficient extraction. The MIBK fraction was transferred to a clean separation funnel and washed using 10 mL of 0.24 M sodium bicarbonate. The liquor was mixed by inverting the separation funnel and venting after each inversion to avoid gas build up. This step was repeated until the pH of the solution was greater than pH 6.5. The sample was transferred to a round bottom flask of known oven-dry weight and the solvent was removed using a rotary evaporator. This round bottom flask and the residue were placed in a vacuum drying oven (Weiss-Gallenkamp, Loughborough U.K.) at 40 ^oC for a minimum of 12 hours. The flask was removed from the oven and allowed to cool in a desiccator. The weight of the flask and sample were recorded and placed back in the drying oven. This step was repeated until there was no weight loss from the flask. Residual lignin was subsequently dissolved in ethanol and transferred to a volumetric flask of known weight. The flask was then transferred to a vacuum drying oven and the ethanol was evaporated. This flask was weighed so as to determine the lignin carried over from the round bottomed flask. Lignin was then re-dissolved in a known volume of ethanol. Using this solution a

standard curve was constructed to determine the concentration of soluble lignin in the samples.

2.2.8.2 Acid hydrolysis and sample preparation

Samples were hydrolysed and analysed according to a method described by Sluiter *et al.* (2008). Filtering crucibles (CoorsTek 60531) were placed in a muffle furnace (Carbolite AAF 1100) at 575 ^{o}C for a minimum of 4 hours following which, the crucibles were removed and cooled in a desiccator for 1 hour. The weights of the crucibles were recorded and placed back in the furnace and ashed to a constant weight. Each crucible was subsequently moved to a desiccator and stored until further use. Sample material (300 mg) was transferred to a test tube and 3 mL of 72 % (v/v) sulphuric acid was added to each tube and vortexed for 20 seconds. Sample test tubes were moved to a water bath and incubated at 30 ^{o}C for 1 hour, mixing intermittently throughout the incubation. Upon completion of the hydrolysis, the tubes were removed from the water bath and decanted to pressure tubes. Each sample was diluted to a final 4 % (v/v) acid concentration and sealed using a crimper (Wheaton corporation, Millville, New Jersey). Vessels were inverted to mix the sample so as to avoid separation between high and low concentrations of acid layers. The test tubes were autoclaved for 1 hour at 121 ^{o}C. Standards containing known concentrations of D-(+) glucose, D-(+) xylose, D-(+) galactose, L-(+) arabinose, D-(+) mannose and D-(+) cellobiose were also prepared using 4 % (v/v) sulphuric acid and autoclaved for 1 hour at 121 °C in pressure tubes.

2.2.8.3 Sample analysis and lignin determination

The pressure tubes from Section 2.2.8.2 were allowed to cool and the samples were vacuum filtered through the filter crucibles (Section 2.2.8.2). A 50 mL aliquot of the resulting liquor was transferred to a storage bottle for carbohydrate and soluble lignin determination. Deionised water was used to transfer any remaining material from the pressure tubes to the filter crucibles. Deionised water (50 mL) was also used to rinse the solids and they were heated in a drying oven (Genlab limited, Cheshire U.K.) set at 105 ^{o}C until a constant weight was achieved. Samples were then moved to a desiccator and cooled. The weights of the crucibles were recorded and the crucibles were placed in a muffle furnace at 575 ^{o}C for 24 +/- 6 hours. After ashing, the crucibles were moved to a desiccator and cooled for 45 minutes. This

was repeated until the crucibles were at a constant weight. This weight was recorded and the insoluble lignin was determined.

Insoluble lignin % was determined as:

$$\text{Insoluble lignin \%} = \{[(W_1\text{-}W_2) – (W_3 – W_2)] / W_4\} \times (100/1)$$

where, W_1 is the weight of the air dried sample following hydrolysis and crucible, W_2 is the weight of the crucible, W_3 is the weight of the crucible and ash and W_4 is the oven dry weight of the sample.

2.2.8.4 Acid soluble carbohydrate determination

Acid soluble carbohydrate was determined using a procedure described by Sluiter *et al*. (2008). Briefly, an aliquot (10 mL) of sample liquor from Section 2.2.8.3 was transferred to a 50 mL centrifuge tube and calcium carbonate was added to neutralise each sample to between pH 5-6. The samples were allowed to settle and subsequently filtered through a 0.2 µM filter (Chromafil RC) to a HPLC vial. Samples were run on an Aminex HPX-87P column with appropriate guard column at 0.6 mL min^{-1} for 35 minutes, using dH_2O as the mobile phase. The column was maintained at 80 ^oC throughout the analysis in a column heater. A Waters 2410 refractive index detector was used to determine the change in the refractive index of the sample, which was used to quantify sugar concentration. De-ashing guards were placed before the guard column so as to prevent artificial peaks in the chromatogram, which can be caused by excess salt concentrations. The standards prepared in Section 2.2.8.2 were filtered and used to quantify carbohydrate concentration.

2.2.8.5 Acid soluble lignin determination

Sample hydrolysate prepared in Section 2.2.8.3 was diluted to obtain an absorbance in the range of 0.7- 1.0 at λ_{320nm}. Each sample was read against a blank of deionised water. The acid soluble lignin was determined using the soluble lignin standard curve prepared in Section 2.2.8.1.

2.2.8.6 Determination of dry matter and dry matter loss

Dry matter was determined using a moisture analyser (Sartorius MA150). An appropriate amount of sample was weighed to a pre-tared aluminum dish and spread evenly. The initial sample weight was noted and the sample was heated until there was no change in the sample weight. The total moisture was determined as:

$$\text{Moisture \%} = [(W_{wet} - W_{dry})/W_{wet}] \times (100/1)$$

where, W_{wet} was the weight of the wet sample and W_{dry} was the weight of the dry sample.

2.2.9 Determination of forage composition using the detergent system

2.2.9.1 Preparation and standardisation of hydrochloric acid standard solution

An acid standard solution was prepared using a procedure described by the Association of Official Analytical Chemists (AOAC) (1990e). This solution was prepared by diluting 430.1 mL of 38 % (v/v) HCl in 9.57 L of CO_2-free deionised water. An aliquot of HCl (40 mL) was transferred to a 250 mL Erlenmeyer flask that had been previously rinsed with CO_2- free water. A Schellbach buret was rinsed with standardised NaOH (Section 2.2.9.2), filled with NaOH and the volume was noted. Methyl red indicator was prepared by dissolving 1 g of methyl red sodium salt in 100 % (w/v) ethanol. An aliquot (3-4 drops) of this solution was added to the acid and the NaOH was titrated to an orange end point. The side of the Erlenmeyer flask was washed down with CO_2 –free H_2O to see if the colour persisted and the quantity of NaOH used was determined from the buret. The normality of the solution was determined and rechecked using the method described above.

2.2.9.2 Preparation and standardisation of sodium hydroxide standard solution

Sodium hydroxide standard solution was prepared using a method described previously by the Association of Official Analytical Chemists (AOAC) (1990f). Sodium hydroxide was prepared by dissolving 1 part reagent grade NaOH in 1 part of CO_2- free water by weight. A quantity (270 mL) of this solution was added to CO_2- free water to make a final volume of 10 L. Potassium hydrogen phthalate (0.4 g) was dissolved in 50 mL of CO_2 free water. NaOH solution (approximately 40 mL) was titrated to the potassium acid phthalate solution until a pH of 8.6 was obtained, taking precautions to exclude CO_2 and using phenolphthalein as an indicator. A pH 8.6 reference end point was prepared by adding 3 drops of phenolphthalein to 50 mL of a pH 8.6 buffer. The volume of NaOH required to produce the endpoint of the blank was determined by matching solution colour to another flask which was prepared by adding 3 drops of phenolphthalein to 50 mL of CO_2 -free water. The volume required to titrate the blank was subtracted from the volume required to titrate the potassium hydrogen phthalate and the normality was

determined. The concentration of the sodium hydroxide standard solution was adjusted and the standardisation was rechecked.

2.2.9.3 Nitrogen and crude protein determination

Crude protein was determined using the Kjeldahl method described by the AOAC (1990d). Approximately 1 g of ground sample, 15 g of potassium sulphate, 0.04 g anhydrous copper sulphate and 0.5 g of boiling chips were weighed to a digestion flask and 20 mL of concentrated sulphuric acid was added. A reagent blank and high purity lysine-HCl were included to check the correctness of the digestion parameters. The flask was placed in a heated burner and heated until white fumes cleared the bulb of the flask. The flask was heated for a further 90 minutes and subsequently cooled carefully to 25 ^{o}C with the addition of 250 mL of dH_2O. A titration flask was prepared by adding appropriate amounts of acid standard solution from Section 2.2.9.1 to an amount of water so that the condenser tip was immersed. A reagent blank was prepared by adding 1 L of acid and 85 mL of water. Methyl red (3 to 4 drops) (Section 2.2.9.1) was added to the titration flask, while 2 or 3 drops of tributyl citrate were also added to the digestion flask to reduce foaming. Approximately 80 mL of 45 % (w/v) sodium hydroxide solution was added to the digestion mixture to make the solution strongly alkali. Both flasks were connected to the distillation apparatus and boiled until about 150 mL of distillate was collected in the titrating flask. The excess acid was titrated with normal volume sodium hydroxide to an orange endpoint and the volume was recorded. The reagent blank was titrated in the same manner. Crude protein was determined as:

Crude protein = percentage nitrogen (DM basis) x F

where F is 6.25 for all forages except wheat grains.

The percentage nitrogen was determined as:

$$\% \text{ N (DM basis)} = \{[(V_A - V_B) \times N_{HCl} \times 1.4007] / [W \times (\text{Lab DM}/100)]\}$$

where, V_A is the volume in mL of the standard HCl required for the sample, V_B is the volume in mL of the standard HCl required for the blank, N_{HCl} is the normality of standard HCl, 1.4007 is the milliequivalent weight of N x 100 and W is the sample weight in grams.

2.2.9.4 Determination of acid detergent fibre and lignin by refluxing

Acid detergent fibre (ADF) was determined using a method described by the AOAC (1990c). Samples were dried and ground to pass through a 2 mm sieve prior to analysis. Glass crucibles were dried in an oven overnight and their weight was subsequently determined. These crucibles were stored in a desiccator until they were needed. Approximately 1 g of sample was weighed into a beaker and the weight was recorded. Following this, 100 mL of acid detergent solution (1 L of 1 N sulphuric acid and 20 g of CTAB) was added. Next the beaker was heated to boiling for 5-10 minutes under a cold water condenser, with the temperature reduced to avoid foaming as boiling began and refluxed for 60 minutes at the onset of boiling. After refluxing, the beaker contents were filtered through the tared crucible. The mat at the end of the crucible was soaked twice with boiling water with the vacuum off and twice with acetone for 2 minutes to rinse the mat. The acetone was removed and the sample was allowed to dry overnight in an oven. The following day the weight of the sample was recorded. Percentage ADF was determined as:

$$[(W_3-W_1)/ (W_2 \text{ x Lab DM}/100)]/100$$

where, W_1 is the tared weight of crucible in grams, W_2 is initial weight of sample in grams and W_3 is the weight of crucible and dry fibre in grams.

The crucibles were subsequently placed in a beaker for support and the contents of the crucible were covered in 72 % H_2SO_4 and stirred with a glass rod. Next each crucible was filled half way up with H_2SO_4 and the contents were stirred at hourly intervals. After 3 hours the acid was filtered off with a vacuum filter and the contents of the crucible was rinsed with hot water to remove any remaining acid. Following this, the crucible was dried at 105 °C for 8 hours and cooled in a desiccator prior to the weight of the crucible being determined to the nearest 0.1 mg. The crucible was subsequently transferred to a muffle furnace and ignited at 500 °C for 2 hours. This crucible was cooled in a desiccator again to a constant weight and weighed to the nearest 0.1 mg. Acid detergent lignin was determined as:

% ADL= (crucible weight after acid soak – crucible weight after ignition)/(sample weight x lab DM)

2.2.9.5 Determination of amylase neutral detergent fibre by refluxing

Neutral detergent fibre (NDF) was determined using a method described previously by Van Soest *et al.* (1991). Neutral detergent solution was prepared by mixing 17.82

L of dH_2O with 540 g sodium lauryl sulphate, 335 g EDTA, 122.6 g sodium borate decahydrate, 82.1 g sodium phosphate dibasic and 180 mL of triethylene glycol. Samples were dried and ground to pass through a 1 mm screen. Fritted glass crucibles were dried overnight, weighed and stored in a desiccator until use. Exactly 0.5 g of sample, 0.5 g sodium sulphite and 50 mL of neutral detergent solution were added to a beaker and swirled until the sodium sulphite and sample were completely suspended. The extraction heating reflux unit was heated and the beaker was placed on the unit under a cool water condenser. Samples were boiled for 5 minutes and 2 mL of standardised amylase solution was added. The beaker was returned to the reflux unit, brought to the boil and allowed to reflux for 60 minutes. Following this, the samples were removed from the heating unit and allowed to settle prior to filtration through the pre-weighed crucibles. Each crucibles was subsequently filled with boiling water and 2 mL of amylase was added and allowed to react for 1 minute. The concentration of the amylase solution needed was determined in Section 2.2.9.6. This solution was removed and the residue was washed twice with boiling water and acetone, allowing the residue to soak for 2 minutes between rinses. The crucibles were allowed to dry overnight and cooled in a desiccator. Each crucible weight was recorded to determine the NDF of the sample. NDF was determined on a dry matter basis as:

$$[(W_3-W_1) / (W_2 \text{ x Lab DM} / 100)] / 100$$

where, W_1 is tared weight of crucible in grams, W_2 is initial weight of sample in grams and W_3 is the weight of crucible and dry fibre in grams.

2.2.9.6 Standardising α-amylase activity for neutral detergent fibre determination

Determination of α-amylase activity for solutions to be used in the neutral detergent fibre procedure was carried out using a method described by Undersander *et al.* (1993). A dilute α-amylase solution (3 % v/v) was prepared by diluting α-amylase solution (Spezyme®) in dH_2O. Hominy corn (0.5 g) was weighed into six Berzelius beakers. Neutral detergent solution (50mL) prepared in Section 2.2.9.5 was added to each beaker and swirled. Each beaker was transferred to the preheated refluxing apparatus at exactly 1 minute intervals. After the samples begin to boil, an aliquot of α-amylase from between 0.5-8 mL was added to each beaker and allowed to reflux for 10 minutes. The beakers were removed from the refluxing unit in 1 minute intervals and a second dose of the same amount of α-amylase was added to each

beaker. The sides of the beaker were rinsed down and the amylase was allowed to react for 60 seconds before being cooled in an ice bath for 5 minutes. Each beaker was placed on a white sheet of paper and 0.5 mL of Burke's iodine solution (2 g potassium iodide, 1 g of iodine dissolved in 100 ml of H_2O) was added to each beaker. After 90 seconds, the efficacy of each solution on raw corn starch was determined where purple was not adequate, amber was not adequate and yellow was adequate. Thus, the amount of enzyme needed in 2 mL of standardised amylase solution can be determined.

2.2.9.7 In-vitro true digestibility

The *in vitro* true digestibility was determined using the Daisy^{II} method described by Holden (1999). F57 filter bags (Ankom, New York, U.S.A.) were rinsed in acetone for 5 minutes and allowed to air dry. The dry weight of the bag was recorded (W_1) and 0.25 g of sample was weighed (W_2) directly into the bag. Each bag was heat-sealed and placed in a Daisy^{II} incubator jar (Ankom, New York, U.S.A.). A blank was included in each jar to determine a correction factor. Two buffers were prepared in dH_2O: Solution A contained 10 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1} NaCl, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 0.5 g L^{-1} urea, while Solution B contained 15 g L^{-1} Na_2CO_3 and $Na_2S \cdot 9H_2O$. These two solutions were preheated and stored at 39 °C. An aliquot of each solution was mixed to contain a final ratio of 1:5 solution A to solution B and the pH was adjusted to pH 6.8 at 39 °C. Exactly 1600 mL of this solution was added to each digestion jar which were placed in a Daisy^{II} Incubator (Ankom, New York U.S.A.) and allowed to equilibrate for 30 minutes. Approximately 2 L of rumen fluid and fibrous mat from the rumen was blended for 30 seconds at 39 °C to ensure a representative microbial population. The rumen fluid was filtered through layers of cheesecloth and collected in a preheated beaker. Subsequently, 400 mL of rumen fluid was added to each digestion jar and purged with CO_2 to obtain an anaerobic atmosphere. Each jar was incubated for 30 hours at 39 °C and removed and drained. Afterwards, the bags were rinsed once with cold H_2O and microbial debris was removed from each bag using the neutral detergent solution from Section 2.2.9.5. The bags were once again rinsed with water and the fibre content was determined using the procedure for determining NDF (W_3) (Section 2.2.9.5). Percentage IVTD was determined as:

$$[100 - (W_3 - (W1 \times C_1)/ W_2] \times 100$$

where, W_1 is the bag tare weight, W_2 is the sample weight, W_3 is the final bag weight after in vitro and sequential neutral detergent treatment and C_1 is the blank bag correction (final oven-dried weight/ blank bag weight).

2.2.9.8 Ethanol soluble carbohydrates

Ethanol soluble carbohydrates were determined using a method described previously by Hall (1999). The sample material was dried and ground to pass through a 1 mm sieve. Exactly 0.2 g of sample and 40 mL of 80 % (v/v) ethanol were transferred to a screw cap tube for extraction at room temperature at 300 rpm for 4 hours. Next the sample was filtered through a 50 mL coarse porosity gooch crucible and the filtrate was retained to determine ethanol soluble carbohydrates. The filtrate was again filtered through Whatman #541 filter paper to remove any remaining fine particles and a 1:10 dilution of each sample was prepared in dH_2O. Sample (0.5 g), 0.5 mL of 5 % (v/v) phenol solution and 2.5 mL of concentrated H_2SO_4 were added to a test tube and incubated at 30 ^oC in a water bath for 20 minutes. The samples were then vortexed and transferred to acid resistant cuvettes, with the absorbance of each sample determined at λ_{490nm} on a UV-vis spectrophotometer. A standard curve of appropriate sucrose concentrations was prepared to determine ethanol soluble carbohydrate concentration.

2.2.9.9 Crude fat (ether extract) determination in forages

Crude fat was determined using a method previously described by the AOAC (1990b). An aliquot (2 g) of sample (W1) was weighed to a small filter cone and extracted with five 20 mL portions of deionised water to remove soluble carbohydrates, glycerol, lactic acid and other water soluble components. Both the sample and filter were moved to an extraction thimble and dried for 5 hours at 100 °C. A beaker for fat determination was also dried in the oven at 100 °C for 1 hour and placed in a desiccator to cool before the weight (W2) was recorded. After 5 hours, the sample was removed from the drying oven and placed in a desiccator to cool. An aliquot (40 mL) of diethyl ether was added to the sample beaker before attaching the beaker to the extractor. Next, the heating element was turned on and the sample was extracted for a minimum of 4 hours. The thimble was removed from the beaker leaving a small amount of diethyl ether in the sample so as to avoid oxidation of the fat. Following this the beaker was placed in a fume hood and the

remaining diethyl ether was evaporated at room temperature. Once all the ether was evaporated the sample was placed in a drying oven at 102 °C and allowed to dry for 30 minutes. The sample was removed to a desiccator and allowed to cool before the weight was determined to the nearest 0.1 mg. % crude fat was determined as:

% crude fat (DM basis) = [(W3-W2) x 100 / (W1 x lab DM) / 100]

where, W1 is the initial weight of the sample in grams, W2 is the tare weight of the beaker in grams and W3 is the weight of the beaker and the fat residue in grams.

2.2.9.10 Determination of total ash

The total ash content was determined using the method previously described by the AOAC (1990a). The crucibles and cover, which were dried for at least 2 hours in an oven at 100 °C were placed in a desiccator and allowed to cool before their weight (W_1) was recorded. An aliquot of sample (2 g) was weight to the crucible and the weight of the crucible with the cover and sample (W_2) was recorded. Next the crucible was transferred to a furnace at 600 °C and ashed for 2 hours. Samples were subsequently removed from the furnace and allowed to cool in a desiccator before the weight (W_3) was recorded. Percentage ash was determined as:

% Ash (DM basis) = [{($W_3 – W_1$) x 100} / {($W_2 – W_1$) x Lab DM / 100}]

where, W_1 is the tare weight of the crucible, W_2 is the crucible and sample weight on grams and W_3 is the weight of the crucible and ash in grams.

2.2.9.11 Non-fibre carbohydrate

NFC was determined as:

100 % - (CP % + (NDF % - NDI-CP) + EE % - Ash %)

where, CP is crude protein, NDF is neutral detergent fibre, NDI-CP is neutral detergent insoluble crude protein and EE is ether extract.

2.2.9.12 Total digestible nutrient determination

% Total digestible nutrients (TDN) = [digestible protein + digestible carbohydrate + (digestible fat x 2.25)]

2.2.10 Statistical analysis

Data was analysed using Minitab statistical software package version 16 (Coventry U.K.). All statements of significance were based on the probability of less than 0.05.

3. Hydrolytic Enzyme Production

3.1 Cellulase and xylanase production in SSF

Solid state fermentation (SSF) is generally viewed as a more appropriate technique for enzyme production in comparison to SmF. This is due in part to the higher titres that can be achieved as a consequence of the filamentous fungi operating in a fed-batch state, while also being allowed to grow in their natural environment (Viniegra-Gonzalez, 1997; Viniegra-Gonzalez *et al.*, 2003). A wide variety of lignocellulosic materials can be used for solid state fermentation including agricultural and forestry wastes, although wheat bran is currently the most widely utilised material for fungal growth and secondary metabolite synthesis, with it providing a solid support matrix and sufficient nutrients to produce high enzyme titres (Pandey, 2003).

In this study corncobs were used as the lignocellulosic substrate of choice for enzyme production due to the vast quantity of the material available globally, in addition to it being an alternative substrate to the traditional solid state fermentation substrates like wheat bran, which has been extensively studied. Currently over 60 million tons of corncobs are produced annually in China and the US alone (Bai *et al.*, 2008), with the majority of this material being used as low cost forage feed or left unutilised, resulting in issues such as soil erosion and water contamination (Miura *et al.*, 2004). Corncob represents a good substrate for SSF as it contains high quantities of both hemicellulose (~35 %) and cellulose (~45 %), while also providing a solid support matrix for fungal growth (Saha, 2003).

Initial screening of a fungal bank of approximately 223 strains focused on identifying fungi which were adept at growing on corncob and capable of producing hollocellulosic enzymes. Strains of interest were to be considered on the basis of low levels of sporulation on the substrate and a rapid colonisation rate (i.e. 5 days). Subsequently, selected strains were assessed for their capacity to produce xylanase, cellulase and protease enzymes on corncobs.

Suitable strains were selected and the production of xylanase and cellulase was optimised to produce maximal titres of these enzymes. The detection and stability of the enzymes was also optimised to assess the ideal parameters for crude enzyme production and detection. Each fermentation was analysed for cellulase activity (Section 2.2.2.2), where a carboxymethylcellulose substrate was used to determine the

endoglucanase activity of the fermentation, which was thought to be the predominant cellulase enzymes produced by filamentous fungi. Total xylanase activity was analysed with a Birchwood xylan substrate by monitoring the release of reducing sugars from the substrate (Section 2.2.2.1). Protease activity was also monitored (Section 2.2.2.3), as low protease activity was optimal to reduce the hydrolysis of xylanase and cellulase enzymes. Haab *et al.* (1990) previously demonstrated that endogenous proteases from *T. reesei* on cellulose were involved in the degradation of cellulases in the medium, where high protease yields correlated with the formation of cellulase degradation products.

3.1.1 Screening of filamentous fungi

Approximately 223 different fungal strains were screened through cultivation on a 1mm diameter ground corncob substrate. A range of fungal genera and species were screened including *Trichoderma* spp., *Rhizopus* spp., *Penicillium* spp., *Myceliopthora* spp. and *Aspergillus* spp. fungi as well as selected other fungal species. Initial screening of the growth of fungal strains on corncobs resulted in the selection of 21 strains of fungi which were subsequently analysed for residual cellulase, xylanase and protease activity. Selected strains were propagated using the method described previously in Section 2.2.1.2 and subsequently used to ferment 20 g of corncobs for 5 days at 30 °C and 80 % RH. Exogenous enzymes were then extracted from the material using the method outlined in Section 2.2.1.4 and analysed for residual enzyme activity.

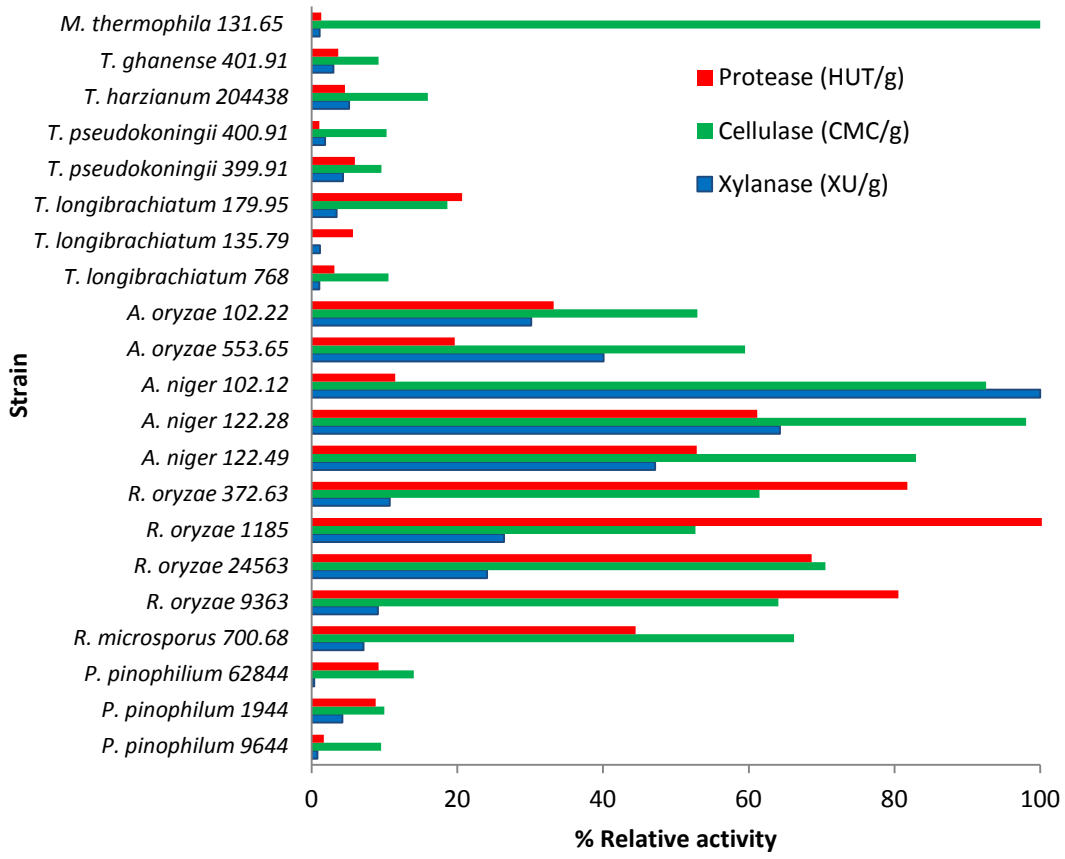


Figure 3.1 Screening of filamentous fungi for xylanase (XU), cellulase (CMCU) and protease (HUT) production.

As can be determined from Figure 3.1, *Myceliophthora thermophila* 131.65 produced the highest cellulase activity of the 21 strains tested at 18.6 CMCU g^{-1}, although it exhibited extremely low xylanase yields, while *Aspergillus niger* 122.28 displayed only slightly lower cellulase activities of 18.27 CMCU g^{-1}. By contrast, *Trichoderma longibrachiatum* 135.79 produced extremely low levels of protease and xylanase, while it did not produce any detectable quantities of cellulase from corncob. Interestingly, Trichoderma strains in general produced relatively low enzyme titres from corncob, with low cellulase activities produced by all Trichoderma strains tested. Trichoderma species, particularly *Trichoderma reesei* are well known producers of cellulolytic enzymes at high levels (Kim *et al.*, 1997; Miettinen-Oinonen *et al.*, 2002) and as a result, higher enzyme yields were expected using these fungal strains. It is likely that the corncob substrate does not provide the optimal substrate for enzyme production from Trichoderma, which is as a consequence affecting the activity of the enzymes. The substrate used for enzyme production is known to have a significant

effect on the production of enzymes from a particular strain. For example, Juhasz *et al.* (2005) monitored the effect of different lignocellulosic substrates on the production of enzymes from *Trichoderma reesei*. The authors determined that the highest endoglucanase activity was observed during fermentation of the corn stover, whilst solka-floc fermentation produced the lowest enzyme activity, indicating that the substrate used during fermentation is important for optimal enzyme production.

Generally, cellulase activities produced on corncob were dramatically lower than those obtained from substrates such as wheat bran. Kang *et al.* (2004) produced between 118-130 U g^{-1} of cellulase from a strain of *A. niger* on a 4:1 mix of wheat bran and oat straw, equating to a 10-fold increase in cellulase production in comparison to the titres of cellulase from *A. niger* strains exhibited in this study. Additionally, Bansal *et al.* (2012) monitored the production of cellulase from a range of substrates and determined that the CMCase activity from *Aspergillus niger* was 31 times greater in wheat bran fermentations than the activity obtained from corncob fermentations.

Xylanase activity of 643 U g^{-1} produced by *Aspergillus niger* 102.12 corresponded to over 200 units more than any of the other strains analysed. In general, the highest xylanase yields were predominantly obtained from Aspergilli which are well known producers of high xylanase titres (Kim *et al.*, 1997; Siedenberg *et al.*, 1998). Strains of Trichoderma produced minimal xylanase activity during the fermentation, which was not unexpected as certain Trichoderma strains, specifically *T. reesei* are generally recognised as poor xylanase producers (Kim *et al.*, 1997), although strains of Trichoderma have been known to produce high xylanase concentrations.

Rhizopus spp. primarily produced the highest protease activities from the fungal species investigated, with *R. oryzae* 1185 producing notably high protease yields of 5123 HUT g^{-1}. *Trichoderma* spp. again produced some of the lowest yields of the fungal strains tested, although *T. longibrachiatum* 179.95 produced relatively high protease titres of 1056 HUT g^{-1}, which were substantially greater than the other Trichoderma strains assayed.

As can be observed in Figure 3.1, *A. niger* 102.12 produced some of the highest yields of both xylanase and cellulase and as a consequence, was selected for further investigation. In addition it also produced one of the lowest titres of protease of the fungi tested which was also desirable. This would reduce the hydrolysis of the other enzymes in the mixture allowing them to act on the lignocellulosic substrate when utilised during different process, such as in a saccharification process. Enzyme

cocktails from the selected strains were to be used in a simultaneous saccharification and fermentation system during ethanol production in Section 4.2.

The selection of a second strain was more difficult due to the wide variety of strains producing high enzyme titres. Initially *Trichoderma* and *Penicillium* spp. were disregarded due to the low activities that were achieved in comparison to the other strains analysed, whilst *Myceliophthora spp.* was also eliminated due to low xylanase activity, despite it producing significant quantities of cellulase. *Aspergillus oryzae* 553.65 was chosen from the remaining strains for a number of reasons. Although it did not produce comparatively high enzyme activities, it produced reasonable amounts of both cellulase and xylanase, with little or no sporulation during fermentation. Despite *A. niger* 122.28 producing higher enzyme titres than *A. oryzae* 553.65, the selection of another *Aspergillus niger* strain was dismissed as one strain had already been selected and two different species were desirable. Furthermore, *A. niger* 122.28 produced a visibly higher spore content than *A. oryzae* 553.65 during fermentation, whilst it also produced a higher protease activity. Finally *Rhizopus* spp. were eliminated due to higher spore formation and higher protease activities, which could interfere with the cellulase and xylanase activity during fermentation.

3.2 Optimisation of enzyme production in SSF

Over the last number of years, focus has shifted to solid state fermentation as a method of producing enzymes due to the high enzyme titres that can be achieved (Bhargav *et al.*, 2008) and the potential to reduce the production and initial capital costs (Castilho *et al.*, 2000). Enzyme synthesis in solid state fermentation is typically governed by process parameters such as temperature, moisture content, pH, oxygen supply and fermentation time, each of which can be optimised to yield maximal enzyme titres. In submerged fermentation, these parameters are generally easy to control due to the homogeneity of the system as a consequence of mixing. However, in SSF they are much more difficult to control due to the heterogeneity of the substrates used and the lack of moisture in the system, which affects both heat and oxygen transfer, thus limiting fungal growth (Durand, 2003).

Following the initial screening, fermentation parameters were optimised to maximise xylanase and cellulase production. These parameters included initial moisture content, carbon and nitrogen source, fermentation duration and temperature.

The optimal for each parameter was adopted as the standard for subsequent fermentations.

3.2.1 Influence of carbon and nitrogen source on cellulase and xylanase production

The substrate has a significant influence on fungal growth and as a consequence the production of enzymes. In SSF, an ideal substrate is required to supply a solid support matrix and sufficient nutrients which are required for fungal growth (Raimbault, 1998). The carbon and nitrogen composition of the growth matrix have been known to affect the production of metabolites, such as enzymes and antibiotics, during fermentation (Ooijkaas *et al.*, 2000). However, in SSF the potential to alter the substrate composition is limited due to the use of natural lignocellulosic substrates. As a consequence, some of the nutrients that are required for fungal growth and metabolite formation may be present at sub-optimal concentrations or in some cases not at all, requiring them to be supplemented to the medium. The source of nitrogen in a substrate has been shown to not only influence the concentration and type of enzymes produced but also the proportion of isoenzymes secreted (Levin *et al.*, 2010). Levin *et al.* (2008) determined that the supplementation of peptone increased the production of ligninolytic enzymes from *Trametes trogii* in solid state fermentation, while Jha *et al.* (1995) noted that a 2.5-fold increase in cellulase formation from *Phanerochaete chrysosporium* occurred through the addition of urea to the soyhull substrate.

The carbon source can also directly influence the production of a specific enzyme of interest. Generally, the production of an enzyme is induced by the availability of the substrate on which that enzyme acts, for instance xylanase is produced if xylan is available in the substrate. Seyis *et al.* (2005) determined that xylanase production was significantly higher when xylan was used as the carbon source in comparison to other substrates. Corncob is generally thought to be a good substrate for fungal growth and lignocellulolytic enzyme formation due to the availability of high concentrations of both cellulose and xylan in the substrate, which are influential in inducing hollocellulosic enzyme production.

3.2.1.1 Effect of carbon source on cellulase and xylanase production

In the present study, the effect of varying concentrations of carbon on xylanase and cellulase production from ground corncob material was determined. A number of

carbon sources (listed in Section 2.2.4.1) were dissolved in deionised water, sterilised and mixed with the fungal inoculum prior to them being added to the substrate to give a final concentration of between 1-5 % (w/v). Following fermentation of the corncobs, cell free extracts were obtained from triplicate flasks and analysed for cellulase and xylanase activity. Unsupplemented control fermentations were included, with the activity of the supplemented samples expressed as a relative percentage of the control.

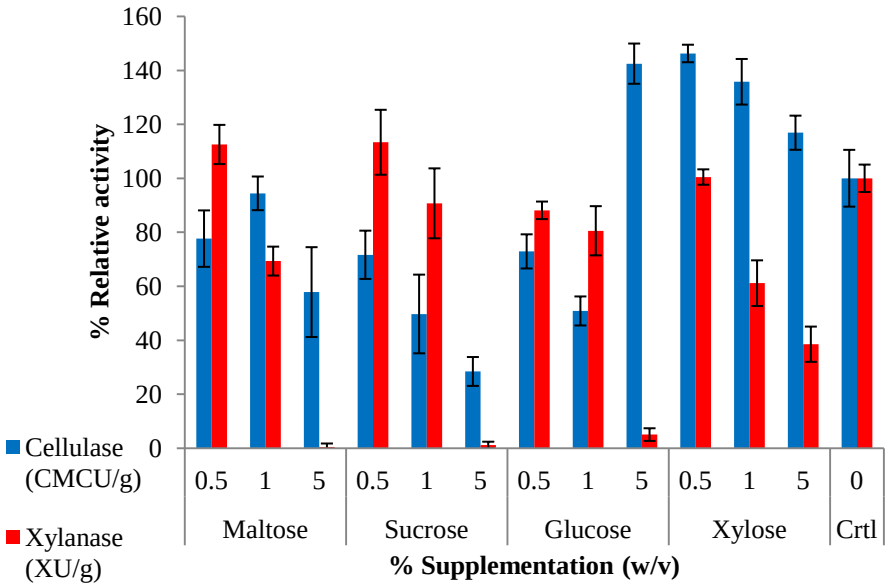


Figure 3.2 Effect of carbon supplementation on cellulase and xylanase production from *A. oryzae* 553.65.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars.

In general, no appreciable increase in cellulase or xylanase activity was observed through the addition of maltose or sucrose to *Aspergillus oryzae* 553.65 fermentations, although at a concentration of 0.5 % (w/v) sucrose and maltose a 13 % increase in activity was detected. However, when the standard error was accounted for the difference between these samples and the control fermentation was far less. At higher concentrations of 1-5 % (w/v) a decrease in xylanase activity was observed through the addition of these carbohydrates, with less than 1 % of relative activity noted when 5 % (w/v) sucrose and maltose was added to the substrate. Cellulase activity was reduced in all cases by the addition of maltose and sucrose to the fermentation medium, although not as significantly as xylanase activity.

The xylanase activity was also decreased with increasing xylose activity. As the concentration of the xylose in the fermentation is increased the xylanase activity is

decreased, with a relative activity of 38 % detected when 5 % of xylose was added to the substrate. This decrease in activity observed through the addition of carbohydrate supplements is most likely as a result of catabolite repression which can occur when concentrations of certain sugars are present in the fermentation above a specific concentration. These sugars function by repressing the expression of genes which are required for enzyme production as enough of the preferred carbohydrate is present in the medium (Stülke *et al.*, 1999). Kamra and Satyanarayana (2004) observed xylanase repression when xylose was present in the fermentation of wheat bran with *Humicola lanuginosa* at concentrations above 3 % (w/v) in SSF. In contrast to xylanase the cellulase activities of the fermentations were increased through the addition of xylose to the fermentation, with a maximal relative activity of 146 % observed when 0.5 % (w/v) xylose was added to the substrate. It is worth noting however, that as the xylose concentration increased the cellulase activity of the fermentation decreased.

Both xylanase and cellulase activities were reduced with the addition of low concentrations of glucose (0.5-1 % w/v) to the medium, with cellulase yields tending to be the worst affected. Again this is most likely due to catabolic repression where the presence of readily metabolizable sugars like glucose can repress cellulase biosynthesis. Niranjane *et al.* (2007) noted that the addition of glucose to the fermentation media suppressed cellulase production from *Phlebia gigantea* in submerged fermentation to a point where no cellulase activity was detected. Despite a reduction in cellulase activity with the addition of 0.5-1 % (v/v) glucose, cellulase activity was increased by 20 %, with the addition of 5 % (v/v) glucose to the media. This was unexpected as glucose repression is usually noted in enzyme production where easily metabolizable sugars are available (Suto *et al.*, 2001). This however, could be due to the high glucose concentrations in the fermentation extracts that may influence the background glucose levels in the DNS assay which may influence the detection of cellulase. In saying this, a blank was utilised in each sample to account for the residual glucose in each sample.

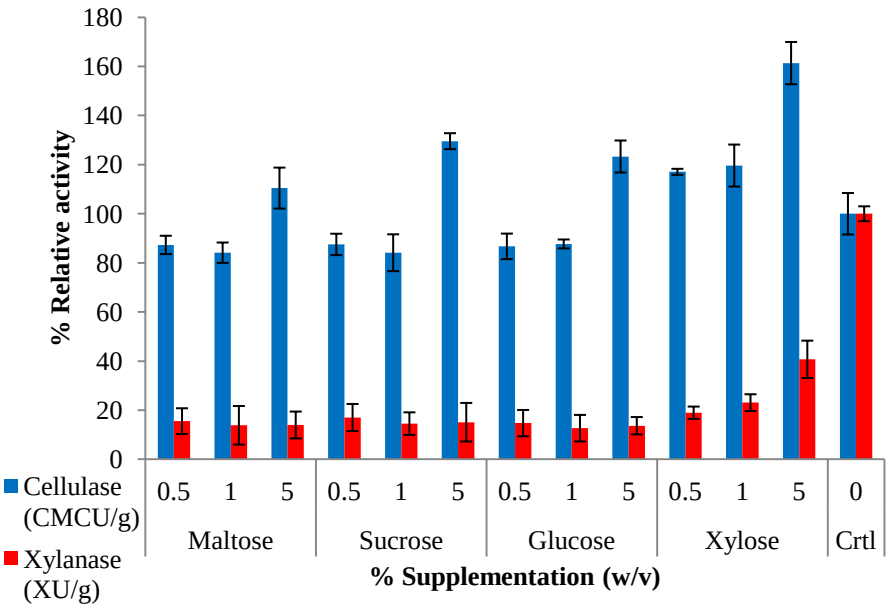


Figure 3.3 Effect of carbon supplementation on cellulase and xylanase production from *A. niger* 102.12.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars.

Typically, xylanase production from *A. niger* 102.12 (Figure 3.3) was repressed by the addition of easily metabolizable sugars with yields being dramatically reduced (by over 60 %), through the addition of supplemental carbon. Farani de Souza *et al.* (2001) studied the effect of easily metabolizable carbohydrates on xylanase production by *Aspergillus tamarii* in SSF using wheat bran, corncobs and sugar cane baggase. The authors concluded that metabolizable sugars such as glucose, sucrose and maltose, at a concentration of 1 % (w/v), severely repressed xylanase production from corncobs and sugarcane baggase. In the case of wheat bran; however, xylanase production was not inhibited by the addition of easily metabolizable carbon sources. The authors also noted that in the wheat bran fermentations, less than 5 % of the initial concentration of glucose remained in the substrate after 4 days whereas in corncob and sugar cane baggase fermentations, more than 60 % of the initial glucose remained. This residual glucose is thought to be the cause of the repression in corncob fermentations in comparison to wheat bran systems, although it was not fully understood why the glucose was so readily utilised in one substrate in relation to the other.

SSF systems have long been known to reduce catabolic repression in comparison to an equivalent submerged fermentation (Ramesh *et al.*, 1991; Nandakumar *et al.*, 1999); however, most of the studies of catabolic repression have

used wheat bran as the substrate rather than other lignocellulosic materials. It is not well known why catabolic activity is suppressed in SSF, although it is thought to be as a result of a number of physico-chemical factors such as diffusion (which is significantly reduced in SmF as a result of agitation) and the low water activity of the substrate (Nandakumar *et al.*, 1999). In saying this it was apparent that this was not the case in these fermentations with substantial catabolic repression observed.

Cellulase production was not as substantially affected as xylanase by the addition of easily metabolizable sugars such as xylose (Figure 3.3). In fact, cellulase production was increased by the addition of xylose concentrations to the substrate, with an increase of up to 60 % in cellulase yield determined through the addition of 5 % (w/v) xylose to the medium. This was also observed by other authors, for instance, Petitdemange *et al.* (1992) who concluded than the addition of xylose to the medium promoted cellulase production in submerged fermentation of *Clostridium celluloyticum.* Furthermore, Jørgensen *et al.* (2005) achieved a 3-fold increase in cellulase filter paper activity by the addition of Birchwood xylan to the substrate in comparison to a substrate with just Solka-flok.

Cellulase activity was reduced by the addition of between 0.5 -1 % (w/v) of glucose, sucrose and maltose to the media, although at higher concentrations of 5 % (w/v), cellulase production was increased (Figure 3.3). It was not fully appreciated why this occurred, although it is thought that it could be due to the metabolizable sugars inducing initial fungal growth. The fungi utilise the available carbon source leading to induction of cellulase production due to low concentrations of readily fermentable carbohydrates such as glucose, which can inhibit cellulase production (Szabó *et al.*, 1996).

3.2.1.2 Effect of nitrogen supplementation on cellulase and xylanase production

The effect of solubilised nitrogen on enzyme synthesis was determined by the addition of different concentrations of the nitrogen containing sources listed in Section 2.2.4.1. Each nitrogen source was solubilised in deionised water and mixed with the culture inoculum prior to the inoculation of the corncob medium. Following the specified incubation period, cell free extracellular extracts were obtained and analysed for cellulase and xylanase activity.

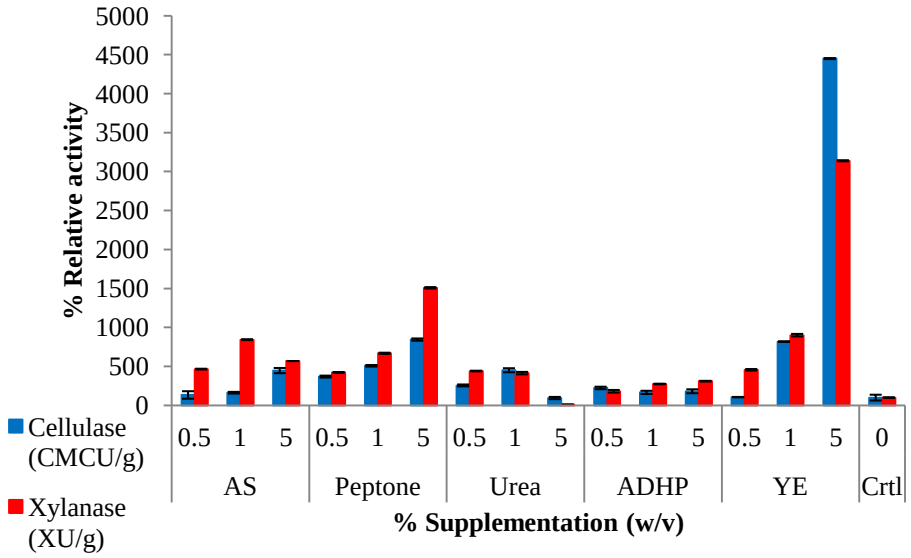


Figure 3.4 Effect of supplemented nitrogen source on cellulase and xylanase activity from *A. oryzae* 553.65.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars
Abbreviations: AS : ammonium sulphate, ADHP; ammonium dihydrogen phosphate, YE; yeast extract, Crtl; control.

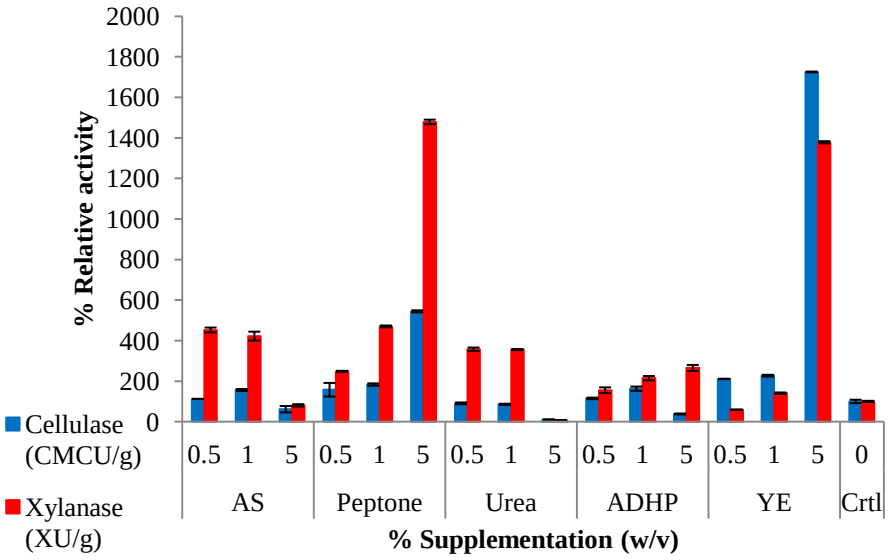


Figure 3.5 Effect of nitrogen source on xylanase and cellulase activity from *A. niger* 102.12.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars.
Abbreviations: AS; ammonium sulphate, ADHP; ammonium dihydrogen phosphate, YE; yeast extract, Crtl ; control.

Good growth was observed in each fermentation of corncobs with *A. niger* 102.12 and *A. oryzae* 553.65, that were supplemented with exogenous nitrogen sources. One exception was noted however, where the addition of 5 % (w/v) urea

resulted in poor growth, thus impacting on enzyme yields, which were negligible in comparison to the yields realized from the control. It is conceivable that the fungi were unable to grow as a result of an increase in the pH of the substrate due to hydrolysis of the urea which can cause alkalinisation and inorganic acid assimilation (Raimbault, 1998). Gowda *et al.* (2004) remarked that the addition of urea to the medium inhibited the growth of *Aspergillus parasiticus* in solid state fermentation on potato dextrose agar. At concentrations as low as 0.1 % (w/v), the spore count of the fungus was reduced by 36 %, while increasing the concentration up to 0.5 % (w/v) inhibited spore concentration by 86 %. In this present study at 0.5 % (w/v), fungal growth was notably reduced when compared to the control fermentations, although the cellulase and xylanase activities were still increased despite the reduced fungal growth, which is likely due to the induction of enzyme production by the nitrogen.

The highest concentration of xylanase from the fermentation of corncob with *A. oryzae* 553.65 (Figure 3.4) was achieved with the addition of 5 % (w/v) yeast extract (YE), where a concentration of 3104 U g^{-1} was obtained, which constituted a 31-fold increase on the control fermentation. Bakri *et al.* (2003) investigated the production of xylanase from the SSF of wheat straw with *Penicillium canescens.* They also determined that optimal xylanase production was obtained through the addition of yeast extract to the substrate. In this present study maximal xylanase production from *A. niger* 102.12 (2602 U g^{-1}) activity was achieved with the addition of 5 % (w/v) peptone. This was in agreement with Qinnghe *et al.* (2004), who determined that the addition of peptone substantially increased xylanase production from *Pleurotus ostreatus* during submerged fermentation of corncobs and wheat bran. Similarly, Chander Kuhad *et al.* (1998) obtained optimal xylanase titres of 20 U mL^{-1} with peptone when *Fusarium oxysporum* was grown in SmF. It is worth noting, however, that considerable xylanase yields of 2425 U g^{-1} were also obtained with the addition of 5 % (w/v) yeast extract to the *A. niger* 102.12 fermentation medium, constituting a 14-fold increase relative to the control fermentation.

Optimal xylanase activity from the two inorganic nitrogen sources was obtained using 1 % and 0.5 % (w/v) ammonium sulphate (AS) from *A. oryzae* 553.65 and *A. niger* 102.12, respectively. This was in agreement with Seyis *et al.* (2005) who found that of the inorganic nitrogen sources tested for xylanase production from *Trichoderma harzianum*, optimum specific activity was displayed when ammonium sulphate was supplemented to the SmF medium.

The lowest increase in relative xylanase activity in comparison to the control was identified when ammonium dihydrogen phosphate (ADHP) was utilised as the nitrogen source. An increase in xylanase activity of 81 % was observed in this fermentation, which was relatively low in comparison to the increases in activity achieved with peptone and yeast extract.

Optimal cellulase production (165 U g^{-1}) from *A. oryzae* 553.65 (Figure 3.4) was obtained with the addition of 5 % (w/v) yeast extract to the fermentation. The addition of peptone also profoundly impacted on the production of cellulase, with a 10-fold increase in cellulase activity observed, although this was still substantially lower than the activity detected with yeast extract as a nitrogen source. Ammonium sulphate again performed best amongst the inorganic nitrogen sources tested (Figure 3.4), although in this case a higher concentration of 5 % (w/v) $(NH_4)_2SO_4$ was required. Liu *et al.* (2011) examined the effect of nitrogen sources on cellulase production from solid state fermentation of agricultural wastes with *Aspergillus fumigatus.* They determined that organic nitrogen sources such as yeast extract and peptone influenced cellulase production the most, while ammonium sulphate was the inorganic nitrogen source that yielded the greatest increase in cellulase activity, which were in agreement with the results presented in this study. Despite the low enzyme yields achieved with urea concentrations of 5 % (w/v), reasonable cellulase titres were obtained with urea at a dosage rate of 1 % (w/v) in the medium. These results were in accordance with Deswal *et al.* (2011) who similarly produced cellulase activities of 81 U g^{-1} with urea supplementation of a solid state fermentation with the brown rot fungus *Fomitopsis spp*; however, in their experiments cellulase production was higher with urea supplements than with yeast extract or peptone supplementation which was not the case in this present study. Similarly, Soni *et al.* (2010) produced relatively high β-glucosidase activity through the supplementation of an *Aspergillus fumigatus* fermentation of ground rice with concentrations of urea, although the endoglucanase activity for this treatment was notably lower than the other nitrogen supplements tested.

Optimal cellulase activity from *A. niger* 102.12 (315 U g^{-1}) was obtained when propagated in the presence of 5 % (w/v) yeast extract (Figure 3.5), which was substantially greater than the highest yields that were obtained through fermentation with *A. oryzae* 553.65 (165 U g^{-1}). With respect to inorganic nitrogen supplements, 1 % (w/v) ammonium phosphate supplementation produced the greatest increase in

cellulase activity, approximately 62 % higher than the control fermentations, while 1 % (w/v) ammonium sulphate also increased cellulase production by 58 %. Kalogeris *et al.* (2003) investigated the effects of nitrogen sources on the production of cellulolytic enzymes from *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes and determined that as the ammonium sulphate concentration increased up to 1 % (w/v), cellulase production also increased; however, above 1 % (w/v), cellulase activity gradually reduced, which was in agreement with the results displayed in the present study.

From these results, it can be concluded that the addition of 5 % (w/v) yeast extract to the fermentation medium had the most considerable impact on enzyme production from both *A. niger* 102.12 and *A. oryzae* 553.65, whilst peptone was also efficient at promoting cellulase and xylanase production. It is worth noting that although optimal xylanase production from *A. niger* 102.12 was achieved with 5 % (w/v) peptone there was nearly a 10-fold decrease in cellulase yield from fermentations with 5 % (w/v) peptone (33 U g^{-1}) in comparison to fermentations incorporating 5 % (w/v) yeast extract (315 U g^{-1}), with only a marginal difference between the two xylanase yields attained. As a consequence 5 % yeast extract was used as the optimal nitrogen source for enzyme production.

Limited literature is available on the rationale as to why nitrogen can be such an effective inducer of enzyme activity in solid state fermentation, although a number of hypotheses exist. The addition of nitrogen is thought to considerably affect the substrate pH which may result in alteration of the metabolic activity, enzyme production and catalytic activity (Haapala *et al.*, 1994). Although urea is a relativity good nitrogen source at lower concentrations (< 1 %), at high concentrations urea can inhibit growth of both fungi tested in this study. This is probably due to the formation of ammonia and can result in a rise in the pH of the medium, thus inhibiting fungal growth. The addition of ammonia in the form of ammonium sulphate to the substrate in this study may also have impacted on the pH, as it is transported as ammonia by fungi leaving behind the hydrogen ion resulting in an increase in pH, that can lead to fungal proliferation at a non-optimal pH, reducing enzyme activity (Lemos *et al.*, 2001). Lemos *et al.* (2001) investigated the effect of pH on xylanase production from *Aspergillus awamori* in solid state fermentation and determined that β-xylosidase activity was greatly increased in media containing citrate phosphate buffer (pH 5), whilst the endoxylanase activity remained the same throughout the fermentation. The

authors concluded that the endoxylanase was active and more stable over a wider pH range than β-xylosidase. Organic nitrogen sources, such as yeast extract, are thought to be good enzyme inducers as they contain a mixture of peptides of varying chain length, which are necessary for fungal growth (Marlida *et al.*, 2000; Bakri *et al.*, 2003), whilst they also contain other growth factors such as vitamins, trace elements and sugars that may influence enzyme production (Grant *et al.*, 1962).

Arst *et al.* (1973) determined that the *areA* gene product is required for the expression of genes encoding enzymes involved in nitrogen metabolism under ammonia depressed conditions. Additionally, Lockington *et al.* (2002) investigated whether the *areA* gene controls the total production of cellulase in *Aspergillus nidulans*. The authors determined that cellulase activity was clearly reduced when comparing a strain with a loss of function of the *areA* allele compared to a constitutively activating *areA* allele when grown in medium containing ammonium phosphate. It can thus be concluded that this gene plays an important role in regulation of nitrogen assimilation and despite it being beyond the scope of this project, further research into induction and regulation of *areA* may elucidate the effects of nitrogen supplementation found in this study.

In conclusion, it is apparent that nitrogen supplementation is a complex affair which depends on the ability of the organism to synthesise the required enzyme and also to respond to induction. Additionally, it can also depend on the effect of the nitrogen on the fermentation pH, which can be influenced by nitrogen assimilation (Lemos *et al.*, 2001).

3.2.2 Effect of initial moisture content on enzyme production

As remarked previously in Section 1.4.1.3, the moisture content in the solid state process can substantially influence a number of key factors such as fungal sporulation, growth rate, germination, metabolite production, heat transfer and gaseous diffusion (Gervais *et al.*, 2003). The moisture level in the fermentation can also be affected by substrate utilisation and the microorganism of interest. Generally in solid state fermentation, bacteria prefer higher moisture contents of over 70 % (w/v), while filamentous fungi require moisture levels of between 20-70 % (w/v). In solid state fermentation of filamentous fungi, the use of moisture contents over 70 % (w/v) can lead to increased competition from bacteria resulting in a fermentation which is more susceptible to contamination (Krishna, 2005). Control of the moisture level in the

fermentation can be carried out through the control of relative humidity (RH) within the fermentation chamber (Pandey, 1992). Without the control of the relative humidity within the system, the substrate can lose moisture affecting fungal growth and subsequent metabolite formation.

In the present study, the influence of initial moisture content on cellulase and xylanase production was investigated by altering the initial inoculum volume. Triplicate flasks of 20 g corncobs were inoculated with volumes ranging from 10-20 mL of diluted seed culture, as described in Section 2.2.4.2. The dilution of each inoculum was prepared so as to take into account the changes that can occur in mycelial biomass concentration as a result of an increased inoculum volume. Following this, flasks were incubated at 30 °C and 80 % RH for 5 days. Each flask was supplemented with 5 % (w/v) yeast extract in accordance with results that were achieved during optimisation of the media through nitrogen supplementation. Carbon sources were not used due to the relatively minor increases in activity compared to those achieved through nitrogen supplementation.

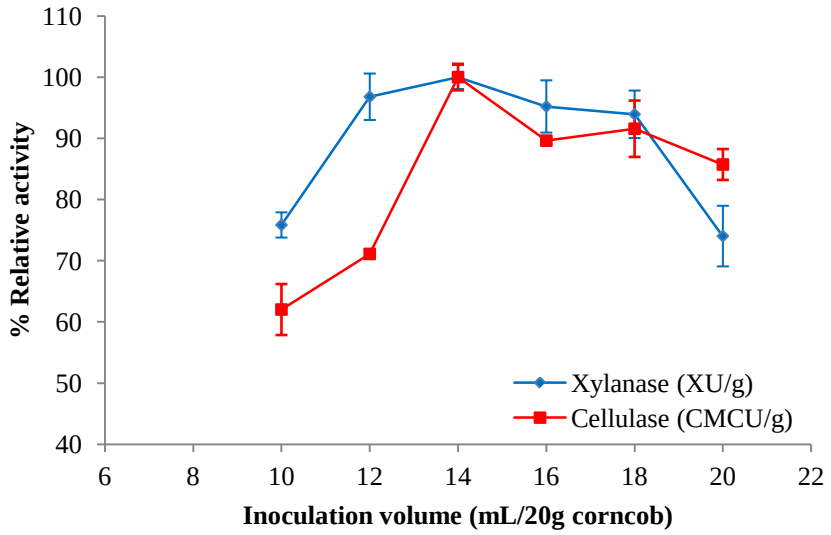


Figure 3.6 Effect of initial moisture content on cellulase and xylanase production from *A. niger* 102.12 in SSF.

Results plotted are the mean of triplicates with the standard deviation represented by error bars.

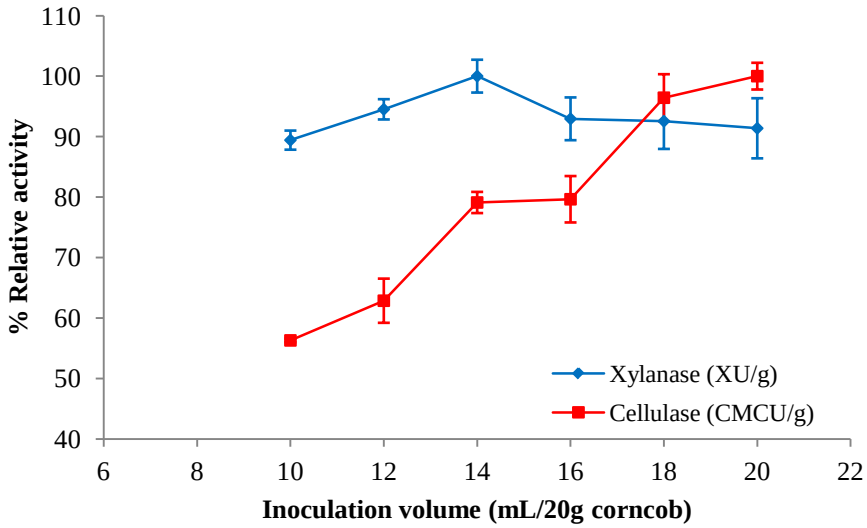


Figure 3.7 Effect of initial moisture content on cellulase and xylanase production from *A. oryzae* 553.65 in SSF.

Results plotted are the mean of triplicates with the standard deviation represented by error bars.

It is evident from Figure 3.6 that the optimal inoculum volume for cellulase and xylanase production from *A. niger* 102.12 was 14 mL per 20 g of corncob. With respect to *A. oryzae* 553.65 (Figure 3.7) the results are less clear. Optimal xylanase production occurred at 14 mL per 20 g, whereas optimal cellulase production was at 20 mL per 20 g. Consequently, an intermediate point where near optimal production of both enzymes could be achieved was desirable. The most suitable inoculum volume was 18 mL/20 g corncob, as less than 8 % of the optimal xylanase activity was lost, whilst only 3.5 % of the optimal cellulase activity was forfeited. At inoculum volumes lower than this, the xylanase activity was increased; however, cellulase production was reduced considerably, by up to 17 % of the overall cellulase activity. Furthermore, when the standard error of the means were taken into account for the xylanase samples there was minimal difference between the lowest activities obtained using 14 mL and the highest value achieved with 18 mL. With respect to both strains, cellulase activities tended to be most susceptible to the initial moisture level in the medium. In each case, the lower moisture level of 10 mL/20 g resulted in a loss of between 38-44 % of the overall relative activity. Xylanase activity from *A. oryzae* 553.65 was less variable with regard to changes in moisture content, with less than 11 % deviation in relative activity from the maximal xylanase attained. By comparison, xylanase production from *A. niger* 102.12 was far more variable, with a reduction of up to 25 % in relative activity on either side of the optimal inoculum volume.

These results were in agreement with other authors who confirmed that moisture content can have a considerable impact on the formation of enzymes in SSF, possibly as a result of early initiation of enzyme production due to more rapid growth than at low initial moisture levels (Bakri *et al.*, 2003; Gervais *et al.*, 2003; Pal *et al.*, 2010). Pal *et al.* (2010) concluded that 70 % initial moisture was the optimal for xylanase production from *Aspergillus niger* on a wheat bran/soybean substrate. This was notably higher than the optimal initial moisture of 53 and 59 % observed in *A. niger* 102.12 and *A. oryzae* 553.65 fermentations, respectively. The optimal initial moisture content can vary considerably depending on the substrate and strain of fungi used in the fermentation. For instance, the moisture level required for fermentation of *Aspergillus niger* on cassava and wheat bran was considerably lower than those required for growth on coffee pulp (Raimbault, 1998).

A range of between 10-20 mL per 20 g was chosen as the moisture range for analysis as this is the range that it was thought would be optimal for fungal growth (Krishna, 2005). Below 10 mL per 20 g, fungal growth can be severely inhibited due to lack of moisture, whereas above 20 mL per 20 g (which would equate to about 62 % moisture taking into account the moisture content of the corncob) the fermentation would generally be more susceptible to bacterial contamination. It was also worth noting that the moisture in the fermentation also affected fungal sporulation. At lower inoculum volumes, the sporulation of the fungi was visibly reduced in comparison to the fermentations containing a higher moisture content.

3.2.3 Effect of fermentation time on xylanase and cellulase production in SSF

Ultimately, the success of solid state fermentation for the production of enzymes is primarily dependent on the fermentation time. If the fermentation requires a lengthy period of time to produce significant quantities of enzyme, the fermentation would not be deemed economically viable. Due to the costs involved with regard to temperature control, pH manipulation, sterilisation and aeration, shorter fermentation times are typically favoured for a more economically feasible fermentation.

To determine the optimal fermentation period for maximum cellulase and xylanase production, triplicate flasks were harvested each day for a period of between 2-6 days and the xylanase and cellulase activities were determined from the cell free extracts using the methods described in Sections 2.2.2.1 and 2.2.2.2. The rate of

enzyme production was expressed as a relative percentage of the highest yield obtained.

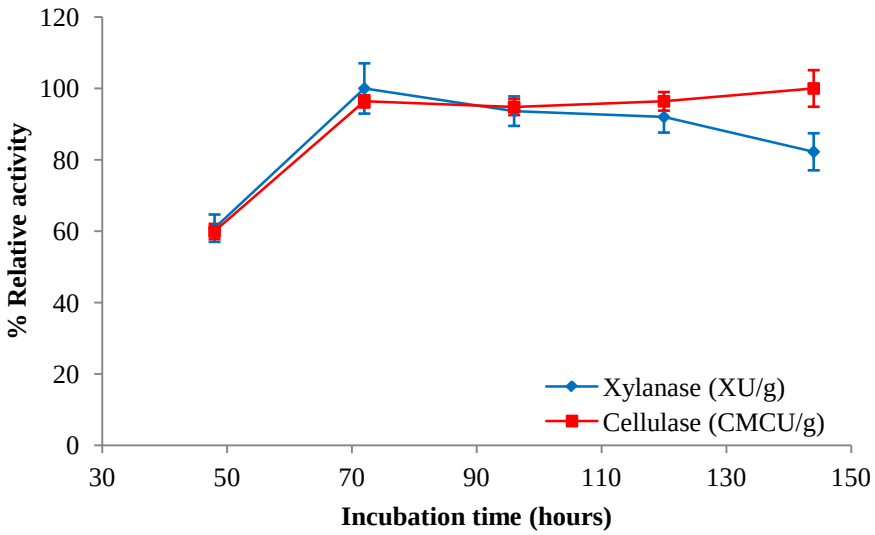


Figure 3.8 Time course of cellulase and xylanase production by *A. oryzae* 553.65 in solid state fermentation.

Results plotted are the mean of triplicate flasks with the standard deviation represented by error bars.

Optimal xylanase production from *A. oryzae* 553.65 (Figure 3.8) was produced after 72 hours of fermentation. Following this, xylanase gradually declined to just over 80 % of the optimal activity after 144 hours of fermentation. Pal *et al.* (2010) investigated the effect of fermentation time on the production of xylanase by *Aspergillus niger* in SSF. The authors concluded that optimum xylanase production was attained after 144 hours whereby production decreased considerably thereafter. They hypothesised that this reduction in xylanase yield could be due to the degradation of the xylanase by non-specific proteases which were secreted by the fungus, making optimisation of fermentation time a significant factor in enzyme production. Accordingly, the reduction in xylanase production observed after 72 hours of fermentation (Figure 3.8) could also be as a result of non-specific protease hydrolysis, with appreciable protease production observed during fermentation of corncobs with *A. niger* 102.12 (Figure 3.1).

Optimal cellulase production was accomplished after 144 hours of fermentation; however, during the final 72 hours of the fermentation an increase of only 4 % activity was noted, whilst a 20 % decrease in xylanase activity was observed over the same period. A useful parameter to assess the efficacy and economic viability

of the fermentation is volumetric productivity, which is measure of the amount of enzyme produced per hour of fermentation (Haltrich *et al.*, 1996). During fermentation, the volumetric productivity between day 2 and 3 was 1.74 U g h^{-1} whereas during the last three days of the fermentation the volumetric productivity was reduced to 0.05 U g h^{-1}. Accordingly, we can deduce that it is not economically viable to continue the fermentation for an additional 72 hours due to the low cellulase productivity of the fermentation. As a consequence, a 72 hour fermentation was chosen as the optimal day for harvesting koji so as to achieve near maximal cellulase and xylanase production.

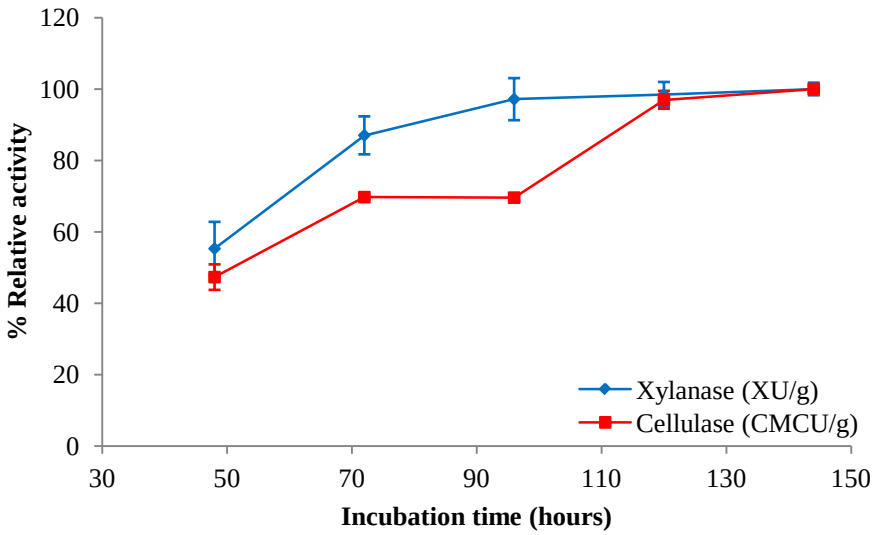


Figure 3.9 Time course of cellulase and xylanase production by *A. niger* 102.12 in solid state fermentation.

Results plotted are the mean of triplicate flasks with the standard deviation represented by error bars.

Optimal cellulase and xylanase activity from *A. niger* 102.12 (Figure 3.9) was attained after 144 hours of fermentation. In actuality, cellulase and xylanase activities were still increasing up until the 6^{th} day of fermentation and if the scope of this experiment was extended, higher concentrations may have been produced after 144 hours. However, when we assess the volumetric productivity of the fermentation for cellulase accumulation we can see that the productivity of the fermentation is reduced from 3.75 U g h^{-1} to 0.41 U g h^{-1}during the last 24 hours of the fermentation. Similarly, the volumetric productivity of xylanase is reduced from 12.79 U g h^{-1}during the third day of fermentation to 3.58 U g h^{-1} during the final days of fermentation. Consequently, it was concluded that continuing the fermentation after 5 days would

not be economically viable in a production scale facility, with 5 days being selected as the optimal time for enzyme production from *A. niger* 102.12.

The inoculum type can dramatically affect the fermentation length. In this experiment, vegetative cells were used instead of spores. Spore inocula can also be used as they offer longer stability during storage as well as enhanced resistance to mishandling (Krishna, 2005); however, as spores are dormant, metabolic activity must be induced before it starts to utilise the substrate and grow, meaning that a fermentation with a spore inoculum requires longer to initiate growth and enzyme production (Mitchell, 1992).

3.2.4 Effect of fermentation temperature on cellulase and xylanase production

Temperature is generally regarded as one of the most important physical parameters that can affect fungal growth and metabolite formation (Krishna, 2005). Despite fungi being able to grow over a wide range of temperatures, the optimum temperatures can differ substantially between mycelial growth and efficient metabolite production (Yadav, 1988).

Heat in SSF can be created through the control of the bioreactor temperature and through the formation of heat by fungal growth and metabolic activity (Raimbault, 1998). The temperature of the vessel in SSF is generally controlled by sparging humidified air through the system to either remove excess heat or to heat the fermentation if required. Without the removal of heat, thermal gradients can be formed, potentially resulting in denaturation of the product of interest. However, in laboratory scale fermentation this accumulation of heat is not as significant and the control of the temperature is more manageable (Muller dos Santos *et al.*, 2004).

The effect of temperature on cellulase and xylanase production from *A. niger* 102.12 and *A. oryzae* 553.65 in SSF was determined by cultivating these strains at different temperatures, ranging between 25-40 °C. Previously optimised fermentation conditions for enzyme production of 72 hours, 18 mL of inoculum per 20 g and 5 % (w/v) yeast extract were used for fermentation with *A. oryzae* 553.65, whilst fermentation conditions of 120 hours, 14 mL per 20 g and 5 % (w/v) of yeast extract were used for enzyme production from *A. niger* 102.12. As 30 °C was used for each of the previous optimisation experiments, the relative activity of each fermentation was determined against this temperature. Triplicate flasks were propagated and the enzyme activity of each set of flasks was plotted (Figures 3.10 & 3.11).

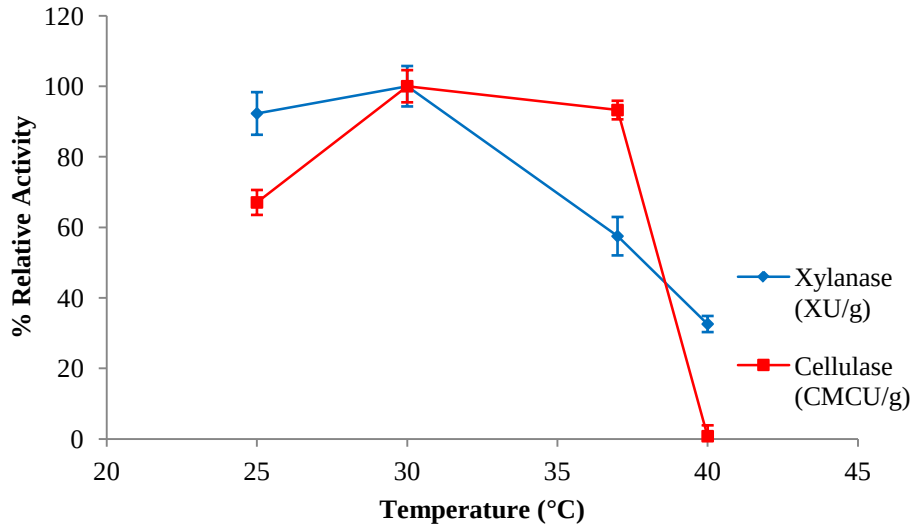


Figure 3.10 Effect of fermentation temperature on cellulase and xylanase production by *A. oryzae* 553.65.

Results plotted are the mean of triplicate samples where the standard deviation is represented by error bars.

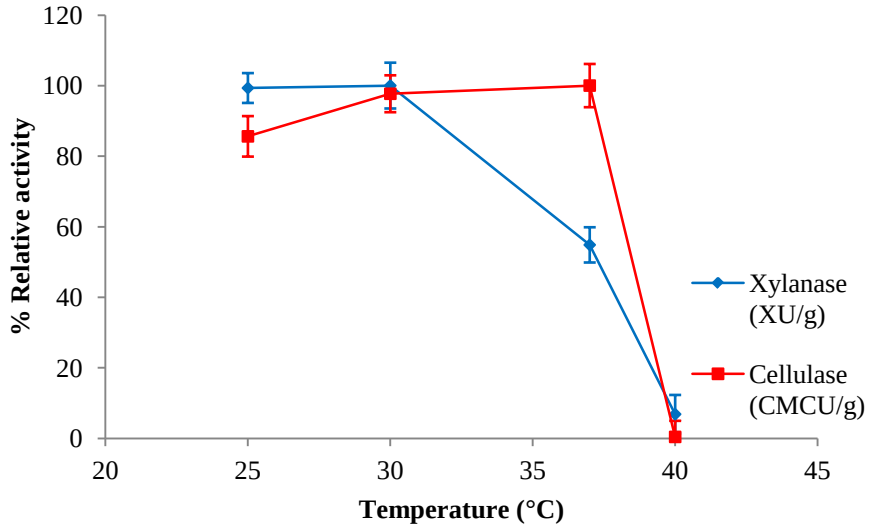


Figure 3.11 Effect of fermentation temperature on cellulase and xylanase production by *A. niger* 102.12.

Results plotted are the mean of triplicate samples where the standard deviation is represented by error bars.

As illustrated in Figure 3.10, the optimum temperature required for maximum cellulase and xylanase production from *A. oryzae* 553.65 was 30 °C. A steady rate of decline in xylanase production was noted after this point where the activity was reduced by over 60 % as the temperature was increased by 10 °C. With regard to

cellulase production, an increase of 7 °C, from 30 °C to 37 °C, resulted in minimal loss in cellulase activity; however, enzyme activity was considerably reduced when the temperature was increased to 40 °C, with the cellulase activity being decreased by over 99 %.

A similar trend was observed in Figure 3.11, where the production of cellulase and xylanase from *A. niger* 102.12 was determined. Optimal xylanase production was observed at 30 °C, after which levels decreased considerably, resulting in a 93 % reduction in relative xylanase activity at 40 °C. Conversely, optimal cellulase production was noted at 37 °C, with relative cellulase production being reduced by over 99 % at 40 °C or above. It is worth noting however, that although maximal cellulase production was noted at 37 °C, xylanase titres were reduced by nearly 50 % in comparison to its optimal at this temperature. As a consequence, 30 °C was selected as the optimal temperature for enzyme production as only a 3 % decline in cellulase activity was noted in comparison to when xylanase production was at sub-optimal temperatures.

These results are generally in agreement with relevant published literature available which assessed the production of cellulase and xylanase from SSF. For instance, Jecu (2000) analysed the production of endoglucanase from the solid state fermentation of wheat straw and wheat bran with *Aspergillus niger* 38. The author determined that the optimal enzyme activity was between 30 and 34 °C, whilst as the temperature approached 37 °C a significant decrease in activity was observed. Similarly, Guoveia *et al.* (2011) examined the production of cellulase from *Trichoderma reesei* in SSF. They also determined that optimal cellulase production was at 30 °C, with activity being significantly reduced thereafter to a point where cellulase production was completely inhibited at 40 °C. Despite cellulase production being almost completely eradicated in this present study, xylanase production was not as considerably affected, which was broadly in line with Lu *et al*. (2003). Lu *et al.* (2003) perceived that the optimal temperature for xylanase production from *Aspergillus sulphureus* was between 30 and 33 °C; however at 40 °C the xylanase activity was reduced by nearly 40 % and by over 90 % at 47 °C.

It is thought that the reduction in enzyme activity above 37 °C could be as a result of poor growth from the fungi at higher temperatures (Lu *et al.*, 2003). In the present study when harvesting flasks during the experiment, the growth of the mycelia on corncob at 40 °C was visibly lower than that in the flasks propagated at 30 and 37

°C, while at lower temperatures of 25 °C the fungal growth was similarly poor. Smits *et al.* (1998) measured the influence of temperature on the radial growth rate of *T. reesei* and determined that above 37 °C the growth rate of the fungus was severely inhibited, while the optimal temperature for radial growth was observed at between 32-37 °C.

3.3 Crude profiling of xylanase and cellulase activity

The conditions of an assay can have a discernible impact on the measurement of enzyme activity. Bailey *et al.* (1992) investigated the difference in xylanase activity of a specific enzyme preparation from *Trichoderma reesei* by a number of different laboratories around the world using their own in-house method for detecting endo-1,4-β-xylanase activity. When the results were correlated from each lab using different methods, a standard deviation of 108 % was noted between the results obtained. However, when the assay conditions, including the substrate were standardised, the standard deviation was reduced to 17 % between the 17 different laboratories.

It can be difficult to compare results of experiments between published works as there is no universal standard method for detection and quantification of a large number of enzymes. For instance, when determining the cellulase activity from solid state samples a number of methods can be used like the DNS method, the release of a substrate from a dye, loss in weight of a cellulosic substrate or the decrease in viscosity of a soluble cellulose suspension (Highley, 1997). In order to effectively compare samples of interest, a method of analysis must first be chosen depending on the application of the enzyme of interest. In this body of work, the DNS method was used because of the ease of the assay and the potential high throughput of samples allowing for rapid determination of cellulase and xylanase activity.

Cellulase and xylanase enzymes are produced by a diverse number of microorganisms in which the characteristics of these enzymes are inherently different. For instance, extremophiles can produce enzymes which are stable at above 100 °C, which would not be the case with enzymes from mesophilic fungi (Iyer *et al.*, 2008). As such, the optimal conditions for cellulase and xylanase determination were assessed, as these conditions can influence the quantitation of the enzyme. To improve the detection capacity of the assay, this work focused on the effect of assay temperature and pH on the cellulase and xylanase activity, while the substrate concentration and reaction time remained constant.

3.3.1 Effect of assay pH on cellulase and xylanase activity

To assess the effect of assay pH on activity, crude enzyme extracts were appropriately diluted in buffered solutions, with each solution prepared at a different pH. The substrate for each sample pH was also prepared using the same buffer at the specified pH. A glycine-HCl buffer was employed to prepare buffers at pHs of 2.0-3.0, a sodium citrate buffer was adopted in the preparation of solutions between pH 3.0-6.0 and sodium phosphate was used to prepare buffers between pH 5.7-8.0.

To further assess the effect of pH on the activity of the enzyme, overlapping pH values were created between each different buffer set. For example, two samples were prepared to analyse the enzyme activity at pH 3, one with a glycine buffer and one with a citrate buffer. Buffers with overlapping pH were used to assess the influence of the buffer type on cellulase and xylanase activity during detection. Cellulase and xylanase activities were determined using the DNS method for analysing reducing sugars, as detailed in Sections 2.2.2.1 and 2.2.2.2. Each sample was expressed as a relative percentage of the maximum activity achieved from each enzyme. It is worth noting that although the cellulase and xylanase activities are expressed as one activity they could potentially contain multiple cellulase and xylanase enzymes.

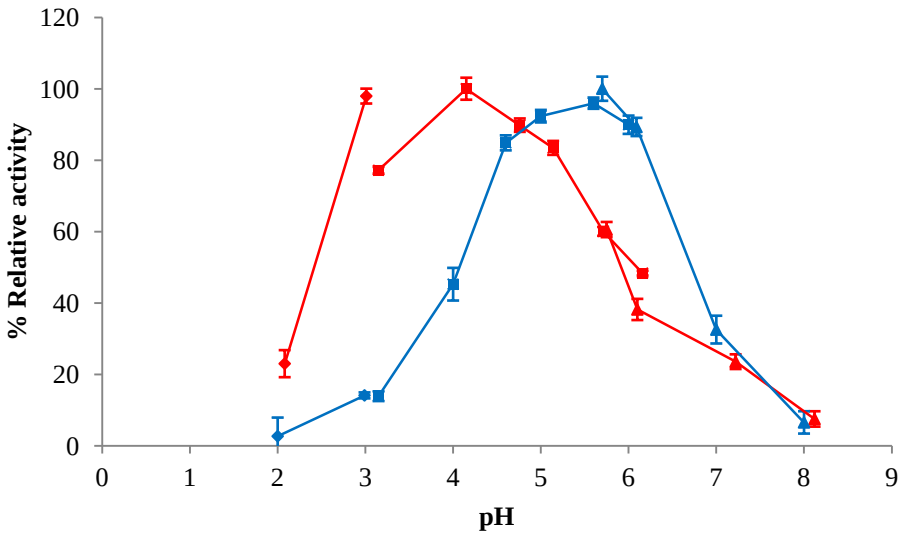


Figure 3.12 pH versus relative activity profiles for cellulase and xylanase enzymes produced from *A. oryzae* 553.65.

Results plotted are the mean of triplicate flasks with the standard deviation represented by error bars. CMCU g^{-1} is displayed using red data points, while XU g^{-1} is represented in blue. Buffers used were; glycine-HCl (♦), sodium citrate (■), sodium phosphate (▲).

As indicated in Figure 3.12, the optimal pH for cellulase activity was observed at pH 4.15 with the relative activity declining steadily thereafter. At pH 8.0 for instance, the relative activity of the enzyme was reduced by over 92 % of the optimum. Similarly at lower pH values, the activity of the enzyme was also profoundly reduced. For example, the activity at pH 2.0 was only 20 % of the optimal activity; although, as the pH increased, the relative activity was also significantly increased. It is worth noting however, that the solutions are at the extremes of their buffering capabilities at low pH values and this is likely to impact on the activity of the enzyme.

Maximal xylanase activity was detected at pH 5.7, which was markedly higher than the pH at which optimal cellulase activity was observed. At lower (pH 2.0) and higher (pH 8.0) pH values the relative activity of xylanase is reduced by over 93 % in comparison to the optimal activity at pH 5.7. This clarifies the importance of the usage of enzymes at the appropriate pH, as the activity of the enzyme activity can be severely reduced at sub-optimal pH. Cellulase appeared to be most active between pH 3.0 and 5.14, with only 17 % of the relative cellulase activity lost over this range. Xylanase tended to be most active between pH 4.6 and 6.1, with a reduction of 16 % of relative activity noted between these values. With the exception of the enzyme samples assayed for cellulase prepared in two different buffers at pH 3, the relative activity of the other buffers prepared at the same pH produced similar relative activities for the crude enzyme extracts.

It was apparent through analysis of Figure 3.12 that an appreciable difference (~20 %) can be detected between the relative activity of the enzyme assayed at pH 3.0 using glycine-HCl compared to the enzyme samples prepared using sodium citrate at pH 3.0. Salts can affect the secondary and tertiary structure of the enzyme as well as the protein-protein interactions in multimeric enzymes. In addition they can also affect the pKa of the catalytic residue through either non-specific ionic strength effects or through non-specific interactions (Bowers *et al.*, 2007). Bowers *et al.* (2007) investigated the effect of salt on β-glucosidase activity and determined that most of the inhibition caused by salt is thought to be as a result of shift in the enzymatic pKa and not as a result of an effect on the pH independent second rate constant (Bowers *et al.*, 2007). They also concluded that the salt concentration had a significant impact on the secondary and tertiary structures of the enzyme activity. The effects of salts generally follow the Hofmeister series which is a classification of ions in order of their ability to salt in or out proteins (and is especially evident with anions) (Bauduin *et al.*, 2006).

Franz Hofmeister (1888) discovered a number of salts which had a consistent effect on protein solubility and the stability of its secondary and tertiary structure. The ions in the Hofmeister series ordinarily affect the performance of the enzyme by acting as the substrate, the cofactor, or the inhibitor of the enzyme as well as altering the bulk water structure, thus affecting the protein water interactions (Yang, 2009). At low concentrations of up to 10 mM, ions tend to affect the performance through various electrostatic interactions; however, at concentrations above this, the Hofmeister ion effect becomes more important as the ionic dispersion forces tend to dominate (Yang *et al.*, 2010). It can thus be concluded that the difference in enzyme activity is as a result of the ions in the sodium citrate buffer affecting the performance of the enzyme by affecting the secondary and tertiary structures of the enzyme.

The results from this study are to be in agreement with other studies that have examined the effect of pH on enzyme activity. Fang *et al.* (2008) purified and characterised xylanase produced from *Aspergillus carneus* and determined that optimal xylanase activity was at pH 6.0. Similarly, Christov *et al.* (1999) observed that the maximal xylanase activity produced from *Aspergillus oryzae* was also at pH 6.0.

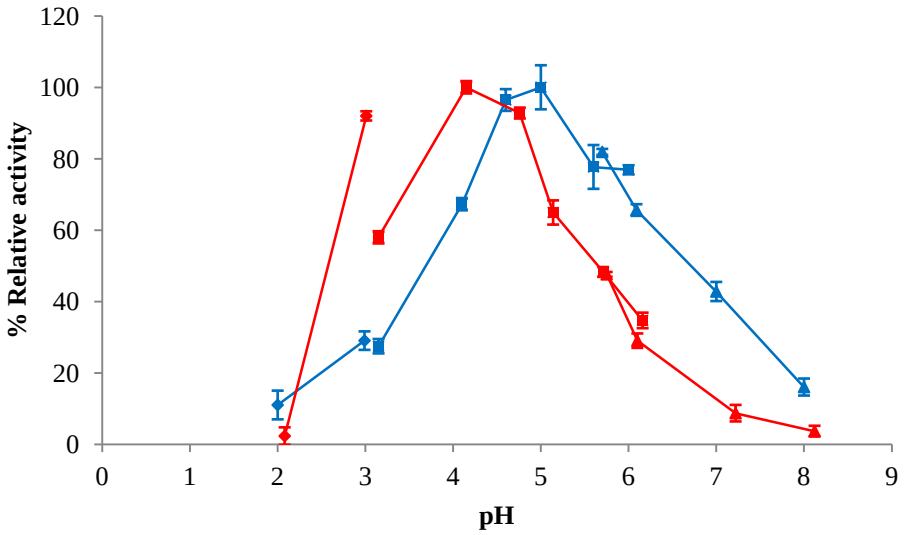


Figure 3.13 pH versus relative enzyme activity profiles for cellulase and xylanase enzymes produced from *A. niger* 102.12.

Results plotted are the mean of triplicate flasks with the standard deviation represented by error bars. CMCU g^{-1} is displayed using red data points, while XU g^{-1} is represented in blue. Buffers used were; glycine-HCl (♦), sodium citrate (■), sodium phosphate (▲).

The affect of assay pH on the activity of cellulase and xylanase produced from *A. niger* 102.12 (Figure 3.13) was largely in line with the results obtained from *A.*

oryzae 553.65 (Figure 3.12). Optimal pH for cellulase activity was also noted at pH 4.15, whereas for xylanase it was observed at pH 5.0, which was marginally lower than the optimal pH for xylanase produced from *A. oryzae* 553.65. Lui *et al.* (2006), investigated the production of xylanase from a strain of *Pichia pastoris* expressing xylanase from *A. niger* and concluded that the optimum pH was at pH 5.0 which was similar to results shown in this study from *A. niger* 102.12. Meanwhile, Hurst *et al.* (1977) analysed the effect of pH on cellulase activity produced from *A. niger* and they determined that the optimal pH for cellulase activity was at pH 4.0, with the activity being reduced to less than 5 % of the maximal yield at pH 7.0, which was in strong agreement with the results from Figure 3.13.

In contrast to the enzymes produced from *A. oryzae* 553.65, cellulase and xylanase activities from *A. niger* 102.12 were stable over a narrower pH, with an approximately 40 % reduction in relative cellulase activity when the pH was reduced or increased by one unit either side of the optimal pH. Similarly, the relative activity of xylanase was reduced by 33 % when the pH was decreased to pH 4.1 from the optimum of pH 5.0, while the relative activity was decreased by between 24 and 34 %, depending on the buffer, when the pH was increased to pH 6.0. It should also be considered that when analysing the relative xylanase activity of two different buffers at the same pH (pH 6.0) a difference of 11 % of relative activity was noted.

The pH dependent activity of an enzyme is set primarily by the pK_a values of one or a number of ionisable groups which function as nucleophiles, carboxylic acids, electrophiles or general acid/base catalysts that are situated within the active site cleft (Joshi *et al.*, 2000). A comparison of the sequence of a number of low molecular mass endo-β-(1,4)-glycosidases from eukaryotic and bacterial species revealed that enzymes with optimal activity in the alkaline pH range, had asparagine bound to the general acid/base catalyst. However, when the optimum pH was in the more acidic range, the asparagine was replaced by aspartic acid (Torronen *et al.*, 1997). Fushinobu *et al.* (1998), investigated this development through mutational analysis of *Aspergillus kawachii* xylanase C. The authors determined that substitution of asparagine with aspartic acid increased the pH optimum of the xylanase from pH 2 to pH 5. Similarly Joshi *et al.* (2000) studied how a single amino acid substitution could modulate the optimum pH of an alkaline xylanase from *Bacillus circulans*. They noted that the active site of this enzyme contained two acid residues Glu78 (pKa 4.6) and Glu172 (pKa 6.7) which function as a nucleophile and as the general acid/base catalyst,

respectively. When they substituted the asparagine residue which resided beside the general acid/base with an aspartic acid they found that the optimum pH of the enzyme was reduced from 5.7 to 4.6.

3.3.2 Thermal activity profiling of cellulase and xylanase activity

The optimal temperature under which an enzyme can operate is essential to determine whether the enzyme is suitable for the application of interest. For instance, in applications such as bioethanol production a cellulase or xylanase enzyme which is optimally active at temperatures between 30 °C and 60 °C would be ideal for use in simultaneous saccharification and co-fermentation.

The optimal temperature for cellulase and xylanase activity from *A. niger* 102.12 and *A. oryzae* 553.65 was determined by performing the standard DNS assay described in Sections 2.2.2.1 and 2.2.2.2 under different assay temperatures ranging between 25 and 90 °C, with respective optimal pHs, as determined in Section 3.3.1. The results were plotted as a function of the assay temperature versus relative activity, where 100 % relative activity was defined as the maximum activity achieved (Figures 3.14 & 3.15).

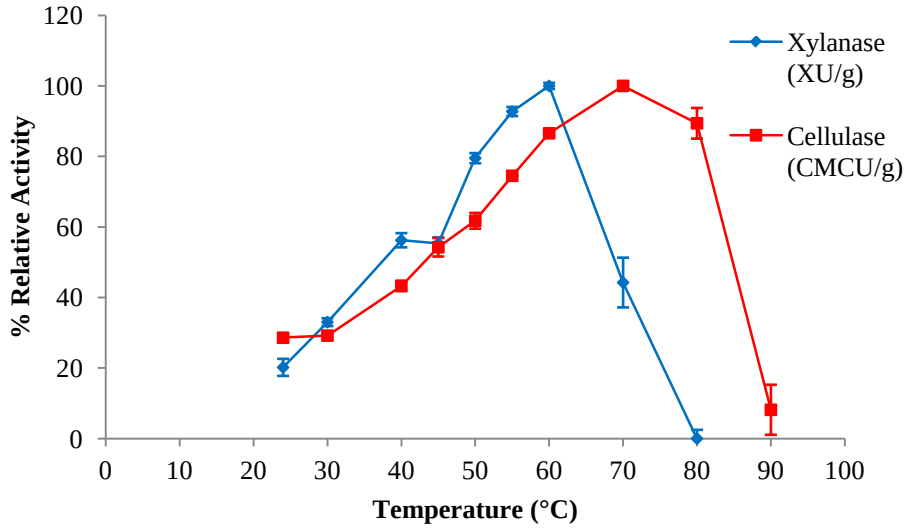


Figure 3.14 Temperature versus relative enzyme activity profiles for cellulase and xylanase enzymes produced from *A. oryzae* 553.65.

Results plotted represent the mean of triplicate flasks with the standard deviation being represented by error bars.

From the results presented in Figure 3.14 it was concluded that the optimal assay temperature for xylanase produced from *A. oryzae* 553.65 was 60 °C. Above this temperature, the activity of the enzyme is dramatically reduced with no activity detected at 80 °C and above. This considerable reduction in activity is likely due to the low conformational stability of the enzymes. The weak forces that are responsible for maintaining the secondary, tertiary and quaternary structure of the enzymes can be denatured by heat, through alteration of the balance of these weak non-bonding forces (Voet *et al.*, 1990a). Typically when a protein in solution is heated, properties which are conformationaly sensitive such as the viscosity and the optical rotation change which result in the unfolding of the protein. Furthermore, the remaining structure which was not unfolded is destabilised resulting in the loss of the biological activity of the protein or enzyme (Voet *et al.*, 1990a). This denaturation of protein is not just induced through heating but can also be as a result of exposure to strong mineral acids/bases, ionic detergents, chemotropic agents, heavy metals and organic solvents.

Results obtained in this study are broadly in line with other authors such as Christov *et al.* (1999), who determined that xylanase produced by *A. oryzae* was optimally activity at 60 °C, while the range in which the enzyme retained over 85 % of its residual activity was similarly narrow.

Optimal cellulase activity from *A. oryzae* 553.65 (Figure 3.14) was achieved at 70 °C, while over 86 % of the activity was retained over a range of elevated temperatures from 60-80 °C. This enzyme would be ideal for industrial processes which require thermally active cellulase enzymes such as in the animal feed industry where pelleting can denature the enzyme and in bioethanol production where thermotolerant enzymes would be suitable for the saccharification process. It is worth noting that cellulase was substantially more thermotolerant than xylanase with over 86 % relative activity remaining at 80 °C, whilst no relative xylanase activity was detected at this temperature as the enzyme was denatured. Mawadza *et al.* (2000) produced cellulase from two strains of *Bacillus spp.*, both of which were optimally activity at 70 °C. Typically, the optimal temperature for cellulase produced from a mesophilic fungus such as *A. oryzae* would be between 50 and 60 °C (Hurst *et al.*, 1977; Gawande *et al.*, 1998; Ghanem *et al.*, 2000; Fang *et al.*, 2008 ; Lee *et al.*, 2008; Li *et al.*, 2010; Liu *et al.*, 2011), with the optimal for bacterial cellulases usually higher at about 70 °C. It was thus somewhat unexpected to note that the optimal temperature for cellulase from *A. oryzae* 553.65 was at 70 °C.

As the temperature of the assay approaches 25 °C, the activity of both cellulase and xylanase from *A. oryzae* 553.65 was reduced by as much as 80 % of the optimal activity produced. It is also worth noting that at 80 °C the cellulase enzyme still retained 90 % of the maximum activity; however, when the temperature was increased by 10 °C the activity was reduced by 80 %, which illustrates the sensitivity of enzymes toward changes in temperature.

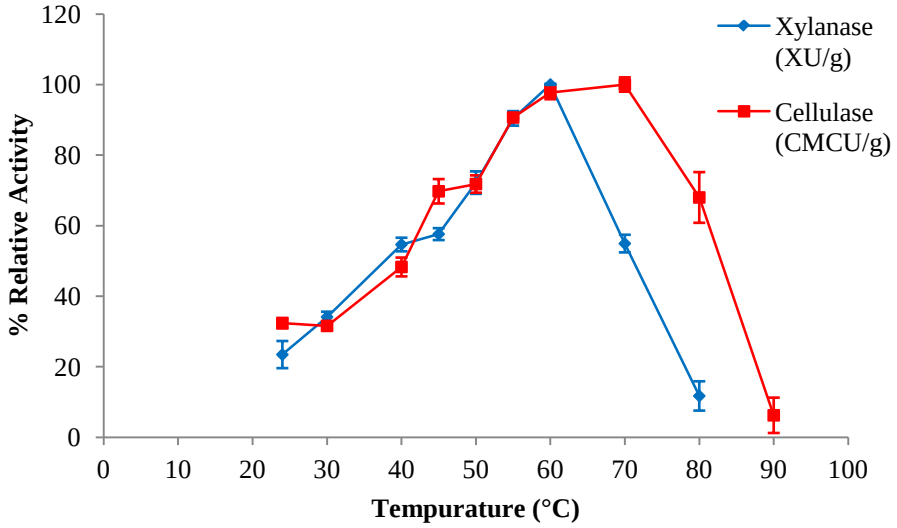


Figure 3.15 Temperature versus relative enzyme activity profiles for cellulase and xylanase enzymes produced from *A. niger* 102.12.

Results plotted represent the mean of triplicate flasks with the standard deviation being expressed by error bars.

When analysing the results from Figures 3.14 & 3.15 it was evident that the temperature profiles for cellulase and xylanase largely followed the same trend as those displayed by *A. oryzae* 553.65, where the maximal activity of cellulase was obtained at 70 °C, while maximal xylanase activity was observed at 60 °C. It was also observed that the cellulase enzyme produced from *A. oryzae* 553.65 tended to be more thermotolerant with relative cellulase activity from *A. niger* 102.12 being reduced dramatically after 70 °C.

3.4 Stability of cellulase and xylanase enzymes

In many applications the stability of the enzyme of interest is of great importance as inherent advantages such as high specificity, unparalleled activity under mild conditions, high turnover number and biodegradability can be overshadowed by

instability which can cause difficulties in production, processing and storage (Iyer *et al.*, 2008).

Enzymes are complex proteins that have many roles in biological processes, with almost all functions in biological cells requiring enzymes to proceed. Proteins in their native state are only slightly stable and depend on various non-covalent influences to confer structural stability such as hydrogen bonding and hydrophobic forces (Voet *et al.*, 1990b). Proteins possess a highly complex three dimensional structure with which the properties of the protein are determined. The primary structure is associated with the covalent bonds which bind the atoms in the protein together, while the secondary structure involves the hydrogen bonds which are found between the atoms that form the α-helix and β-sheets structures. The tertiary structure is its three dimensional structural arrangement which forms as a result of the folding of its secondary structures. Finally, the quaternary structure is the conformational fitting of two protein molecules together to carry out a specific function (Bischof *et al.*, 2006). When this structure is altered to inhibit the action of the protein, unravelling of the structure by alteration of the hydrogen bonds that hold the tertiary structure together occurs in a process known as denaturation (Bischof *et al.*, 2006). This denaturation can be caused by a number of conditions and substances such as a change in the pH of the liquid, heat treatment, detergents, solvents and salts (Voet *et al.*, 1990b).

To assess the pH and thermal stabilities of the xylanase and cellulase produced from the two fungal strains, an aliquot of each crude enzyme extract was appropriately diluted (1/110) with 50 mM sodium citrate (pH 3.0-6.0) or 50 mM sodium phosphate (pH 6.1-8.0) depending on the pH required for the solution. Each mixture was subsequently incubated at different temperatures ranging between 24 to 60 °C, with samples taken at specific intervals and analysed for cellulase and xylanase activity using the assays outlined previously in Sections 2.2.2.1 and 2.2.2.2. The activity of the samples were displayed as a relative percentage of the maximal activity achieved from each sample set at a specific pH and temperature.

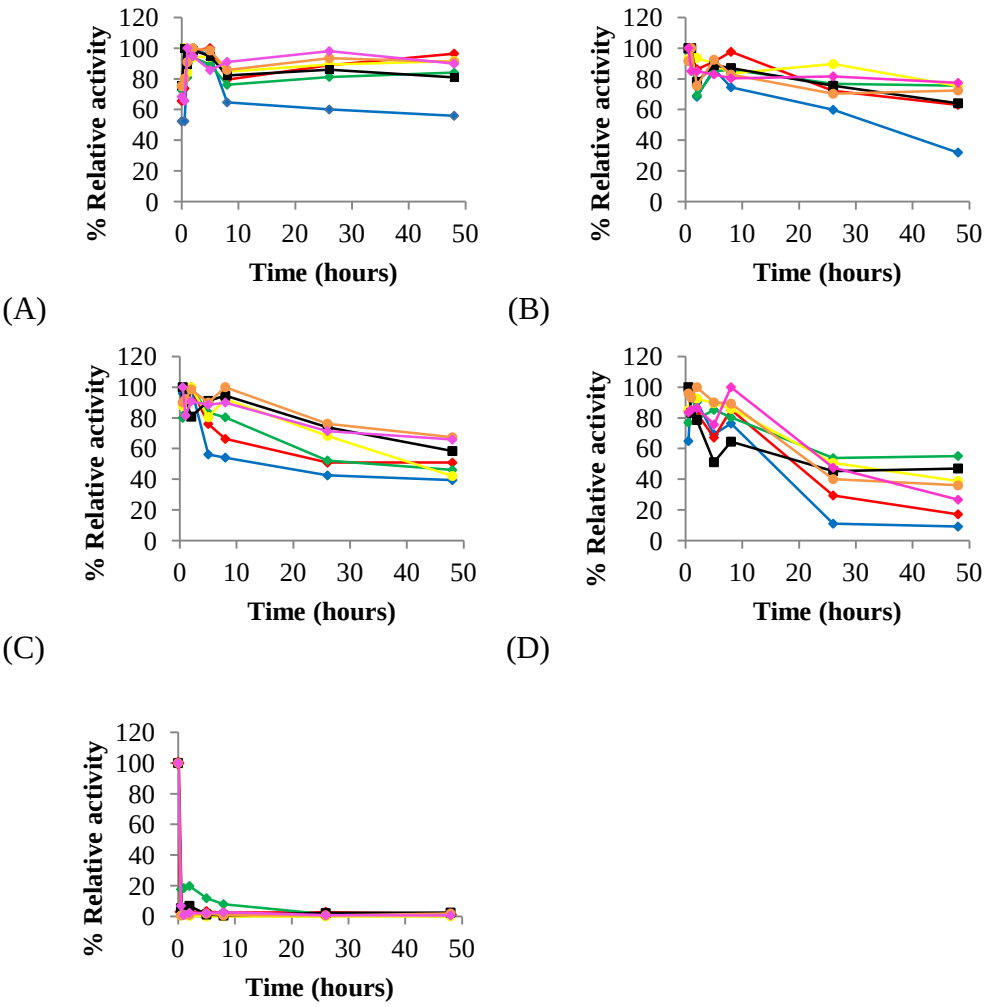


Figure 3.16 Stability of xylanases produced by *A. niger* 102.12 incubated at a range of different temperatures and pHs.

Legend: pH 3.1 (♦), pH 4.1 (♦), pH 5.1 (♦), pH 6 (●), pH 6.1 (■),pH 7 (●), pH 8 (♦)
(A): 24 °C, (B): 30 °C, (C): 40 °C, (D): 50 °C, (E) 60 °C

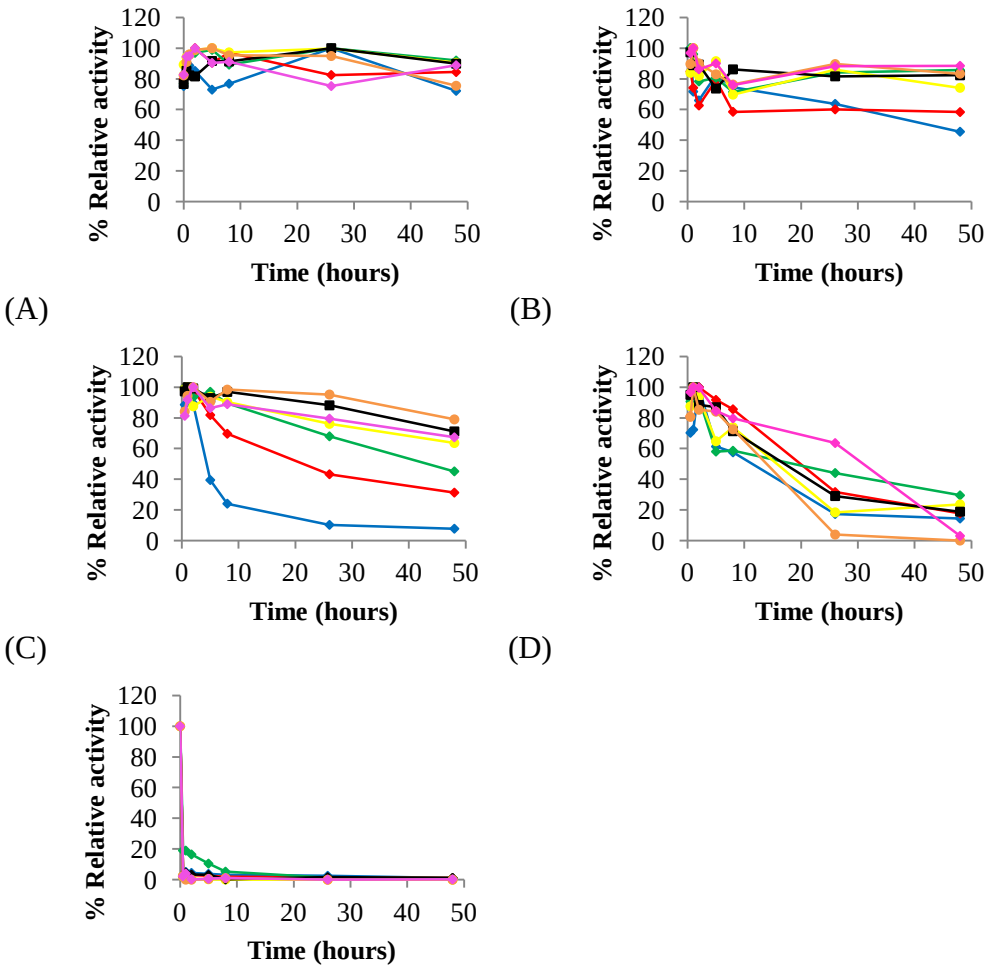


Figure 3.17 Stability of xylanases produced *A. oryzae* 553.65 incubated at a range of different temperatures and pHs.

Legend: pH 3.1 (♦), pH 4.1 (♦), pH 5.1 (♦), pH 6 (●), pH 6.1 (■),pH 7 (●), pH 8 (♦)
(A): 24 °C, (B): 30 °C, (C): 40 °C, (D): 50 °C, (E) 60 °C

As can be determined from Figures 3.16 & 3.17 (E), the xylanase activity was significantly affected when the temperature was increased to 60 °C, where with the exception of the samples stored in buffer at pH 5.1, the xylanase activity from both strains was completely denatured after 30 minutes of incubation. This was possibly as a result of thermal denaturation of the samples where the bonds responsible for maintaining the secondary and tertiary structure of the enzymes are broken resulting in protein unfolding (Rodwell, 1988; Voet *et al.*, 1990b). At pH 5.1, (60 °C) the xylanase from *A. niger* 102.12 and *A. oryzae* 553.65 were marginally more thermostable than at any other pH, with the activity being reduced to between 18-20 % of the optimal activity after 30 minutes. Furthermore, after 8 hours the xylanase was reduced to between 5-8 % of the maximal activity achieved. It is also worth noting that although

the optimal assay temperature for detection of xylanase activity from *A. niger* 102.12 (Figure 3.15) and *A. oryzae* 553.65 (Figure 3.14) was at 60 °C, almost complete denaturation occurred after 30 minutes at this temperature (Figures 3.16 & 3.17 E). It is likely that although this is the optimum temperature for the enzymes, denaturation can also occur at this point. Barton (1979) investigated mathematically, the denaturation of proteins at their optimum temperature and demonstrated that denaturation not only causes the optimum temperature but that protein degradation can also occur at this point.

At 24 °C the xylanase from both strains (Figures 3.16 & 3.17 A) was relatively stable, with the exception of xylanase produced from *A. niger* 102.12 at pH 3.1 (Figure 3.16 A), which was moderately unstable, with the activity decreasing to 55 % of the maximal activity attained. At 30 °C, xylanase from both strains (Figures 3.16 & 3.17 B) was relatively unstable at pH 3.1, where the activity decreased by 70 % and 55 % for *A. niger* 102.12 and *A. oryzae* 553.65, respectively after 48 hours of incubation. Xylanase from *A. niger* 102.12 was also somewhat unstable at pH 4.1 where a reduction of 40 % in the activity was noted over the course of the incubation. Xylanase from *A. oryzae* 553.65 (Figure 3.16 B) tended to be most stable between pH 5.1 and pH 8 with over 74 % of the enzyme activity being retained following incubation for 48 hours. Xylanase from *A. niger* 102.12 (Figure 3.17 B) was typically less stable than that from *A. oryzae* 553.65. For instance, at pH 5.1 xylanase activity from *A. niger* 102.12 was reduced to 76 % of the maximal activity, whilst the activity from *A. oryzae* 553.65 at pH 5.1 was only reduced to 86 % of the maximal.

At 40 °C the xylanase for *A. oryzae* 553.65 (Figure 3.17 C) tended to be most stable at higher pHs of between 6-8 with over 63 % of relative activity being retained after 48 hours, while xylanase from *A. niger* 102.12 (Figure 3.16 C) was also the most stable between pH 6-8 with over 60 % of activity being preserved. At 50 °C xylanase produced from both strains (Figures 3.16 & 3.17 D) tended to be most stable at pH 5.1, where the activity of xylanase from *A niger* 102.12 was reduced by 40 %. Furthermore, xylanolytic activity from *A. oryzae* 553.65 was decreased by 70 % after 48 hours in comparison to 90 % of activity being lost when stored at pH 3.1 at the same temperature.

It is worth noting that during the first number of hours an increase in activity was observed in many denaturation curves before a reduction in activity due to denaturation occurred (Figure 3.16 & 3.17). A number of factors may have been

responsible for this. Buffers can confer a conformational change in the stability of the enzyme or protein of interest during freezing. Prior to diluting the sample in buffer at the appropriate pH the crude enzyme extracts were stored in a sodium citrate buffer at pH 7 at 4 °C to retain the catalytic activity of the enzyme. Once all the samples were collected the samples were thawed and assayed for enzyme activity. When freezing a buffered protein, one constituent of the buffer, for example the salt can freeze before the other constituents such as the base or acid which can result in severe changes in the pH of the solution causing denaturation of the protein (Wang, 2000). For instance, lactate dehydrogenase was denatured during freezing owing to crystallisation of the ammonium sulphate which resulted in a drop in pH of the solution from 7.5 to 4.5 resulting in denaturation (Anchordoquy *et al.*, 1996). Additionally changing the buffer constituents can often decrease the pH that occurred during freezing. As appears to be the case, the majority of the first set of samples appear to have lower relative activity than the samples taken after 30 minutes which could be due to denaturation of this sample set due to non uniform mixing and gradient formation which has led to enzyme denaturation. Hellerbrand *et al* (1998) determined that replacing the sodium with potassium in a phosphate buffer could reduce the shift in pH during the freezing process prior to lyophilisation. Alternatively, partial unfolding of the enzyme structure may have occurred as the temperature increased from 4 °C to the actual incubation temperature. This enzyme was subsequently frozen and may have remained in a partially unfolded state and thus more readily formed enzyme substrate-complexes during the assay following thawing, which lead to the spike in activity. Vieira *et al.* (2009) determined that in the absence of xylobiose, the thumb region of the substrate binding cleft from a xylanase exhibited temperature dependent movement, which at optimum temperatures, adopted a more open conformation, facilitating binding in the active site. As the enzyme was incubated beyond 1 hour in this present study, these activity increases were negated by the onset of enzyme denaturation.

A number of thermal stability studies have been carried out assessing the stability of xylanase from various microorganisms. Shah *et al.* (2005) examined the thermostability of a partially purified xylanase from *Aspergillus foetidus* over a range of different pH values for 3 hours. Similar to this present study, the crude xylanase extract was extremely stable at lower temperatures; however at 60 °C, 80 % of the activity was lost after 30 minutes of incubation, while the xylanase was completely denatured after 1 hour. Furthermore, Christov *et al.* (1999) investigated the thermal

stability of crude xylanase from *A. oryzae* and *Gliocladium viride* incubated at between 30-60 °C, in a buffer at pH 5. They concluded that at 30 °C, the xylanase extract was extremely stable with 100 % of activity still present after 24 hours of incubation. At 60 °C however, the enzyme was completely denatured after 8 hours although, it is worth noting that no time points were taken prior to 8 hours and the xylanase could have been completely denatured prior to this point.

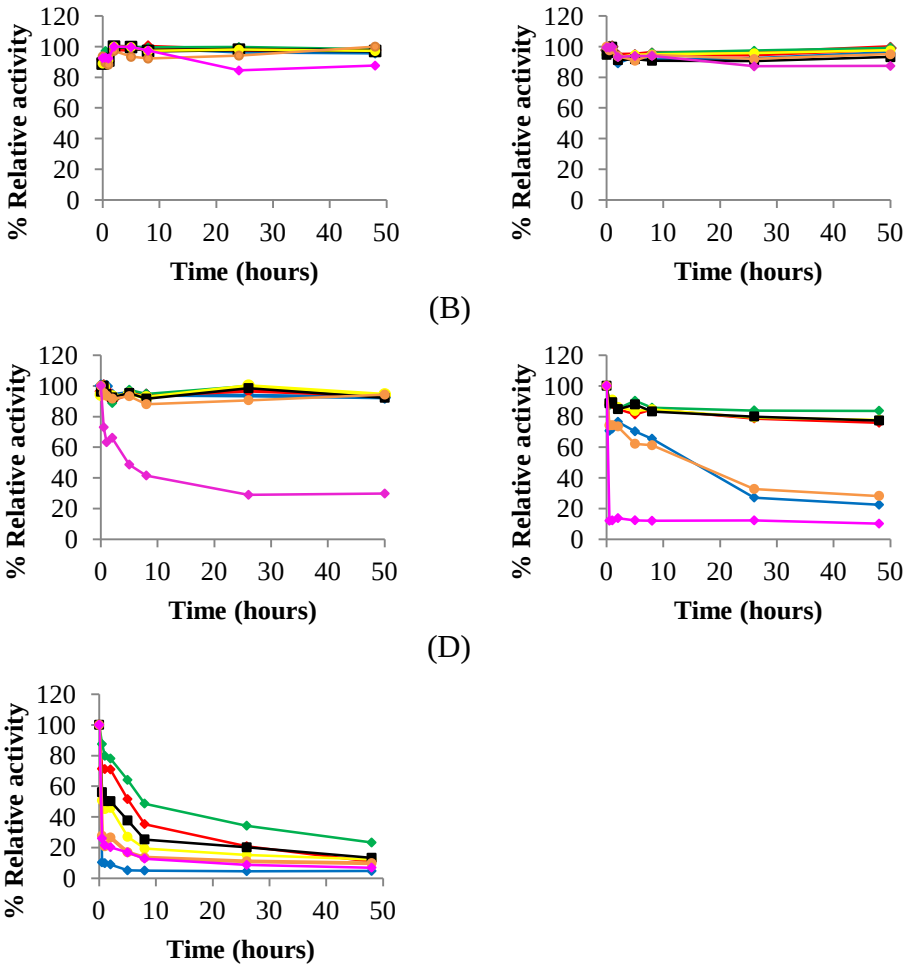


Figure 3.18 Stability of cellulases produced by *A. niger* 102.12 incubated at a range of different temperatures and pHs.

Legend: pH 3.1 (♦), pH 4.1 (♦), pH 5.1 (♦), pH 6 (●), pH 6.1 (■),pH 7 (●), pH 8 (♦)
(A): 24 °C, (B): 30 °C, (C): 40 °C, (D): 50 °C, (E) 60 °C

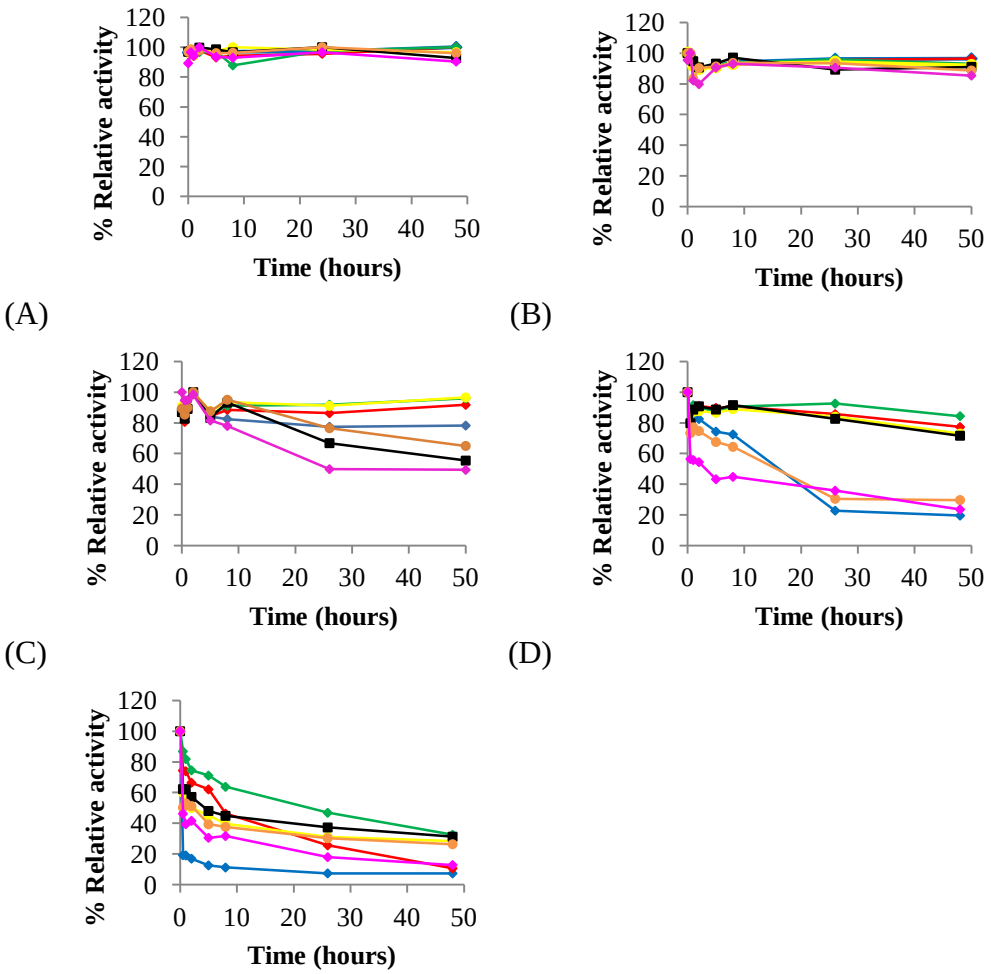


Figure 3.19 Stability of cellulases produced by *A. oryzae* 553.65 incubated at a range of different temperatures and pHs.

Legend: pH 3.1 (♦), pH 4.1 (♦), pH 5.1 (♦), pH 6 (●), pH 6.1 (■),pH 7 (●), pH 8 (♦)
(A): 24 °C, (B): 30 °C, (C): 40 °C, (D): 50 °C, (E) 60 °C

In general, cellulase (Figures 3.18 & 3.19 E) tended to be more thermostable than xylanase (Figures at 3.16 & 3.17 E) at 60 °C, with between 26-32 % of the activity still present, when cellulase from *A. oryzae* 553.65 was prepared in buffers ranging in pH from 5.1 to 7.0, after 48 hours. Cellulases from *A. oryzae* 553.65 (Figure 3.19 E) were least stable when stored in buffer at pH 3.1, with over 80 % of activity being lost during the first 30 minutes of incubation. Cellulase from *A. niger* 102.12 (Figure 3.18 E) was less tolerant than those produced from *A. oryzae* 553.65 (Figure 3.19 E) where, with the exception of buffer pH 5.1, which retained 23 % of its activity after 48 hours, cellulase activity was reduced by over 87 % when stored at any other pH.

At lower temperatures of between 24-30 °C, the cellulases produced from both *A. niger* 102.12 (Figures 3.18 A & B) and *A oryzae* 553.65 (Figures 3.19 A & B) were exceptionally stable across the range of pH values with over 85 % of cellulase activity still present after 48 hours of incubation.

At 40 °C, cellulase from *A. oryzae* 553.65 (Figure 3.19 C) was extremely stable at pH 5.1 and 6.1, with over 97 % of activity retained after 48 hours of incubation. By comparison, when cellulase was incubated at pH 8.0 the cellulase activity was reduced by over 50 % during storage. Another point to note is that when comparing the citrate buffer at pH 6.1 to the phosphate buffer at pH 6.0 there was a 41 % difference in activity between the two buffers with the citrate buffer performing more favourably, indicating that citrate was the better buffer for cellulase stability. This is likely due to the effect of the ions on the structural stability of the enzyme, as explained previously in Section 3.3.1. The buffer used in an enzyme assay can have a considerable effect on the activity that is determined from the enzyme of interest (Criquet, 2002). Ferreira *et al.* (2009) assessed the effect that the buffer system can have on the cellulase activity from sclerophyllous forest litter. They determined that at pH 6.0, there was a profound difference between the cellulase activities when sodium citrate was used as the buffer in comparison to sodium acetate being used. In addition, Criquet *et al.* (2002) monitored the difference between cellulase activity from forest litter using two different buffers. They attributed the difference in activity between the two buffers to the lignin degradation products such as phenolic compounds which can stimulate endoglucanase activity. Citrate is known to be a powerful extractant of phenolics, which would be present in the corncobs and could have influenced the cellulase activity.

In contrast to *A. oryzae* 553.65, the cellulase from *A. niger* 102.12 was more stable over a wider pH range of between pH 3.1-7.0 at 40 °C (Figure 3.18 C), where over 90 % of enzyme activity was retained after incubation. However, at pH 8.0 the enzyme was extremely unstable with 70 % of activity lost. Up to 60 % of this activity was lost during the initial 8 hours of incubation, with the activity stabilising somewhat thereafter.

At 50 °C the two enzyme preparations followed the same trend with the cellulase enzyme being the most stable between pH 4.1 and 6.1 (Figures 3.18 & 3.19 D). At pHs above or below these values, the cellulase enzyme was relatively unstable with over 65 % of activity being lost during 48 hours of incubation. At pH 8.0 the

cellulase from *A. niger* 102.12 (Figure 3.18 D) was extremely unstable with 90 % of activity denatured during the first 30 minutes of incubation, although after this the enzyme was relatively stable with 10 % of activity being retained throughout the rest of the incubation. This dramatic degradation of the enzyme over the initial stages of incubation could be due to the effect of the pH of the buffer in conjunction with the incubation temperature, where the change in pH is destabilising the enzyme leading to further denaturation by heat. Similarly, the degradation of cellulase prepared in buffers at pH 3.1 and 7.0 (Figure 3.18 D) occurred over the first 24 hours of incubation with the activity of the enzyme levelling off thereafter. This levelling off effect seen in Figures 3.18 & 3.19 could be as a result of a number of different endoglucanases present in the crude extract with one enzyme being more thermostable than the other. Tahara *et al.* (1997) studied the characteristics of cellulases produced an *Acetobacter xylinum* and postulated that at least two different cellulases were present, with one cellulase stable at a lower pH, while the residual activity of the other faded away at pH 3.

Like xylanase, cellulase thermal stability from a number of different microorganisms including both fungi and bacteria produced in solid state and submerged fermentation has been previously investigated (Heidorne *et al.*, 2006; Lee *et al.*, 2008; Wang *et al.*, 2009; Liu *et al.*, 2011). Liu *et al.* (2011) investigated the effect of both pH and temperature on the stability of cellulase produced from *Aspergillus fumigatus* Z5 and observed that the cellulase was relatively stable between 20 and 60 °C over 20 hours; however, as the temperature increased, the activity was notably reduced over the same time period. To assess pH stability, they measured the effect of pH at 50 °C over 20 hours and determined that cellulase was most stable at pH 5.0 which was in agreement with the results presented in Figures 3.18 & 3.19 (D).

Thermal stability of an enzyme can be inferred by a number of structural differences and interactions between amino acids through disulphide bridges and hydrophobic and electrostatic interactions. Turunen *et al.* (2001) determined that the introduction of a disulfide bridge in the α-helix of a XYN11 endo-1,4-β-xylanase from *T. reesei* increased the half-life of the enzyme from 1 minute to 14 minutes at 65 °C. Furthermore, an additional mutation in the C-terminus of the α-helix further increased the half-life to 63 minutes. Similarly, Xiong *et al.* (2004) introduced disulphide bridges into the N-terminal region and the single α-helix of the mesophilic *T. reesei* xylanase. They determined that the most stable mutant which contained 2 disulphide

bridges had a half-life of 56 hours at 65 °C and was 5000 times more thermostable than the wild type xylanase. A study on the structure of rubredoxins from a thermophilic Pyrococcus revealed that the structure was very similar to that of mesophilic rubredoxins, although the thermophillic form contained additional hydrogen bonds within the β-sheet and extra electrostatic interactions on the protein surface, which they hypothesised could be the reason for the increased thermostability of the protein (Blake *et al.*, 1992 ; Day *et al.*, 1992).

It is also thought that the differences between amino acid structures of the enzyme can also influence its thermostability. A number of studies have shown that although most point mutations of amino acids in proteins have little influence on the stability of the enzyme, some mutations can (Hecht *et al.*, 1983; Reidhaar-Olson *et al.*, 1988; Matthews, 1993). For instance, of over 2000 point mutations that were made to lysozyme, 91 % of them had little influence on the thermal stability of the enzyme. However, of the 9 % that did affect thermal stability, all the mutations occurred at amino acids with limited mobility (Matthews, 1993). These amino acids are likely to be on the interior of the protein thus affecting the hydrophobic interactions, which are thought to be one of the most significant interactions in thermal stability (Daniel *et al.*, 1996). As a consequence of this, it is thought that structurally the cellulase from *A. oryzae* 553.65 may contain more structural bonds within the enzyme making it a more thermostable enzyme than the cellulase produced from *A. niger* 102.12, although to confirm this the cellulase genes would have to be isolated and sequenced.

The stability of an enzyme to pH is dependent on the pI of the specific enzyme and electrostatic interactions which are determined by the ionisation states of the side chains of the amino acids and the dipoles of the main chain (Nielsen, 2007). Charge-charge interactions, which are the interactions between anions and cations in a protein, are optimised better in enzymes than in proteins which do not contain catalytic activity meaning that salt or buffer mediated electrostatic binding effects are likely to more pronounced in enzymes in comparison to proteins (Spassov *et al.*, 1994). The more pronounced the difference between the pI and the pH of the buffer, the greater the net charge of the protein implying that the ability of an ionic compound to cause stabilisation or destabilisation of the protein by binding to specific residues should increase as the difference between the pI and the pH is accentuated (Ugwu *et al.*, 2004). From Figures 3.16 - 3.19 it can be determined that as the pH of the storage buffer is changed to a lower pH of 3.1 or a higher value of 7.0 and 8.0 the stability of

the enzyme is substantially reduced in comparison to when the pH of the buffers were closer to the pKa of the enzyme. It may be possible to increase the enzyme stability by increasing the buffer concentration which has been shown to influence the stability at higher temperature. Kalisz *et al.* (1997), showed that increasing the buffer concentration of an acetate or phosphate buffer from 50 mM to 1 M resulted in a 10-fold increase in the thermal stability of glucose oxidase from *Pencillium amagasakiense* at pH 6.0 and 8.0.

3.5 General conclusions

The cellulase and xylanase enzymes produced from *A. niger* 102.12 and *A. oryzae* 553.65 were typically mesophilic, with both cellulase and xylanase enzymes becoming less stable above 50 °C. However, the cellulase enzymes from both strains were typically more thermotolerant that xylanase during storage at different pHs. At 60 °C up to 33 % of the relative activity of the cellulase from *A. oryzae* 553.65 was retained following 48 hours of incubation. In comparison, at 60 °C the xylanase from both strains was generally denatured during the first 30 minutes of incubation. At lower temperatures of 24 and 30 °C, both cellulase and xylanase appeared to be relatively thermostable. At these temperatures the cellulase enzymes retained over 85 % of activity during storage at all pH values tested. Xylanase in comparison was moderately less thermostable, with xylanase from both strains tending to be the least thermostable at pH 3.1.

Table 3.1 Optimal fermentation conditions for cellulase and xylanase production

Strain	Fermentation time (hours)	Fermentation temperature (°C)	Yeast extract % (w/v)	Inoculum volume (mL)
A. niger 102.12	120	30	5	14
A. oryzae 553.65	72	30	5	18

The optimal conditions to obtain maximal enzyme production from both *A. niger* 102.12 and *A. oryzae* 553.65 are presented in table 3.1. Through utilisation of these fermentation conditions the cellulase and xylanase production from the two strains was significantly improved in comparison to the control fermentation. The highest degree of improvement in enzyme yield was noted through the addition of 5 % yeast extract to the media, with a 31 and 44-fold increase in xylanase and cellulase,

respectively, during fermentation with *A. oryzae* 553.65. The addition of yeast extract to the *A. niger* 102.12 fermentation did not have as significant an impact on the cellulase and xylanase production, with a 14 and 17-fold increase in the xylanase and cellulase activity noted, respectively. The fermentation time also had a discernible effect on enzyme production, with *A. oryzae* 553.65 achieving maximal cellulase and xylanase production 48 hours quicker than *A. oryzae* 102.12. This was due to the production of cellulase from this strain being relatively slow in comparison to the xylanase production, in which near maximal production occurred after 96 hours.

The effect of assay temperature and pH was also assessed to determine the optimal conditions for enzyme detection. The results indicated that the optimal temperature for cellulase activity from both fungal strains was at 70 °C, with the activity being reduced above and below this point. The optimal temperature for xylanase detection was moderately lower, with the maximal activity observed at 60 °C from both fungal extracts. Optimal pH for cellulase activity was at pH 4.15 for both fungal extract. Meanwhile, maximal relative xylanase activity was observed at a higher pH than that of cellulase, with pH 5 and 5.7 being optimal for production of xylanase from *A niger* 102.12 and *A. oryzae* 553.65, respectively.

These enzymes would be suitable for saccharification of lignocellulosic substrates in a simultaneous saccharification and co-fermentation system. Hydrolysis of the substrate would be required to proceed at temperatures of around 35 °C to facilitate yeast growth as well as enzyme hydrolysis. These enzymes would also be suitable for separate hydrolysis and fermentation, where the saccharification of the substrate typically takes place at temperatures of 40-50 °C.

The enzyme preparations may also be used in animal feed to improve the digestibility of the feed by removing anti-nutritional factors (Bedford, 1993; Galante *et al.*, 1998a). However, in many animal feed processes, the feed is pelleted to improve handling, palatability and in many cases to improve feeding results as pelleted feed reduces ingredient segregation (Lundblad *et al.*, 2011). Temperatures of between 75-95 °C are commonly used in the pelleting process (Puder *et al.*, 2009), which may result in inactivation of xylanase enzymes as the tended to be less thermostable than cellulase. Furthermore, the cellulases enzyme may find use in the detergent industry, while the xylanases may be used in the pulp and paper industry.

It is evident that the production of cellulase and xylanase through SSF offers an alternative method of producing enzymes than SmF, as high enzyme titres can be

achieved during fermentation in relatively short periods of time. Furthermore, at present one of the principal costs in enzyme production via submerged fermentation is the substrate. This expense can be substantially reduced through using lignocellulosic substrates such as corncobs in a solid state fermentation system, as these substrates can be obtained relatively cheap (Aachary *et al.*, 2009), making this an ideal method for enzyme production.

4. Biological pretreatment and bioethanol production

4.1 Biological pretreatment of lignocellulosic biomass

Lignocellulosic material, which is the major component in plant biomass comprises over half of the matter produced through photosynthesis. Furthermore, it is one of the most abundant sources of potential renewable energy, which can be used as an alternative to fossil fuels, in the form of bioethanol (Sánchez, 2009). It is comprised of lignin, cellulose and hemicellulose and varies in concentration depending on the source of the material. The cellulose microfibrils are bound together through hydrogen bonds, hemicellulose and other polymers and wrapped in lignin, making it extremely difficult to access the carbohydrates (Menon *et al.*, 2012). As a consequence, the biomass needs to be pretreated to release the cellulose microfibrils, used in the production of bioethanol. Biological pretreatment was chosen as the method of choice in the present study due to the mild conditions required, the selectivity of the fungi towards lignin, the low energy demands and the reduction in the formation of toxic compounds and inhibitors (Alvira *et al.*, 2010), in comparison to other chemical and physico-chemical methods outlined in Section 1.1.2.

Wood rotting fungi are traditionally employed in biological degradation of lignocellulosic materials and are generally classified depending on the type of rot that they evoke; namely brown, white and soft rot. In general, white rot fungi are the most studied due to their unique enzyme systems and their potential to selectively degrade the lignin polymer leaving the cellulose and hemicellulose fractions relatively untouched (Eriksson *et al.*, 1990). A wide selection of white rot fungi have been employed in the degradation of various agricultural residues including *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Cyathus stercoreus*, *Ceriporiopsis subvermispora* and *Ganoderma oregonense.* *P. chrysosporium* is one of the most thoroughly studied white rot fungi due mainly to its capacity to degrade pollutants, its good ligninolytic properties, fast metabolic cycle and ease of handling in culture (Barclay *et al.*, 1993; Asgher *et al.*, 2006). However, due to the abundance of literature available investigating this fungus and the fact that when it was grown in SSF it sporulated heavily, other fungi were investigated in the present study. Common agricultural wastes such as corn stover, corncobs, oat straw and cottonseed hulls were selected for investigation due to the relative abundance of these materials, which have little use

currently, except as forage feed in ruminant diets. As a consequence of the excess of lignocellulosic waste available, soil erosion and water pollution have occurred, as the materials are left to decompose in fields (Miura *et al.*, 2004). Bioethanol production offers a potential use for these residues whereby the cellulose and xylan can be utilised as a substrate, following pretreatment to remove the lignin fraction. The production of ethanol using lignocellulosic biomass offers a cheaper alternative to materials such as molasses and corn kernels, which typically require government subsidies to compete with the petroleum industry (Gírio *et al.*, 2010).

A selection of white rot fungi were propagated on a range of different lignocellulosic wastes. *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086 were selected as they produced a nominal quantity of spores and germinated rapidly on a range of substrates at 24 °C, in comparison to other strains tested which typically propagated at a slower rate than these two strains. These other strains included, *Phanerochaete chrysosporium* 1547, 1556, 6909, 24725 and 32629, *Pleurotus eryngii* 9619, *Trametes versicolor* 3086, *Phlebia radiata* 5111, *Pleurotus ostreatus* 1833, 32783 and Oyrm 1, *Pleurotus salmoneostramineus* 5338, *Pleurotus pulmonarius* 9558, *Hericium erinaceus* 52487 and *Lentinula edodes* 66784. For the rest of this discussion, the fungal strains *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086 will be referred to as Oyrm 1 and TV 3086, respectively.

To monitor the degradation of lignocellulosic agricultural wastes by Oyrm 1 and TV 3086; corn stover, oat straw, cottonseed hulls, coconut fibre, pine wood shavings and corncobs were fermented for up to 240 days, with samples collected every 30 days to monitor the breakdown of the lignocellulosic material over time. Each sample was analysed for dry matter loss, soluble and insoluble lignin content and carbohydrate composition. This was accomplished through hydrolysis of the lignocellulosic samples with concentrated sulphuric acid (72 % w/v) and subsequent analysis via HPLC, as outlined in Section 2.2.8. Due to issues associated with the absorbance of furfural and hydroxymethyl furfural, which can cause interference when measuring soluble lignin, 320 nm was selected as the wavelength for soluble lignin analysis so as to reduce the interference from these compounds (Hyman *et al.*, 2008). Each fermentation was carried out in sealed sterile polypropylene bags (Unicorn bag, Garland, Texas) that contained a filter membrane to allow the gas exchange which is required for fungal growth and heat removal.

Both strains grew adequately on most of the waste substrates tested with the exception of coconut fibre and pine wood shavings, where the fungi failed to colonise the material sufficiently. Consequently, coconut fibre was mixed with cottonseed hulls to investigate the potential of using a mixed substrate. It is likely that TV 3086 and Oyrm 1 grew poorly on pine wood shavings as white rot fungi preferentially degrade syringyl units in lignin which are mainly found in angiosperms, whereas guaiacyl units which are found in gymnosperms (softwood) are more resistant to degradation (Hatakka, 2005).

4.1.1 Biological pretreatment of corncob

As remarked earlier in Section 3.1, corncob is thought to be a favourable potential substrate for ethanol production due to the availability of sizable quantities of hemicellulose (~35 %) and cellulose (~45 %). Around 60 million tons of corncobs are produced from the US and China alone, with the majority of this being used as a low cost agricultural feed or left unutilised in the harvest fields. In China, corncob has gained a lot of attention due in part to its high xylose content which has lead many researchers to investigate the production of a variety of products including xylooligosaccharides, which can be used as a prebiotic (Chapla *et al.*, 2012), xylitol for use as an artificial sweetener (Cheng *et al.*, 2009) and lactic acid through fermentation of an acid hydrolysate of corncob residue (Miura *et al.*, 2004). However, due to the high cellulose content of corncob, it may also have an application in the bioethanol industry as a cellulosic substrate.

Biological pretreatment of corncob was assessed with selected strains of white rot fungi as detailed in Section 2.2.5.2, whereby changes in composition were monitored through acid hydrolysis of the substrate and subsequent analysis via HPLC (described previously in Section 2.2.8). Sugars measured included glucose (which is a measure of the cellulose content), xylose (which is a measure of the xylan content), arabinose, cellobiose, galactose and mannose, which are considered the main constituents in agricultural waste products (Table 4.1). Galactose, mannose and cellobiose were not included in the following tables as they were below the limits of detection.

Table 4.1 Chemical composition and weight loss of corncobs pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss % (w/w)
0	38.06	29.89	3.52	17.10	9.08	0.00
60	37.73	27.80	3.52	14.67*	7.67*	3.36*
90	37.93	29.35	3.03	15.37*	8.47	8.47*
120	39.29*	29.34	2.31*	13.89*	8.82	11.12*
150	41.41*	26.25*	0.06*	15.26*	9.78*	12.99*
180	36.80*	28.55	3.67	16.01	9.84*	14.64*
210	34.06*	30.29	3.59	16.05	9.48	17.56*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Over the course of the 210 day fermentation a considerable amount of weight loss (~18 %) was observed, with the greatest loss (5 % w/w) occurring during the third month of fermentation (Table 4.1). However, it can also be appreciated that there was little reduction in the concentration of both the soluble and insoluble lignin over the course of the fermentation. Despite the change in lignin composition observed being statistically significant, the amount of lignin remaining in the substrate (84 % of the soluble lignin and 86 % of insoluble lignin remaining after 60 days of fermentation) was still relatively high. Similarly, minimal reduction in the concentration of the carbohydrate fraction (cellulose and hemicellulose) was observed during the course of the fermentation. In spite of this, when the material was harvested it was apparent that the fungi colonised the material extremely well, with complete colonisation occurring during the first 30 days of fermentation. Furthermore, the moisture of the fermentation was monitored, with minimal change noted during fermentation, indicating the majority of weight loss was due to substrate degradation (data not shown). Losses may have occurred due to CO_2 evolution; however, accurate quantitation of this loss is beyond the scope of this project. Thus, it was thought that despite the satisfactory fungal growth observed during fermentation, the lignin degradation was relatively minor. This may have been due to poor secretion of ligninolytic enzymes which may impact on lignin degradation. Steffen *et al.* (2007), investigated the degradation of flax, straw and bark in solid state fermentation and noted that although *Marasmius alliacues* and *Mycena epipterygia* grew well on flax and bark, their secretion of enzymes was poor. Furthermore, Hatakka (1994) investigated the role of lignin modifying enzymes in lignin degradation and determined that fungi that produced

lignin and manganese peroxidase degraded the synthetic lignin the most, whilst fungi that produced lignin peroxidase and laccase poorly degraded the lignin.

Maximal insoluble lignin removal occurred after 120 days of fermentation, where a 19 % reduction was detected. A substantial amount of lignin still remained in the fermentation, which would impact greatly on any further processing i.e. ethanol production, as it would impede enzymatic degradation of the substrate. In the case of TV 3086, the lignin concentration increased as the fermentation proceeded, with a final difference of only 7 % relative to the starting point control after 210 days of fermentation.

Minimal change in the arabinan content (represented by arabinose) of the corncob was noted following the initial 90 days of fermentation. However, after 150 days of fermentation the arabinose content was almost completely removed from the substrate. It is possible that the arabinose was completely utilised by the fungi during fermentation, with the arabinose found following 180 days of fermentation (Table 4.1) being due to the arabinose content of the fungal mycelia. The main constituents of fungal mycelia tend to be glucose, mannitol and trehalose, although arabinose has also been found in fungal cell walls (Reis *et al.*, 2012). Tsai *et al.* (2009) investigated the soluble sugar composition of a number of different fungi and detected arabitol concentrations of up to 17.1 mg g^{-1} in *Pleurotus ostreatus*. Alternatively, this may be due to variation in the rates of degradation by the fungi in the different fermentation bags. Eriksson *et al.* (1990) studied the mineralisation of wood components and found variations in degradation within a substrate, when they were fermented by a single fungal strain.

The apparent lack of compositional change noted during fermentation could be due to the method of sample preparation and analysis. As the white rot fungi penetrates the material, it was difficult to remove the fungal mycelium from the substrate and as such, mycelia were incorporated during acid hydrolysis of the material. Therefore, the nominal change in composition could be due to the synthesis of cellulose and hemicellulose by the fungi for cell wall production which are known to contain glucan (Kalač, 2009). Glucose may also be stored as glycogen in the fungi at levels of up to 10 % of dry weight, which when hydrolysed by concentrated sulphuric acid is recovered as glucose (Carlile *et al.*, 2001). Concentrated sulphuric acid (72 % v/v) is used during acid hydrolysis of lignocellulosic material to hydrolyse the material to soluble sugars, which are subsequently detected using HPLC. Despite

the reduction in glucose, xylose and lignin being statistically significant ($p<0.05$) at certain time points during the fermentation, the amount of lignin and hemicellulose degradation observed was still low in comparison to the degradation observed in the other lignocellulosic materials such as oat straw (Section 4.1.5).

It is not understood why the fungi did not efficiently degrade the lignin, although it could be due to a number of factors such as the nitrogen content of the material, which had previously been shown to affect degradation (Kirk *et al.*, 1978). Although it is dependent on crop harvest time and the soil amongst other factors, the nitrogen content of corn can range from between 0.63-0.90 % (Tsai *et al.*, 2001; Cao *et al.*, 2004; Demiral *et al.*, 2012), which would be considered relatively high for fungal growth and ligninolytic enzyme production. Humar *et al.* (2005), investigated the effect of nitrogen content on a number of lignocellulosic fungi and determined that nitrogen concentrations of 0.325% impacted on the growth and consequently the degradation of Norway spruce by *Trametes versicolor*. Other environmental factors such as temperature, humidity, microclimate and compartmentalisation are also thought to influence the production of ligninolytic enzymes (Tuor *et al.*, 1995). Dill *et al*. (1988) examined the factors that influenced degradation of lignocellulose and determined that the optimum conditions for selective degradation by *Ganoderma applanatum* was under low nitrogen conditions with a high carbon dioxide/low oxygen partial pressure and high humidity.

Table 4.2 Chemical composition and weight loss of corncobs pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss % (w/w)
0	38.06	29.89	3.52	17.10	9.08	0.00
60	36.65*	27.16	2.97	16.34*	7.82*	2.10*
90	37.08	26.44*	2.76	15.50*	7.54*	6.07*
120	39.44	24.86*	2.62	14.82*	8.08*	8.43*
150	43.86*	26.38*	5.86	15.67*	8.07*	11.18*
180	43.94*	26.87	3.58	14.70*	8.80	11.41*
210	43.04*	27.52	4.05	15.57*	8.49*	13.45*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

In comparison to pretreatment with *Trametes versicolor* 3086 (Table 4.1), the dry matter loss was marginally lower with Oyrm 1 (Table 4.2), where 13.5 % was lost

during fermentation, in comparison to the 17.6 % lost through pretreatment with TV 3086. Over the first 60 days of incubation the dry matter loss was relatively low (~2 %); however, between 60 and 90 days the degradation increased dramatically (~4 %) with a significant ($p<0.05$) increase in the rate of degradation of the substrate. This is in agreement with relevant literature which states that degradation usually occurs during the secondary phase of microbial metabolism after the substrate is completely colonised (Villas-Boas *et al.*, 2002). The insoluble lignin content of the corncob was reduced significantly ($p<0.05$) (by 13 %) after 120 days of fermentation which was in agreement with the TV 3086 fermentation (Table 4.1), whilst maximal soluble lignin removal of 17 % occurred after 90 days of fermentation. Despite this, it is clear that there was still a substantial concentration of lignin remaining in the substrate after 210 days of fermentation which may impact on the subsequent enzymatic hydrolysis of the substrate. This contrasts with other techniques such as aqueous ammonia, formic acid/aqueous ammonia and dilute sulphuric acid hydrolysis, where the lignin content of corncob was reduced from 17 % to between 10 % and 4 %, depending on the pretreatment method, with a treatment time of between 45 minutes and 12 hours (Zhang *et al.*, 2010). In saying this however, the pretreatment conditions were harsher and the processes tended to be more energy intensive.

During the first 120 days of fermentation the xylan fraction (represented by xylose) tended to be preferentially degraded over the cellulose (represented by the glucose), which remained relatively unchanged. After 210 days a significant increase (13 %) in the concentration of glucose was observed in comparison to the untreated sample. This could be due to a simple concentration effect as a result of the removal of the xylose and lignin from the biomass. As the fungi removes the xylose and lignin in the fermentation the respective concentrations in the substrate are decreased. This results in an apparent increase in the cellulose concentration as the cellulose fraction makes up a larger portion of the lignocellulose material. Samuel *et al.* (2011) examined the pretreatment of switchgrass using different chemical techniques and also noted an increase in the glucan content, which coincided with an appreciable decrease in the xylan content of the switchgrass. Alternatively, the xylose which was utilised by the fungi in this fermentation may have been synthesised by the fungi for cell wall production. This fungal cell wall can contain a substantial portion of glucan, which is recovered as glucose following hydrolysis with concentrated sulphuric acid, as explained previously in this section.

Due to the scarcity of published research dealing with biological delignification of corncobs or lignocellulosic material in general, it was difficult to compare with other published works. However, Chen *et al.* (2010) investigated the pretreatment of corncobs with a strain of *Phanearocheate chrysosporium* and removed 41 % of the total lignin over 20 days, which was notably more than that removed with either strain tested in this study. In contrast to the present study however, this was carried out using submerged fermentation making the control of the fermentation parameters more manageable in comparison to solid state fermentation.

4.1.2 Biological pretreatment of corn stover

Production of corn stover throughout the world is estimated to be around 284 million tons, with the US producing about 150 million tons alone. This is due in part to the dramatic increase in corn production as a consequence of increased bioethanol manufacture as well as an increasing world population (Aziz *et al.*, 2006). Although the composition of corn stover can vary depending on the harvest date and geographical origin, the composition of corn stover is estimated to range between 36-50 % cellulose, 22-27 % hemicellulose and 16-21 % lignin (Aziz *et al.*, 2006; Chen *et al.*, 2009; Xu *et al.*, 2012) making it an ideal substrate for lignocellulosic ethanol production.

As corn stover can vary in composition between different sections of the plant (i.e. the leaves and the stem) the stover was separated into three distinct constituents. These components consisted of the leaf blades (separated at the point of the blade meeting the stem), the upper stem (all herbage above the 5^{th} node, excluding the leaf blade, husks, silks, rachis and grain) and the lower stem (all herbage above 5cm and below the 5^{th} node excluding the leaf blades with the husks, silks, rachis and grains removed). Each constituent was steeped in deionised water for 20 minutes to moisten the material, sterilised and inoculated with fungal mycelia, whereby plugs from a freshly prepared plates of the selected strain was used as the inoculum. Following inoculation the fermentation bags were incubated at 24 °C, with samples harvested every 30 days. The analysis to determine the lignocellulosic composition of each of these samples was carried out using the methods described previously in Section 2.2.8.

4.1.2.1 Lower stem pretreatment

Table 4.3 Chemical composition and weight loss of the corn lower stem pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	41.63	16.07	3.62	17.34	5.33	0.00
60	38.46*	14.71	1.74*	14.74*	7.81*	16.15*
120	42.86*	10.56*	0.88*	10.56*	9.91*	21.12*
150	47.76*	4.32*	0.74*	11.54*	9.53*	32.30*
180	44.81*	8.37*	1.17*	9.01*	9.90*	40.34*
210	46.22*	5.66*	0.73*	8.11*	11.21*	49.83*
240	48.46*	5.26*	0.83*	6.51*	10.28*	54.63*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Typically the weight loss noted from pretreatment of the lower stem material was substantially more extensive than that observed from cob, with over 54 % of weight lost during 210 days of fermentation (Table 4.3). Coinciding with this, a significant ($p<0.05$) (~62 %) portion of the insoluble lignin was removed over the course of the fermentation, which was as a consequence of degradation by the white rot fungi. Furthermore, an increase in the soluble lignin fraction of 92 % in comparison to the untreated control sample was also observed. It was thought that the increase in the soluble lignin is potentially due to degradation products which are formed during the biological pretreatment process. Alternatively, this increase in the soluble lignin fraction could be associated with hydrolysis of the covalent bonds linking both syringyl and guaiacyl which could result in increased syringyl concentration in the soluble lignin fraction.

The soluble lignin fraction is thought to consist of three components; lignin degradation products such as ρ-coumaric acid, ferulic acid and vanillin; the secondarily formed hydrophilic materials such as lignin-carbohydrate compounds and finally, syringyl units which have also been detected in high quantities (Yasuda *et al.*, 1990). Yasuda *et al.* (1995) determined that the acid soluble lignin fraction contained 58 % syringyl units while the insoluble fraction only contained 19 %, with this being attributed to covalent bonds between the guaiacyl and syringyl units. Sjöberg *et al.* (2004) analysed the degradation of spruce needles by white rot and litter decomposing fungi over time under laboratory conditions. They found that the cinnamyl and

vanillyl compounds, which are the main constituents in soluble lignin, were found to increase with increasing fermentation time, where the cinnamyl compounds were represented by ρ-coumaric acid as no ferulic acid was detected. Valmaseda *et al.* (1991) investigated the solid state fermentation of wheat straw with *Pleurotus ostreatus* and *Trametes versicolor* and also noted an increase in the soluble lignin concentration during the course of the fermentation. The degradation of cinnamic acid and syringyl units was observed in addition to an increase in the ρ-hydroxyphenyl and vanillic acid content which could be attributed to the increase in soluble lignin they detected.

A significant (16 %) ($p<0.05$) increase in the glucose content was also noted over the course of the fermentation. This could be due to selective degradation of the lignin and hemicellulose fraction, where a 67 % decrease in xylose and an 80 % reduction in the arabinose content was determined, resulting in an apparent concentration of the cellulose fraction and/or an increase in the cellulose levels within the material, as explained previously in Section 4.1.1. This apparent selective delignification of the substrate which coincided with a perceived increase in the glucose content would make this method of pretreatment suitable for lignocellulosic ethanol production. Loss of part of the cellulose during pretreatment such as that noted through pretreatment of corncob with TV 3086 (Table 4.1) can impact on the efficacy of the fermentation, as the yield of ethanol from the substrate will be reduced. Consequently, strains of fungi that selectively degrade the lignin fraction thus minimising the loss of glucose are preferred.

Table 4.4 Chemical composition and weight loss of the corn lower stem pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	41.63	16.07	3.62	17.34	5.33	0.00
60	42.18	13.96	1.40*	14.23*	7.29*	19.88*
120	40.70*	10.43*	1.47*	13.12*	8.44*	26.09*
150	38.29*	8.53*	2.04*	14.05*	9.72*	33.23*
180	36.59*	9.49*	2.00*	11.67*	10*	40.56*
210	35.93*	8.42*	1.17*	11.91*	11.12*	47.49*
240	35.24*	8.13*	1.66*	11.99*	10.91*	52.63*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

The results determined from the pretreatment of the lower corn stem with *Trametes versicolor* 3086 (Table 4.4) closely resembled (with the exception of the glucose content) that of pretreatment with Oyrm 1, where substantial dry matter loss was observed over the course of the fermentation, whilst insoluble lignin and xylose concentrations were reduced by 30 and 50 %, respectively. Furthermore, a significant reduction in the arabinose concentration was observed, with a reduction of 68 % determined following 210 days of pretreatment. It is also worth noting that after 150 days, the reduction in insoluble lignin plateaued with minimal lignin loss observed after this point. Additionally, the glucose content was significantly reduced (16 %), which was in direct contrast to the results obtained from the Oyrm 1 fermentation, where an increase in the glucose concentration was measured. The effects on lignin were similar to pretreatment with Oyrm 1, where the soluble lignin concentration also increased as a consequence of either lignin degradation products or increased syringyl units in the acid soluble lignin fraction.

It can be concluded from Table 4.4 that TV 3086 non-selectively degraded the substrate with a reduction in both cellulose and hemicellulose as a result of the fungi utilising both fractions during metabolism, which was as expected as *Trametes versicolor* is known to be a non-selective white rot fungus. In contrast *Pleurotus ostreatus* is known to selectively degrade the lignin and hemicellulose during fermentation which was observed in Table 4.3, where a decrease in the xylose and lignin content was detected, whilst the cellulose content increased. It was apparent that pretreatment of the lower corn stem with Oyrm 1 was a more effective pretreatment technique than utilising TV 3086 as an increase in the apparent glucose concentration was noted through pretreatment (Table 4.3), whilst a higher degree of lignin degradation was also observed.

Wan *et al.* (2010) investigated the microbial pretreatment of corn stover with *Ceriporiopsis subvermispora* and found that after 42 days of incubation at 28 °C over 39 % of lignin was removed. Additionally, *C. subvermispora* preferentially degraded lignin over cellulose which correlated with the results from Oyrm 1. In this present study 62 % of lignin was removed from the material during fermentation with Oyrm1, which was an appreciably higher degree of lignin degradation than Wan's study. However, the fermentation time to achieve this higher degree of degradation was considerably longer in the present study. In saying this, a lower fermentation

temperature was used in this study (24 °C), which may have impacted on the fermentation time, whilst other fermentation conditions may also be a factor.

4.1.2.2 Upper stem pretreatment

Table 4.5 Chemical composition and weight loss of the corn upper stem pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	41.71	16.44	4.03	15.13	5.82	0.00
60	45.03*	17.05	5.68	10.14*	9.56*	15.79*
120	46.34*	10.44*	1.82*	9.28*	10.59*	25.86*
150	47.94*	8.63*	2.09*	7.67*	9.96*	33.92*
180	51.50*	7.28*	0.93*	5.88*	10.54*	40.54*
210	48.12*	6.45*	0.47*	7.32*	10.35*	48.65*
240	48.45*	4.33*	0.83*	5.57*	11.21*	53.45*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

In comparison to the lower portion of the corn stem, the insoluble lignin content from the upper stem was ~2 % lower, while the acid soluble lignin fraction was similar to that of the lower segment at ~5.5 %, comprising an overall 2 % difference in the total lignin concentration of the two segments (Table 4.5). The glucose and xylose concentrations from both the upper and lower stems were very similar with minimal (<1 %) difference observed between the fractions.

The decomposition of the upper stem with Oyrm 1 tended to follow the same pattern of degradation as the lower stem fraction, with over 50 % of dry matter loss and an increase in the cellulose content during fermentation. A similar rate of lignin hydrolysis to the lower stem was also observed in the upper stem following pretreatment with Oyrm 1, where 63 % degradation of the insoluble lignin fraction was measured, whilst a significant ($p<0.05$) increase (93 %) in the acid soluble lignin fraction was detected. Substantial xylose degradation was also observed, with 74 % of xylose degraded during fermentation, allowing it to be concluded that hemicellulose and lignin were preferentially degraded over the cellulose fraction. Furthermore, a significant ($p<0.05$) decrease (89 %) in arabinose was also noted, which is generally found bound to the xylose in the form of arabinoxylan.

Darwish *et al.* (2012) studied the effect of pretreatment of maize stalk with *Pleurotus ostreatus* to improve the nutritional value of the material. The authors reported considerable cellulose degradation after 28 days of incubation, which was in direct contrast to the results outlined above. Similarly, the hemicellulose fraction was substantially degraded indicating that non-selective degradation of the material occurred during fermentation. This was surprising considering *Pleurotus ostreatus* is generally thought to be a fungus that selectively degrades lignin (Kerem *et al.*, 1995; Hatakka, 2005; Taniguchi *et al.*, 2005). It is apparent that the strain of *Pleurotus ostreatus* utilised in this present study was a more effective strain for pretreatment than that used by Darwish *et al.*, (2012) as 38 % of the cellulose fraction was degraded in their study, whilst an increase in the apparent cellulose content (represented by glucose) was noted in this study. Furthermore, a higher degree of lignin degradation was observed in this present study (63 %) in comparison to Darwish *et al.* (30 %), although an appreciably longer fermentation time was utilised.

Table 4.6 Chemical composition and weight loss of the corn upper stem pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	41.71	16.44	4.03	15.13	5.82	0.00
60	40.23	14.59	1.87*	12.23*	9.62*	13.29*
120	35.60*	9.71*	2.01*	11.18*	10.40*	27.61*
150	32.24*	6.75*	2.26*	10.65*	10.73*	30.89*
180	35.73*	9.19*	2.77*	10.16*	9.79*	35.56*
210	30.98*	6.25*	1.67*	10.08*	11.88*	41.45*
240	29.44*	6.59*	2.25*	10.62*	12.26*	46.82*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Pretreatment of the upper stem of corn with TV 3086 (Table 4.6) resulted in the non-selective degradation of the substrate with a 30 % reduction in glucose concentration, whilst the xylose content was also decreased by over 60 % of the initial titre. Furthermore, the arabinose fraction was also extensively degraded with 59 % of this carbohydrate being removed during fermentation. This reduction in the arabinose content observed through pretreatment with TV 3086 was not as substantial as that observed through pretreatment with Oyrm 1. This correlated with the rate of removal of the xylose fraction (to which the arabinose in typically bound), where a higher

degree of degradation was also noted during pretreatment with Oyrm 1. This was possibly as a result of the fungus degrading the hemicellulose fraction in which the xylose is closely associated with the arabinose fraction in the form of arabinoxylan.

Insoluble lignin degradation was not as substantial in this fermentation in comparison to degradation by Oyrm 1, with 30 % of the lignin being removed following 240 days of fermentation. The majority of degradation occurred over the first 150 days of fermentation, with 99 % of the degradation occurring during this time. The increase in acid soluble lignin (110 %) was notably higher with TV 3086 than the increase observed when the upper stem was pretreated with Oyrm 1. This was surprising considering the soluble lignin fraction is thought to consist of degradation products from lignin hydrolysis and significantly more insoluble lignin degradation occurred during the Oyrm 1 fermentation. However, it may be due to the degradation profiles of the two fungi in which more degradation products are formed and a larger number of soluble syringyl units are produced as a result of lignin degradation with TV 3086, resulting in an increased soluble lignin content.

4.1.2.3 Corn leaves pretreatment

When examining the composition of corn leaves (Table 4.7), it was determined that considerably less glucose (30 %) was found to be present in comparison to the stem of the plant (Tables 4.4 & 4.6). Furthermore, the xylose content of the corn leaves was of a similar concentration to the stem, while the insoluble lignin fraction was the same as that from the upper stem. Additionally, the corn leaves contained 7.56 % (w/v) of acid soluble lignin which was higher than the quantity obtained from both the upper and lower stem of the corn. These results were broadly in agreement with Li *et al.* (2012b) who determined leaf composition to be 31.3 % cellulose and 21.2 % hemicellulose, with xylose accounting for 12.4 % of this value. Similarly, they determined the lignin content to be 17.4 % for the insoluble lignin and 2.5 % for the acid soluble lignin fraction. Any difference in composition between the two stovers may be attributed to the variation in climate and soil during growth (Templeton *et al.*, 2009), whilst harvest time may also be a factor (Pordesimo *et al.*, 2005).

Table 4.7 Chemical composition and weight loss of corn leaves pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	29.98	16.67	3.29	15.87	7.56	0.00
60	29.05*	12.25*	2.18*	9.54*	11.36*	22.89*
120	28.58*	12.37*	3.12	7.69*	11.57*	27.71*
150	26.74*	7.81*	1.90*	7.01*	11.29*	42.17*
180	19.40*	4.15*	2.29*	8.45*	13.11*	48.56*
210	19.41*	5.28*	1.81*	7.12*	11.48*	53.56*
240	13.22*	4.31*	0.23*	9.17*	13.45*	57.45*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.8 Chemical composition and weight loss of corn leaves pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	29.98	16.67	3.29	15.87	7.56	0.00
60	20.46*	10.43*	2.38*	9.52*	10.58*	20.23*
120	14.31*	7.37*	2.02*	9.09*	10.8*	31.93*
150	13.30*	5.35*	2.31*	7.39*	10.09*	38.56*
180	12.34*	3.53*	1.90*	11.30*	14.55*	45.23*
210	10.88*	3.52*	1.60*	12.87*	13.5*	51.45*
240	10.07*	4.14*	1.56*	10.24*	17.99*	56.73*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Significant degradation of the corn leaves was noted with both fungi during fermentation, with weight loss exceeding 50 % in both cases (Tables 4.7 & 4.8). This weight loss is mainly due to the degradation of the substrate as moisture analysis indicated that minimal moisture loss occurred during fermentation. Furthermore, significant ($p<0.05$) glucose utilisation was also observed, with 55 and 66 % of the original glucose content being removed through pretreatment with Oyrm 1 and TV 3086, respectively. Considerable xylose utilisation was also detected with ~75 % of xylose removed after 240 days of fermentation in both cases. Substantial arabinose removal was also noted during both fermentations, with the arabinose being almost completely utilised during fermentation with Oyrm 1. Following fermentation, only 0.23 % of the arabinose fraction remained in the substrate which was notably less than the amount of arabinose remaining following fermentation with TV 3086, in which

1.56 % was still available. Both strains degraded ~60 % of the insoluble lignin fraction following 150 days pretreatment, with the highest rate of lignin removal occurring during the first 60 days of fermentation, where 40 % of insoluble lignin was removed by both TV 3086 and Oyrm 1. It was worth noting however, that following the initial decrease in the insoluble lignin fraction an increase was detected during the remaining days of the fermentation. This increase in the insoluble lignin content may be as a result of the accumulation of condensed degraded polysaccharides during pretreatment (Samuel *et al.*, 2011). Alternatively, it may be due to the high protein content which is known to influence the determination of insoluble lignin (Sluiter *et al.*, 2008). The soluble lignin content was increased by 77 and 137 % by Oyrm 1 and TV 3086, respectively, which was also observed during fermentation of the other two corn stover fractions (Sections 4.1.2.1 & 4.1.2.2).

Biological pretreatment of corn leaves with either of these fungal strains does not appear to be an effective pretreatment method despite the significant ($p<0.05$) reduction in lignin content. A marked decrease in the glucose content of both fermentations was detected during pretreatment, with up to 66 % being utilised, reducing the cellulose available for ethanol fermentation. Despite the substantial amount of lignin removed during fermentation, this method is unlikely to be economically feasible due to the amount of cellulose and xylan that was lost. This was in contrast to the other fermentations, such as the pretreatment of the upper and lower portion of the stem with Oyrm 1, where no reduction in the glucose concentration was observed.

4.1.3 Biological pretreatment of cottonseed hulls

Across the world, over 116.7 million bales of cotton are produced annually, with the waste from this process (cotton plant stalk, leaves and cottonseed hulls) usually discarded (Isci *et al.*, 2007). In the US alone, over 2.25 million tonnes of cotton waste are produced every year, making it a potentially valuable renewable substrate for bioethanol production (Holt *et al.*, 2000). Furthermore, cottonseed hulls have been shown to be a suitable substrate for fungal cultivation due in part to their high water holding capabilities and their high nitrogen content (Lu *et al.*, 1984). Consequently, it would appear that cottonseed hulls would be an appropriate substrate for biological pretreatment.

Cottonseed hulls (30 g) were moistened with 21 mL of deionised water and inoculated with a plug from a freshly prepared slant of mycelia of an appropriate fungal strain. The flasks were subsequently sealed and incubated for up to 210 days, with sample flasks being harvested every 30 days to monitor the change in composition of the substrate over time, using the methods described previously in Section 2.2.8.

Table 4.9 Chemical composition and weight loss of cottonseed hulls pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	36.13	20.88	3.12	24.12	3.52	0.00
60	33.95	16.45*	3.16*	27.47*	5.59*	4.40*
90	37.78	12.77*	5.00*	27.75*	5.91*	5.98*
120	33.88	11.88*	0.86*	28.25*	6.06*	7.46*
150	33.01	7.78*	1.36*	28.67*	6.52*	8.71*
180	34.87	4.08*	1.07*	28.68*	7.21*	10.29*
210	37.11	5.01*	0.26*	23.38*	6.76*	14.68*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.10 Chemical composition and weight loss of cottonseed hulls pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	36.13	20.88	3.12	24.12	3.52	0.00
60	37.81	21.65	3.00*	21.99*	4.52*	6.84*
90	37.96	21.90	2.56*	22.75*	4.72*	10.28*
120	38.66	16.14	0.77*	22.10*	4.99*	12.48*
150	38.07	16.29	0.81*	22.84*	4.85*	13.29*
180	37.49	16.42	0.81*	21.72*	5.51*	16.53*
210	36.31	16.01*	0.86*	21.50*	5.31*	20.47*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Biological pretreatment of cottonseed hulls with TV 3086 (Table 4.9) and Oyrm 1 (Table 4.10) was not as successful at removing lignin from the substrate as it was with other materials examined previously i.e. corn stems (Section 4.1.2). Despite both strains growing well on cottonseed hulls, the degradation of lignin over the course of the fermentation was relatively low, with 11 and 3 % of the insoluble lignin fraction

being removed by Oyrm 1 and TV 3086, respectively. As witnessed with other pretreated materials, the soluble lignin fraction increased throughout the fermentation, coinciding with decreasing insoluble lignin content. An increase of 50 % in the soluble lignin fraction was noted through pretreatment with Oyrm 1, whilst an increase of 92 % was observed when pretreated with TV 3086. Minimal change in the glucose content was detected when the standard error of each sample was taken into account, with the final glucose concentration being almost the same as the untreated material. For instance, the difference between the untreated sample (36.13±1.3) and the substrate pretreated for 120 days (38.66±1.3) was minimal when the error rate was taken into account. It appeared that Oyrm 1and TV 3086 preferentially degraded the hemicellulose fraction of the substrate, with 24 and 54 % of the xylose being removed from the substrate, respectively after 210 days of fermentation. Furthermore, the arabinose fraction in the cottonseed hulls was almost completely removed by both fungi, with 0.26 and 0.86 % (w/v) remaining following pretreatment with TV 3086 and Oyrm 1, respectively. The TV 3086 fermentation removed moderately more arabinose during fermentation. This corresponded with the xylose use in which substantially more xylose was degraded during fermentation with TV 3086 in comparison to Oyrm 1.

It is not completely understood what affected the rate of lignin degradation, although it could have been a number of factors. The degradation of the lignin may have been affected by the physiological conditions of the fermentation such as pH, nitrogen content and carbon dioxide level. Lignin degradation is known to occur at a low pH. *P. chrysosporium* for example, which is a well known white rot fungus, degrades lignin more efficiently at pH 4, with the activity decreasing towards lower pH values (Kirk *et al.*, 1978). If the pH of the fermentation was less than the optimal pH, this may have affected the production of the ligninolytic enzymes which in turn may have influenced the degradation of the lignin. The pH of these fermentations was not examined; however, as it can be difficult to monitor the pH in SSF, as mentioned previously in Section 1.4.1.4. The inadequacy of the lignin degradation noted during fermentation may also be due to the high nitrogen content present in the cottonseed hulls which, as explained previously in Section 4.1.1, can influence lignin degradation. Carreiro *et al.* (2000) analysed the effect of nitrogen on litter decay and microbial enzyme production and concluded that increased nitrogen content in litter negatively impacted on the production of ligninolytic enzyme activity from white rot fungi.

Alternatively, the inefficiency of lignin degradation in cottonseed hulls compared to corn stover could be due to the complex nature of lignin and the differences in composition of the two materials. Abduazimov *et al.* (1997) examined the content of lignin from the hulls, stems and bolls of the cotton plant and determined that cottonseed hull lignin was the most highly hydroxylated of the lignins studied and contained the highest molecular mass of the lignin fractions analysed from various cotton plants. Biological degradation of lignin by white rot fungi is dependent on the molecular mass of the lignin whereby, in general, the higher the molecular mass the slower the rate of decomposition observed (Abduazimov *et al.*, 1997).

An increase in the insoluble lignin fraction was noted during the fermentation of cottonseed hulls with TV 3086. It is thought that this was possibly due to the condensed degraded polysaccharides which can occur during pretreatment, as mentioned previously in Section 4.1.2.3. A higher degree of carbohydrate degradation occurred during pretreatment with TV 3086 (Table 4.9) in comparison to Oyrm 1 (Table 4.10) which may have resulted in the formation of these degradation products in the TV 3086 fermentation, thus increasing the apparent lignin content. Alternatively, it may have been due to the formation of insoluble lignin compounds during pretreatment. Reid (1991) analysed the production of lignin intermediates during delignification by *Phlebia tremellosa* and found that insoluble lignin compounds can be formed at the expense of water and dioxane-soluble lignin. This increase was attributed to oxidative polymerisation and/or condensation, which was thought to be part of lignin degradation either in the main pathway to depolymerisation or as a side branch.

Li *et al.* (2001) investigated the compositional change of cottonseed hulls through fermentation with *Pleurotus ostreatus* and found that over 45 days of incubation both hemicellulose and cellulose were utilised during the fermentation. In addition, the lignin content was reduced from ~17 % to ~11 %, which was in contrast to the results obtained in this present study, where only a modest amount of lignin degradation was observed over a longer incubation period. The majority of lignin degradation from their study occurred during primordial formation (mushrooms at their earliest growth phase), whereas cellulose utilisation occurred during the formation of the first flush of mushrooms. This may explain the discrepancies between both sets of results, as fungal cultivations in this present study were in mycelial form and fruiting bodies were not stimulated.

4.1.4 Biological pretreatment of a homogenous substrate containing cottonseed hulls and coconut fibre

Coconut fibre has significant potential as a source of biofuels due to its high cellulose content (39-51 %); however, it also contains a considerably high lignin content (38-41 %) and as such, needs to be pretreated prior to use as a substrate for cellulosic ethanol production. Currently, the bulk of research into uses for coconut fibre have concentrated on areas such as the replacement of synthetic fibres, use in the automotive industry (Tomczak *et al.*, 2007) and as an additive to cement (Abdullah *et al.*, 2011).

Originally, coconut fibre was analysed as a sole lignocellulosic substrate in this study; however, due to poor growth, the fibre was mixed with cottonseed hulls to provide a better substrate and support matrix. It is thought that the poor growth observed may have been due to the poor thermal conductivity (Abdullah *et al.*, 2011) of the coconut fibre which could create an inhospitable environment due to the formation of temperature gradients during the initial stages of fungal metabolism. Alternatively, it could be due to the composition of the substrate which creates a sub-optimal environment for fungal growth as a result of insufficient nutrients required for fungal delignification. As a consequence, 1500 g of dried coconut fibre was mixed with 2100 g of cottonseed hulls and allowed to steep in 9 L of deionised water to moisten the material and was subsequently fermented with strains of white rot fungi. Samples were taken every 30 days and analysed to determine the degradation over time, using the methods outline in Section 2.2.8.

Table 4.11 Influence of biological pretreatment with *Pleurotus ostreatus* Oyrm 1 on the composition of a heterogeneous cottonseed hulls and coconut fibre substrate.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble lignin % (w/v)	Weight loss %
0	32.18	9.63	4.09	33.63	2.79	0.00
60	31.53	11.53	1.45*	32.08	4.84*	9.45*
90	33.29	11.17	1.05*	25.39*	5.39*	11.61*
120	34.65	8.48	1.25*	24.75*	5.14*	12.91*
150	35.78*	7.91	0.82*	23.64*	5.58*	14.06*
180	36.62*	8.01	0.94*	21.51*	5.17*	17.88*
210	35.57*	7.90	0.79*	21.97*	6.78*	20.43*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.12 Influence of biological pretreatment with *Trametes versicolor* 3086 on the composition of a heterogeneous cottonseed hulls and coconut fibre substrate.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble lignin % (w/v)	Weight loss %
0	32.18	9.63	4.09	33.63	2.79	0.00
60	29.06*	9.84	0.51*	33.18	4.84*	7.31*
90	29.38*	5.52*	1.15*	34.66*	4.30*	9.47*
120	28.83*	4.71*	1.22*	35.04*	5.09*	11.30*
150	30.73	4.32*	1.13*	33.87	4.00*	13.59*
180	28.66*	3.45*	1.65*	34.81*	4.98*	16.23*
210	27.44*	4.61*	2.34*	34.73*	5.12*	18.99*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Pretreatment of the mixed substrate with Oyrm 1 (Table 4.11) resulted in a considerable reduction (35 %) in the insoluble lignin content in comparison to the untreated sample, while a significant ($p<0.05$) increase (143 %) in the soluble lignin fraction was also observed. Oyrm 1 preferentially removed the hemicellulosic fraction of the lignocellulosic material as evidenced by the arabinose being almost completely utilised during the fermentation, with 65 % being degraded during the first two months of fermentation alone. Xylose was also one of the primary carbohydrates degraded with over 18 % being consumed during fermentation. It is also worth noting that during the initial 90 days of fermentation, the xylose concentration of the substrate was increased by 19 %; however, as the fermentation proceeded the xylose content was reduced again with this xylose being utilised during the fourth month of fermentation. This initial increase in the xylose content may be as a result of a concentration effect on the xylose fraction, in which the other fractions in the fermentation were degraded, as was observed with the substantial removal of arabinose (65 %) during fermentation.

In contrast to Oyrm 1, minimal lignin degradation occurred during pretreatment with TV 3086 (Table 4.12) despite satisfactory growth and colonisation of the substrate. It was apparent that TV 3086 non-selectively degraded the material with glucose, xylose and arabinose contents being reduced by 15, 53 and 43 %, respectively during pretreatment. *T. versicolor* is known to require a nitrogen or carbon deficient environment to produce the lignin and manganese peroxidase enzymes required for lignin degradation (Swamy *et al.*, 1999). It is feasible that perhaps due to the relatively nitrogen rich environment provided by the cottonseed hulls in the substrate (Lu *et al.*,

1984; Li *et al.*, 2001) that the enzymes required for lignocellulosic degradation were produced in low quantities by TV 3086, thus minimal lignin degradation occurred, as also demonstrated in Table 4.9. If this is in fact the case, it would appear that TV 3086 is more sensitive to high nitrogen concentrations than Oyrm 1. The nitrogen content of the substrate is thought to affect the production of ligninolytic enzymes from fungi in different ways. For instance, Tekere *et al.* (2001) determined that *Trametes cingulata* and *T. elegans* produced the highest MnP activity in a medium containing low nitrogen conditions, whilst *Lentinula velutinus* and *Irpex* spp. favoured high nitrogen conditions for MnP production. Furthermore, Elisashvili *et al.* (2008) investigated the effect of nitrogen supplementation on laccase and MnP production from *T. versicolor*. They determined that supplementation with nitrogen increased laccase activity, while it tended to decrease the manganese peroxidase activity in most cases. This indicated that nitrogen can influence ligninolytic enzymes from the same fungus differently which can subsequently impact on the degradation of the substrate.

Composition of lignin found in both cotton seed hulls and coconut fibre may also have an impact on the degradation, where the increased molecular mass of the lignin found in cottonseed hulls influenced the degradation of the substrate by TV 3086. The structural and chemical differences in the lignin substrate can result in the production of a specialised set of enzymes in specific microorganisms, in particular the oxidative enzymes (Tuor *et al.*, 1995). Potentially, the enzymes produced from TV 3086 lacked the capability to sufficiently degrade the lignin from cottonseed hulls and coconut fibre, as white rot fungi produce different ligninolytic enzymes in various quantities and thus degrade lignin a different rates (Hatakka, 1994).

It is evident that fermentation of the cottonseed hulls and coconut fibre with Oyrm 1 was an effective method of pretreatment, with a significant ($p<0.05$) amount of lignin removed during fermentation of the substrate. In addition Oyrm 1 left the cellulose fraction (represent by glucose) relatively undegraded during fermentation, which would impact of the feasibility of using this substrate in bioethanol production, as the majority of the cellulose fraction is still available for hydrolysis to glucose and subsequent fermentation to ethanol.

4.1.5 Biological pretreatment of switchgrass

Switchgrass is a warm season grass which grows natively in North America and central Mexico (Lewandowski *et al.*, 2003). It has been identified as a model

herbaceous energy crop due to its high productivity, suitability for growth on marginal land quality, flexibility for multiple uses and low water and nutritional requirements (McLaughlin *et al.*, 1999). Switchgrass was traditionally cultivated and bred primarily as a forage crop; however, recent focus has shifted to breeding crops with high cellulose content to produce cellulosic ethanol (McLaughlin *et al.*, 1999; Mazarei *et al.*, 2011). Most studies involving the use of switchgrass as an energy crop for bioethanol have concentrated on physico-chemical and chemical techniques for pretreatment such as ammonium fibre expansion (AFEX), acid hydrolysis and aqueous ammonia (Kim *et al.*, 2011b). A relative scarcity of literature exists where biological pretreatment was used as a method for delignification of switchgrass. Keshwani *et al.* (2009) reviewed the current available literature on production of bioethanol from switchgrass and did not detail any published literature which dealt with the subject of biological pretreatment of switchgrass.

To assess its suitability, samples of switchgrass were soaked in deionised water for 45 minutes to moisten the material and subsequently sterilised and fermented with white rot fungi as detailed in Section 2.2.5.2. Samples were taken every 30 days, with the change in composition of the substrate monitored, using the methods outline in Section 2.2.8.

Table 4.13 Chemical composition and weight loss of switchgrass pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	32.96	17.17	5.01	20.98	6.78	0.00
60	37.37*	17.15	3.02	14.33*	7.00	10.77*
90	39.37*	14.38*	2.66	12.51*	7.13	15.46*
120	38.52*	10.61*	1.52*	11.60*	8.57*	17.65*
150	36.78*	9.62*	0.02*	11.11*	8.68*	19.76*
180	36.85*	8.89*	1.49*	11.13*	8.57*	25.68*
210	38.68*	9.43*	1.61*	10.20*	7.98*	27.53*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

As a substrate for bioethanol production, switchgrass appears to be relatively effective due to its high cellulose content (glucose) and relatively good xylan (xylose) concentrations which could be utilised for ethanol generation. This glucose content remained notably high following pretreatment with Oyrm 1 (Table 4.13), where a

significant increase ($p<0.05$) of 17 % was recorded in comparison to the initial value. This was most likely due to a concentration effect on the cellulose due to the degradation of the hemicellulose and lignin during fermentation (as explained previously in Section 4.1.1). Alternatively, the xylose utilised during the fermentation may have been synthesised during cell wall production, with the glucan in the mycelial cell wall known to be recovered as glucose during acid hydrolysis. Papaspyridi *et al.* (2010) investigated the production of *Pluerotus ostreatus* biomass with xylose as the primary carbon source and determined that the mycelium from this fungi contained 14 % glucan in the cell wall of the fungi.

The hemicellulose fraction appeared to be preferentially degraded by Oyrm 1 (Table 4.13) with the xylose concentration being reduced by 45 %. Furthermore, the arabinose content was also significantly ($p<0.05$) reduced with almost 68 % removed during fermentation. This coincided with the substantial reduction in the xylose concentration which is known to be linked to the arabinose units in the hemicellulose fraction. Insoluble lignin was reduced by over 52 % (from 21 % to 10.2 %), with the largest degree of degradation occurring during the first two months of fermentation. The rate of dry matter loss was most pronounced during the first 90 days of fermentation, which coincided with the loss of 40 % of the insoluble lignin. As the fermentation continued, the weight loss from the fermentation almost doubled, with 27 % of the material degraded after 210 days of fermentation, where minimal loss was due to a reduction in moisture in the fermentation.

Wan *et al.* (2011) investigated the pretreatment of a range of lignocellulosic materials including switchgrass with the white rot fungus *Ceriporiopsis subvermispora* and found that over 25 % of the lignin was removed during 18 days of incubation, which was a substantially faster rate of delignification than obtained in the present study. The increased rate of delignification noted by Wan and colleagues could be due to a longer colonisation period. *Pleurotus ostreatus* Oyrm 1 took over 21 days to fully colonise the material in comparison to *Ceriporiopsis subvermispora* in which the length of fermentation was 35 days, with lignin degradation thought not being initiated until complete colonisation of the substrate occurs (Villas-Boas *et al.*, 2002). Furthermore, this difference in the rate of delignification between studies could be due to the difference in the strains of fungi utilised, in which *Ceriporiopsis subvermispora* may be a more effective fungus at delignifying the substrate. Finally, the deviation in delignification rate may also be attributed to the composition of the switchgrass

investigated with the harvest period, herbage maturity as well as environmental factors influencing the composition and digestibility of the switchgrass (Buxton, 1996).

In any case the utilisation of Oyrm 1 as a pretreatment technique appears to be an effective method of removing the lignin from the substrate with 52 % (w/v) removed during fermentation, whilst the glucose fraction remained relatively undegraded, which would impact positively on the amount of ethanol that can be produced from the substrate.

Table 4.14 Chemical composition and weight loss of switchgrass pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Weight loss %
0	32.96	17.17	5.01	20.98	6.78	0.00
60	30.26*	15.36	4.49	15.56*	8.88*	10.75*
90	29.08*	14.02*	4.77	15.46*	9.47*	11.62*
120	31.52	14.80	2.12*	15.32*	9.72*	22.77*
150	33.83	13.28*	1.94*	13.64*	10.93*	27.91*
180	32.82	11.77*	1.91*	13.63*	11.15*	28.89*
210	27.58*	9.37*	3.13*	13.76	11.14*	33.59*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

It appears that lignin degradation of the switchgrass substrate by TV 3086 (Table 4.14) was not as pronounced as that detected with Oyrm 1. The insoluble lignin fraction was reduced by 35 % with TV 3086, where the highest rate of delignification was again observed over the first 60 days of fermentation. As was determined with other fermentations involving TV 3086, selective degradation did not occur, with the glucose content being reduced by 17 %, whilst xylose and arabinose concentrations were decreased by 48 and 38 %, respectively. In contrast to pretreatment with Oyrm 1, TV 3086 fermentation resulted in a substantial increase in soluble lignin (64 %), as a potential consequence of the formation of lignin degradation products (discussed previously in Section 4.1.2.1).

In comparison to some of the leading chemical methods for pretreatment of switchgrass, the results outlined in this study appear to compare favourably with other results reported. However, the length of fermentation time would have to be factored into any financial considerations, as this may impact on the economic feasibility of the fermentation (Pandey *et al.*, 1999). A larger inoculum may be used to potentially

shorten the initial colonisation period considerably, thus substantially reducing the fermentation time. Kim *et al.* (2011b) investigated the use of AFEX, dilute acid, liquid hot water, lime and aqueous ammonia pretreatment on switchgrass and concluded that AFEX pretreatment failed to remove any lignin from the substrate, while dilute acid and liquid hot water only removed 10 % of the overall lignin. This delignification was relatively low in comparison to the 35 and 52 % insoluble lignin degradation which was achieved through biological delignification with the strains investigated in this study. Aqueous ammonia appeared to be the best chemical pretreatment method, with 60 % of insoluble lignin removed and 95 % of cellulose retained during pretreatment. It appears that in most chemical pretreatment methods almost the entire hemicellulosic fraction is degraded in the substrate through treatment with the various techniques which, depending on the type of ethanol fermentation being performed, may be less than ideal. At present, the production of bioethanol is an expensive process and producers find it difficult to compete with petroleum in the commercial market without subsidies (Gírio *et al.*, 2010). As was mentioned previously in Section 1.5.3.2, the conversion of the hemicellulosic fraction to ethanol can help reduce the cost of ethanol production by up to 25 % and thus increase the competitiveness of the process (Hinman *et al.*, 1989).

4.1.6 Biological pretreatment of oat straw

Crop residues represent an important by-product of the agricultural industry due to their lignocellulosic biomass which represents between 50 and 60 % of the total plant biomass (Zafar *et al.*, 1996). Cereal straws such as oat and wheat straw are the most abundant agricultural waste materials and are generally degraded slowly in nature due to the high content of recalcitrant lignin (Tuomela *et al.*, 2000) and their low composition of macronutrients (Stepanova *et al.*, 2003). Delignification of straw can alter the composition of this material leading to a more commercially valuable product for utilisation in a number of applications, such as the bioethanol industry. Oat straw represents an attractive substrate for bioethanol production because of its relatively high cellulose (30 %) and hemicellulose (22 %) content (Graminha *et al.*, 2008). Consequently, oat straw was biologically pretreated with two strains of white rot fungi and the degradation of the substrate monitored using the methods outlined previously in Section 2.2.8.

Table 4.15 Chemical composition and weight loss of oat straw pretreated with *Pleurotus ostreatus* Oyrm 1.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Dry matter loss %
0	38.58	20.04	1.12	23.82	5.18	0.00
60	40.16*	19.75	2.57*	16.92*	8.19*	20.59*
90	42.23*	19.46	2.90*	14.78*	9.13*	30.55*
120	46.41*	16.05*	1.56	12.62*	9.19*	35.56*
150	47.37*	12.60*	1.31	10.24*	9.42*	43.46*
180	46.82*	9.84*	1.14	8.88*	9.49*	43.63*
210	46.39*	7.15*	1.87	5.79*	9.24*	57.25*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

It would appear from analysis of the results obtained in Table 4.15 that the delignification of oat straw with Oyrm 1 resulted in the highest amount of delignification of any of the substrates studied in Section 4.1, with over 75 % of the insoluble lignin removed during 210 days of pretreatment. The most substantial rate of degradation occurred during the first 60 days of fermentation with ~30 % of lignin being broken down during this time. To coincide with this, the soluble lignin fraction was increased by over 78 % of the initially recorded value to 9.24 % of the total composition. As discussed previously, this was most likely due to an increase in the lignin degradation products which are released from the substrate during fermentation, as outlined previously in Section 4.1.2.1.

It would appear that *Pleurotus ostreatus* Oyrm 1 again selectively degraded the xylose fraction of the substrate, with 65 % of xylose utilised during fermentation. In contrast, the cellulose fraction (represented by glucose in Table 4.15) was increased, which is most likely due to the concentration of the cellulose fraction due to the degradation of the xylan and lignin fraction or the utilisation of the xylose by the fungi for growth and cell wall formation, which contains glucan that is recovered as glucose following hydrolysis by concentrated sulphuric acid, as explained previously in Section 4.1.1. The arabinose content was increased during the first 90 days of fermentation. This may also be attributed to a concentration effect, in which the removal of the lignin and xylose has concentrated the arabinose content in the oat straw. However, as the fermentation continued the arabinose was subsequently decreased which coincided with a significant reduction in the xylose content.

A dearth of literature analysing oat straw degradation by white rot fungi exists and no published literature could be found analysing the two strains selected for this study. Nevertheless, Stepanova *et al.* (2003) analysed the degradation of oat straw through solid state fermentation with *Cerrena maxima* for 83 days. The authors determined that 53 % of lignin was removed during this time, which would be in close agreement with the results obtained in this study over a similar fermentation time. However in contrast, nearly 75 % of the cellulose fraction was removed during pretreatment with *C. maxima* which was substantially more than the results obtained in this study.

Biological pretreatment of oat straw with Oyrm 1 also compared favourably with a variety of commonly used chemical methods of pretreatment. Krongtaew *et al.* (2010) investigated the pretreatment of oat and wheat straw with a number of different chemical techniques including acid hydrolysis, alkali and H_2O_2 pretreatment. The authors noted that acid pretreatment removed 12 % of lignin, which was substantially less than the lignin removal achieved through biological pretreatment. Pretreatment with alkali and hydrogen peroxide obtained the highest degree of delignification, with 75 % of lignin removed, which is similar to what was obtained with biological pretreatment in this study. However, the formation of inhibitory products, which need to be removed prior to ethanol fermentation can occur during alkali pretreatment, making it an unfavourable technique.

Table 4.16 Chemical composition and weight loss of oat straw pretreated with *Trametes versicolor* 3086.

Time (days)	Glucose % (w/v)	Xylose % (w/v)	Arabinose % (w/v)	Insoluble Lignin % (w/v)	Soluble Lignin % (w/v)	Dry matter loss %
0	38.58	20.04	1.12	23.82	5.18	0.00
60	36.97*	19.88	2.99*	18.34*	7.44*	15.97*
90	36.10*	19.86	3.22*	17.85*	8.36*	24.17*
120	33.18*	17.83	3.25*	21.71*	8.03*	32.84*
150	35.02*	19.27	2.57*	17.78*	8.83*	45.89*
180	36.68*	17.18*	2.32*	13.67*	9.51*	50.38*
210	32.60*	18.18	3.46*	19.03*	9.33*	53.12*

Results displayed are a representation of the means of triplicate fermentations. Probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

In contrast to Oyrm 1, delignification by TV 3086 (Table 4.16) was considerably less dramatic with only 21 % of the insoluble lignin removed during the

fermentation. As has been noted throughout these experiments, TV 3086 did not selectively degrade the lignin and hemicellulose in the substrate but rather non-selectively degrades the whole substrate. This was again demonstrated, with 16 % of the cellulose (glucose) utilised from the sample, whilst 10 % of xylose (xylan) was also lost during pretreatment. It was evident that the growth of TV 3086 on oat straw was relatively poor, with the substrate colonised slowly throughout the fermentation. This was in stark contrast with *Pleurotus ostreatus* Oyrm 1, which grew rapidly on the oat straw and colonised the substrate heavily. Thus it was concluded that TV 3086 may not be an ideal strain to use for pretreatment of oat straw as it may not supply sufficient nutrients for the TV 3086 to grow adequately.

4.1.7 General conclusions

The use of a biological approach for pretreatment of lignocellulosic material appears to be a suitable method for the degradation of a number of the lignocellulosic substrates examined, namely corn stover, switchgrass, cottonseed hulls/coconut fibre and oat straw. The main problem in using lignocellulosic waste products in their current state is the difficulty in accessing the cellulose fraction due to the presence of lignin; however, in a number of these fermentations substantial substrate delignification was achieved. For instance, 63 % of insoluble lignin was removed from the upper stem of the corn with Oyrm 1, whilst almost 74 % of the xylan was also removed. Efficient removal of the hemicellulose fraction from the cellulose may also be required to enable efficient hydrolysis of the cellulose fraction prior to lignocellulosic ethanol production, due to the interaction between the two fractions which can interfere with hydrolysis (Chesson, 1981; Kuhad *et al.*, 1997). Maximal lignin removal occurred through pretreatment of oat straw with Oyrm 1, which degraded 75 % of the insoluble lignin fraction during fermentation. Biological pretreatment was not an effective pretreatment technique in all cases however. For example, in cottonseed hulls minimal lignin removal was observed, with only 3 and 11 % of the insoluble lignin fraction being degraded with TV 3086 and Oyrm 1, respectively. In addition, both TV 3086 and Oyrm 1 failed to colonise the pine wood shavings and coconut fibre during fermentation, thus limiting the applicability of using biological pretreatment with these substrates.

It was evident that pretreatment with *Pleurotus ostreatus* Oyrm 1 was typically more efficient at removing lignin than TV 3086. In most fermentations Oyrm 1 tended

to remove a higher degree of lignin during fermentation, with up to 3.8 times more lignin being removed in some substrates (oat straw). Furthermore, an increase in the glucose content of substrates was also observed during pretreatment with Oyrm 1. This was attributed to a concentration effect in the sample in which the reduction in hemicellulose and lignin caused an apparent increase in the cellulose fraction.

TV 3086 tended to non-selectively degrade the lignocellulosic substrates, with indiscriminate degradation of the cellulose, hemicellulose and lignin observed through pretreatment. By contrast, *Pleurotus ostreatus* Oyrm 1 tended to selectively remove the lignin and hemicellulose fractions during fermentation, leaving the cellulose relatively undegraded.

A fungal strain such as *Pleurotus ostreatus* Oyrm 1 would be a suitable strain for pretreatment of lignocellulosic biomass for ethanol production as the cellulose fraction remained relatively unutilised during fermentation. Using a strain such as TV 3086 would result in the degradation of the cellulose fraction during pretreatment of the substrate, which in turn would result in a lower ethanol yield, thus affecting the efficiency of the fermentation.

Although there are certain disadvantages to biological pretreatment such as fermentation time and economic viability, on a whole the results in Section 4.1 demonstrated that lignin can be efficiently removed from agricultural wastes without the use of harsh, environmentally damaging chemicals. Furthermore, this method of lignin removal compared favourably with other pretreatment techniques in which comparable amounts of lignin were removed during processing.

4.2 Bioethanol production from corn mash and lignocellulosic wastes

At present, the principal cost in the production of fuel ethanol is the substrate, which can be obtained from agricultural materials such as wheat, corn and barley (Lee, 1997). However, due to the competition between the food and fuel sectors for these residues, this cost has increased further over the last decade (Rathmann *et al.*, 2010). Incorporation of lower value lignocellulosic substrates offers great potential in reducing the cost of production, which would in turn allow bioethanol to be more competitive with traditional fuel sources, such as petroleum.

Lignocellulose is the most abundant biopolymer found on earth (Sánchez *et al.*, 2008) and represents over 50 % of the biomass produced annually in the world, with it being estimated that between 10-50 billion tons are produced every year (Claassen *et al.*, 1999). Accordingly, this material represents a vast potential resource for bioethanol production, which may be able to compete with petroleum as a fuel source, provided some of the current drawbacks are overcome. Presently, the fundamental problem associated with lignocellulosic ethanol production is the efficient hydrolysis of the biomass to ensure adequate lignin removal, whilst retaining sufficient cellulose to ensure an economical fermentation.

A number of methods exist pertaining to lignin and hemicellulose removal from lignocellulosic biomass, including the physical, mechanical and biological degradation methods outlined previously in Section 1.1.2. Despite the number of methods that exist for lignocellulose pretreatment, each one has their inherent disadvantages including pollution concerns, cost and the co-production of inhibitors (Graminha *et al.*, 2008).

In Section 4.1, biological pretreatment was selected as the method of pretreatment due to the low energy cost and low process control required, in addition to the capacity to remove both lignin and hemicellulose fractions (Graminha *et al.*, 2008). This is not the case with other techniques such as steam and CO_2 explosion, where a two-stage process is required to achieve efficient lignin and hemicellulose removal.

Ethanol production globally uses materials containing high starch or simple sugar concentrations as they are easy to use and require little processing prior to fermentation. Currently, the principal substrates used in bioethanol production are sugar cane, corn, wheat and barley grains, whilst in some tropical countries such as Thailand and South America, cassava is also used (Sánchez *et al.*, 2008). However, due to the increased production of ethanol and the competition for these residues with the food industry, the cost of these substrates has risen dramatically over the last number of years (Binod *et al.*, 2010). As a consequence, research has been undertaken to replace these residues with lower cost lignocellulosic materials which can be obtained relatively cheaply (Ohgren *et al.*, 2007; Flandez *et al.*, 2012).

In this section, the pretreated lignocellulosic materials produced for Section 4.1 were utilised by blending the degraded biomass with freshly prepared corn mash. This was to determine whether the final ethanol concentration could be enhanced. Ethanol

production using the pretreated material as the sole substrate was also investigated, where the material was hydrolysed with an enzyme cocktail in a simultaneous saccharification and co-fermentation process. An enzyme cocktail produced using the optimal conditions for xylanase and cellulase production from *Aspergillus niger* 102.12 and *Aspergillus oryzae* 553.65, as determined in Section 3.2, was utilised in these fermentations. Samples were taken at specific time intervals during each fermentation with the carbohydrate, acetic acid, lactic acid, glycerol and ethanol concentrations being determined throughout.

4.2.1 Yeast strain selection

The selection of an appropriate yeast prior to fermentation is essential, as an unsuitable strain can lead to poor yields of the target product of interest i.e. yeast biomass, ethanol or glycerol. Typically, strains of *Saccharomyces cerevisiae* are used in ethanol fermentations due to their robust nature and tolerance to high ethanol concentrations (Gírio *et al.*, 2010). However, research is ongoing in the search for other yeast strains which may offer better efficiency in order to reduce the relative cost of producing ethanol (Nissen *et al.*, 2000; Watanabe *et al.*, 2010; Kasavi *et al.*, 2012).

Strains of yeast for ethanol production require a number of desirable traits in order to be considered for a production scale facility. These traits include efficient aerobic and anaerobic metabolic capabilities, high ethanol tolerance, resistance to killer yeasts, low foam formation and a resistance to high osmotic pressures (Basso *et al.*, 2008). The formation of glycerol during fermentation is also thought to be inhibitory in terms of ethanol production, meaning low glycerol concentrations would be desirable. Basso *et al.* (2008) studied a number of different yeast strains found in the ethanol production industry in Brazil. They determined that yeasts which produced higher ethanol yields also typically produced lower glycerol concentrations and contained a higher cell viability.

To find a suitable strain for ethanol production, a selection of yeasts were propagated in yeast malt (YM) medium for 16 hours. These suspensions were subsequently centrifuged and a dilution of each was carried out to obtain a culture containing 2×10^{10} cells mL^{-1}. These cultures were then used to inoculate corn mash, which was prepared using the method described previously in Section 2.2.6.2. Samples were taken intermittently and analysed for ethanol, carbohydrate and the fermentation acid concentration using the HPLC method described in Section 2.2.6.4.

Fermentations were compared in relation to final ethanol concentration, fermentation efficiency, ethanol production rate and residual sugar concentration remaining after fermentation to determine the optimal strain for ethanol production (Table 4.17 & Figure 4.1). Fermentation efficiency is determined as a percentage of ethanol produced in relation to the amount of carbohydrate remaining in the mash.

The yeast investigated in this test included *Saccharomyces cerevisiae* 72, 358, 88, 7044, 10676, 1026, *Pichia thermotolerans* 7012, *Pichia jardinii* 621, *Saccharomyces pastorianus* 203 and *Candida utilis* 70167. Saccharomyces strains Lab 1 and 2, which were yeast strains kindly donated by Alltech Inc., Nicholasville Kentucky, were also used. A number of other yeast strains were also considered; however, the results from an initial screening (results not shown) indicated that these strains produced mixed products such as lactic acid, acetic acid and ethanol in the fermentation broth and thus were disregarded.

Table 4.17 Compositional analysis of corn mash fermentation liquor after 48 hours of fermentation with a selection of yeast strains.

Yeast strain	Maltodextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Acetic acid % (w/v)	Ethanol % (v/v)	Efficiency %
Lab 1	0.03	0.07	0.01	0.61	0.03	10.03	99.52
Lab 2	0.04	0.01	0.01	0.60	0.00	10.54	99.75
Saccharomyces cerevisiae 72	0.04	0.03	0.01	0.58	0.02	10.03	99.65
Saccharomyces cerevisiae 88	0.02	0.01	0.01	0.59	0.04	10.04	99.83
Saccharomyces pastorianus 203	0.04	0.01	0.04	0.55	0.01	10.68	99.64
Saccharomyces cerevisiae 358	0.04	0.00	0.02	0.52	0.00	10.29	99.75
Pichia jardinii 621	0.04	0.01	0.02	0.53	0.01	10.61	99.71
Saccharomyces cerevisiae 1026	0.04	0.01	0.20	0.59	0.00	10.00	98.96
Saccharomyces cerevisiae 4070	0.04	0.01	0.02	0.55	0.01	10.62	99.71
Saccharomyces cerevisiae 7044	0.04	0.01	0.02	0.55	0.02	10.09	99.70
Pichia thermotolerans 7012	0.04	0.00	0.02	0.53	0.00	10.68	99.76
Saccharomyces cerevisiae 10676	0.04	0.01	0.06	0.46	0.00	10.28	99.54
Candida utilis 70167	0.35	0.13	1.22	0.56	0.00	8.78	92.47

Results displayed are the means of triplicate flasks.
Ethanol efficiency was determined using the formula outline in Section 2.2.6.4

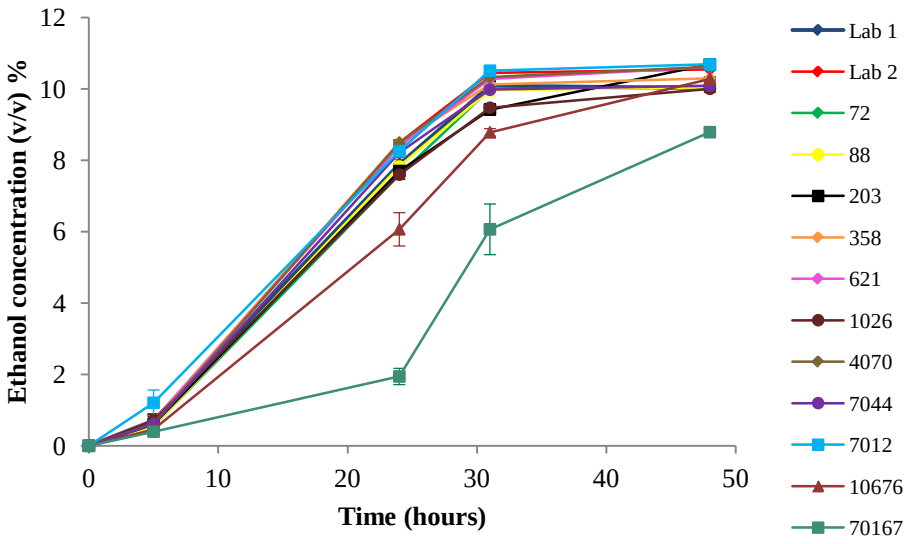


Figure 4.1 Comparison of the ethanol production from a selection of yeast strains over time.

Results displayed are the means of triplicate fermentations with the standard deviation of each fermentation represented by error bars.

Analysing the data in Figure 4.1, it was evident that the majority of the strains examined produced ethanol at a similar rate, with the ethanol fermentation rate curves looking distinctly similar. However, two yeast strains produced ethanol at a slower rate (*Candida utilis* 70167 & *Saccharomyces cerevisiae* 10676) in comparison to the other yeast strains. These two yeast strains were among a group, which also included *Saccharomyces pastorianus* 203 and *Saccharomyces cerevisiae* 4070 that grew visibly slower during initial cultivation of the yeast strains to produce the yeast inoculum. The cell counts from these four strains were lower (<1x 10^6 cells mL^{-1}) during this initial cultivation period in comparison to the cell counts of the other yeast strains (~1 x 10^8 cell mL^{-1}), thus requiring a lower dilution of the yeast cells to obtain the same biomass concentration (2x10^{10} cells mL^{-1}) for the subsequent ethanol fermentations.

Candida utilis 70167 produced the lowest final ethanol concentration of all the strains tested, with the final yield being almost 2 % (v/v) less than the ethanol concentration achieved by *Pichia thermotolerans* 7012 (Table 4.17). When the composition of the liquor was examined it was determined that 1.22 % (w/v) glucose remained in the broth, as well as 0.35 % (w/v) maltodextrin. This was substantially more than the sugar concentrations remaining in the other fermentations, where almost complete utilisation of the available carbohydrates was observed (Table 4.17). If this fermentation was allowed to continue longer, the strain may have produced a higher

concentration of ethanol as it was evident that the growth rate of the yeast was poor in comparison to the other fermentations. On the other hand, this residual sugar may be due to a lower tolerance to ethanol and thus a reduced viability. Yeasts are known to have different tolerance to ethanol depending on the strain which was cultivated. In ethanol production *Saccharomyces* spp. are traditionally utilised as they had a high ethanol tolerance (up to 12 %) and a similarly high tolerance to osmotic stress (Rose, 1993). Pina *et al.* (2004) analysed the difference in ethanol tolerance between *S. cerevisiae* and a number of different yeast strains under a variety of different conditions. They found that *Candida stellata* was the most stable yeast under ethanol stress in all conditions, whilst *Debaryomyces hansenii* was extremely intolerant to ethanol stress, with the yeast viability being reduced to zero after four minutes of exposure to 25 % (v/v) ethanol.

Saccharomyces pastorianus 203 and *Pichia thermotolerans* 7012 produced the highest concentration of ethanol from all strains tested at 10.68 % (v/v). This was marginally (1 %) greater than the next best strain, *Saccharomyces cerevisiae* 4070, although the rate of fermentation was also lower with this strain. Whilst there does not appear to be an appreciable difference in final ethanol concentration between these strains and the *Saccharomyces cerevisiae* strains, if these fermentations were scaled up to a larger volume the difference in the ethanol yield would be substantially more. For example, Basso *et al.* (2008) determined than a difference of 3 % in the ethanol yield from two different yeast strains would equate to an increase of 2.1 million litres of ethanol per crop season in a medium capacity distillery.

Despite *Saccharomyces pastorianus* 203 and *Pichia thermotolerans* 7012 producing the same final ethanol concentration (Table 4.17), the rate of ethanol production by *Saccharomyces pastorianus* 203 was marginally lower. After 31 hours of fermentation 9.41 % (v/v) of ethanol was produced with *Saccharomyces pastorianus* 203 in comparison to *Pichia thermotolerans* 7012 which produced 10.5 % (v/v) over the same time period. It is likely this fermentation would be stopped after this time as very little additional ethanol (0.18 % v/v) was produced during the final 18 hours of fermentation and it would not be economically viable on a commercial scale to continue fermentation.

The glycerol content of each fermentation is also an interesting measurement as it can give an indication of the viability of the yeast since glycerol formation is linked to yeast growth (Oura, 1973). It is thus thought that the fermentations with the highest

glycerol concentration would contain the most vigorous growth. However, glycerol can also be produced during times of stress, most notably osmotic stress (Walker, 1998), suggesting that it may also be formed as a result of this. In these fermentations there did not appear to be any correlation between the ethanol and glycerol content produced by the yeast, with the Lab 2 strain producing one of the highest ethanol yields, while also producing the second highest concentration of glycerol.

The efficiencies of the fermentations tended to be relatively similar in all fermentations, with the exception of *Candida utilis* 70167, where substantially more carbohydrate remained following the fermentation period (Table 4.17). It is thus likely that the differences between the final ethanol concentrations between each yeast strain was as a result of a larger concentration of the carbohydrate being utilised for maintenance, growth and glycerol production in comparison to *Pichia thermotolerans* 7012 (Zhang *et al.*, 2008).

It was therefore apparent that *Pichia thermotolerans* 7012 would be the most suitable strain for ethanol production due to the higher ethanol yields achieved in comparison to the other strains. In addition the efficiencies of the fermentations were all relatively similar, with the exception of *Candida utilis* 70167 which was lower, as the rate of fermentation was slower. Consequently, *Pichia thermotolerans* 7012 was selected as the yeast strain to be used during the remainder of this study.

4.2.2 Corn mash supplementation with pretreated lignocellulosic biomass

As mentioned in Section 4.1, two fungal strains were selected to investigate the degradation of lignocellulose by white rot fungi. Ideally, the chosen fungi would hydrolyse the bound lignin, leaving the cellulose available for enzymatic hydrolysis and subsequent fermentation to ethanol. These two strains included *Pleurotus ostreatus* Oyrm 1, which selectively degraded the lignocellulosic biomass and *Trametes versicolor* 3086 which was found to non-selectively degrade the same material. Biological degradation of lignocellulosic materials causes significant changes in the biomass composition that can lead to the release of nutrients from the biomass, which otherwise would be unavailable. Lignocellulosic residues pretreated with Oyrm 1 were selected for this analysis as the results from Section 4.1 indicated that Oyrm1 offered a greater degree of lignin hydrolysis, whilst also retaining a higher portion of the cellulose content following pretreatment. Consequently, a selection of different lignocellulosic materials pretreated with Oyrm 1 were used to replace an

equal weight of corn mash in different fermentations. The aim was to determine whether the pretreatment process resulted in a significant change in the composition of the pretreated material. This could subsequently influence both the rate of ethanol production and/or final ethanol concentration through the release of nutrients from the lignocellulosic material. Due to difficulties during the harvest season, insufficient corn stover was obtained to carry out substitution fermentations with these residues. However, fermentations that included oat straw, cottonseed hulls, cottonseed hulls/coconut fibre, corncobs and switchgrass were investigated.

To determine the effect of the replacement of corn mash with ground (<3mm) pretreated lignocellulosic biomass, samples were added to the corn mash and autoclaved, as described previously in Section 2.2.6.5. This substrate was subsequently inoculated and incubated at 30 °C for a defined time period. Samples were taken intermittently throughout the fermentation, with the production of ethanol and fermentation acids being monitored throughout (Section 2.2.6.4). Each lignocellulosic biomass was assessed at a number of different time points, with ethanol production compared to a control corn mash fermentation to assess the difference between the fermentation rates and the final ethanol concentrations.

A number of fermentations were also supplemented with exogenous cellulase (2.15 filter paper units (FPU) per g of substrate) from *Trichoderma viride* (Sigma-Aldrich, Tallaght, Ireland) to assess whether sufficient hydrolysis of the lignin had occurred such that the cellulose fraction (which is protected by layers of lignin) would be more available for hydrolysis by cellulase enzymes. Resulting glucose could subsequently be utilised by the yeast for the production of ethanol during fermentation. Typically, 15 FPU of cellulase is added per gram of glucan during ethanol production (Sathitsuksanoh *et al.*, 2010). A concentration of 2.15 FPU mL^{-1} of fermentation liquor would provide a sufficient concentration of cellulase for efficient hydrolysis, as the maximal amount of glucan added was in the corncob fermentations, where 6.6 g (as can be determined by the amount of cellulose present in the corncob after 180 days pretreatment Table 4.2) was added to the fermentation.

4.2.2.1 Ethanol production from corn mash supplemented with pretreated corncobs

To obtain an indication of the hygroscopicity of the substrate, a titration of different volumes of fermented corncob were used to replace an equal amount of corn mash substrate. This indicated that the addition of 6 % (w/w) corncobs to the mash was

adequate to provide a sufficient portion of lignocellulose, whilst not impeding ethanol production and sampling. Dried corncobs were highly hygroscopic and the addition of too much can make it difficult to sample. Each fermentation was analysed for ethanol production from *P. thermotolerans* 7012 (Figure 4.2), as well as sugar utilisation and glycerol formation during fermentation (Table 4.18).

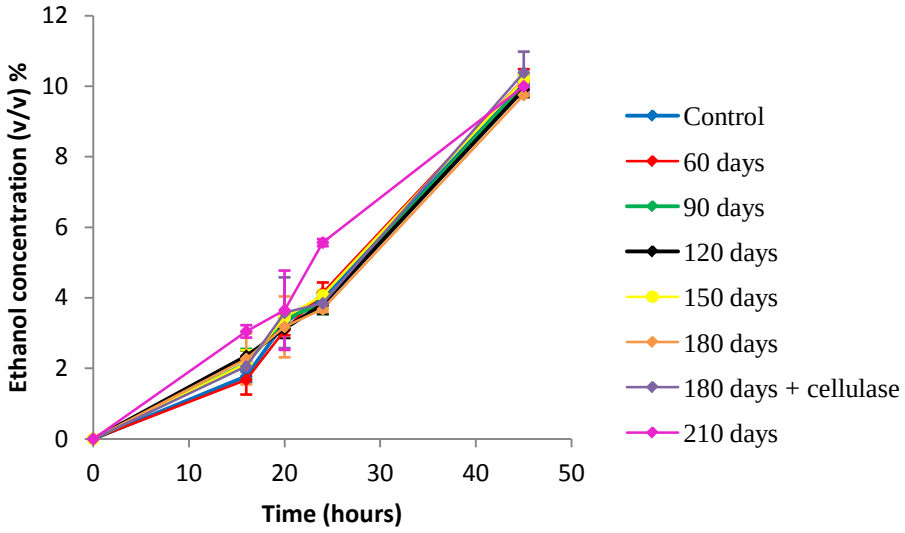


Figure 4.2 Comparison of the rate of ethanol production from corn mash fermentations supplemented with corncobs pretreated with *Pleurotus ostreatus* Oyrm 1.

Results plotted are the mean of triplicate flasks with the standard deviation of each treatment represented by error bars. + cellulase indicates that exogenous cellulase was added to the fermentation.

Table 4.18 End point fermentation composition and efficiency of corn mash liquor supplemented with corncobs pretreated with *Pleurotus ostreatus* Oyrm 1.

Pretreatment Time	Dextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Control	0.00	0.37	0.10	0.70	10.06±0.07	97.99
60	0.00	0.25	0.42	0.74	10.20±0.19	97.28
90	0.00	0.28	0.45	0.68	10.03±0.29	97.04
120	0.00	0.21	0.10	0.84	9.91±0.22	98.69
150	0.00	0.23	0.13	0.64	10.19±0.14	98.47
180	0.00	0.26	0.11	0.59	9.75±0.29	98.34
180 + cellulase	0.00	0.26	0.13	0.82	10.39±0.59	98.43
210	0.00	0.31	0.18	0.27	10.00±0.10	97.92

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*). + cellulase indicates that exogenous cellulase was added to the fermentation.

Analysis of the data in Figure 4.2 indicated that minimal change in the rate of ethanol production occurred through the addition of pretreated corncobs to the medium, with the exception of the fermentation containing corncobs pretreated for 210

days, where a moderate increase in the initial rate of fermentation was noted. However, as the fermentation approached 45 hours, the amount of ethanol produced was similar to the other fermentations as the rate of ethanol production slowed in comparison. In each other fermentation, the rate of ethanol production was relatively similar to that of the control, with almost complete utilisation of the substrate occurring after 45 hours (Table 4.18).

The final ethanol concentration from the control mash was marginally lower than a number of the other fermentations tested. The highest concentration of ethanol (10.39 % v/v) was produced from the fermentation containing corncob pretreated for 180 days and supplemented with cellulase enzyme (Table 4.18). However, considering the standard deviation for individual fermentations, the resultant increase in the final ethanol concentration were not statistically different to that of the control. In saying this, when it is considered that 6 % less corn mash was used to obtain a marginally higher final ethanol concentration, appreciable savings may be possible with regard to the cost of the typical corn mash substrate, although the cost of pretreatment would have to be taken into account.

It was apparent that the reduction in the final ethanol concentration from the control in comparison to a number of the sample fermentations was partly due to the decreased ethanol efficiency in the fermentation, where a larger concentration of unfermented maltose remained (Table 4.18) in comparison those containing corncobs pretreated for 150 days and 180 days (with cellulase). Alternatively, the decreased ethanol yield may be due to the yeast utilising a higher portion of the carbohydrate in the mash for cell growth and maintenance, which would reduce the sugars available for ethanol production, as explained previously in Section 4.2.1.

The fermentation including cellulase contained a marginally higher final ethanol concentration than the fermentation containing corncobs pretreated for 180 days without cellulase. Despite this, when the standard deviation from the fermentations were taken into account, the difference in the final ethanol concentration was minimal. In any case, 15 g of pretreated corncobs was added to the mash, equating to 6.6 g of cellulose being available for hydrolysis by cellulases, providing complete removal of lignin occurred. This was not the case however, with appreciable quantities of lignin remaining in the substrate (Section 4.1.1). Theoretically, if this cellulose was converted to glucose and subsequently fermented to ethanol, an additional 3.4 % (v/v) ethanol could be produced, although evidently this was not the

case. This was not completely unexpected; however, considering the quantity of lignin remaining in the material undegraded. This inability of the exogenous cellulase to sufficiently hydrolyse the cellulose fraction from the corncob substrate is likely due to the insufficient lignin being removed from the substrate (Table 4.2), with the remaining portion of the lignin interfering with the hydrolysis of the cellulose polymer as the lignin wraps itself around the cellulose microfibrils, protecting them from hydrolysis. Pretreatment of corncobs with Oyrm 1 resulted in the removal of 14 % of the lignin from fermentation which leaves a significant portion of the lignin remaining in the material which could inhibit the hydrolysis of the cellulose. Although in saying this, it was thought that as some lignin was removed a portion of the cellulose may be available for conversion to ethanol.

Despite the minimal increase in the ethanol yield observed through the addition of pretreated corncobs to the corn mash, an appreciable reduction in the overall fermentation cost could be achieved through the utilisation of the corncobs in the fermentation, as less corn mash is required to obtain the same yield of ethanol from the fermentation the cost of the substrate could potentially be reduced, although the cost of pretreatment would need to be taken into account.

4.2.2.2 Ethanol production from corn mash supplemented with switchgrass

To obtain an indication of the hygroscopicity of the substrate, a titration of different volumes of pretreated switchgrass was added to the corn mash to determine the optimal inclusion rate for switchgrass. Findings indicated that the replacement of 2 % (w/w) corn mash with switchgrass was adequate to provide a sufficient portion of lignocellulose to the substrate, whilst not impeding ethanol fermentation and sampling. Switchgrass is known to absorb a substantial amount of water, with it being able to absorb as much as three times its weight in water during the pretreatment process. The fermentation containing switchgrass pretreated for 210 days was used to assess the impact of the addition of exogenous cellulase to the substrate as insufficient material from the switchgrass pretreated for 180 days was available. Results presented in Figure 4.3 and Table 4.19 indicate the rate of ethanol production from *P. thermotolerans* 7012 and the composition of the broth containing pretreated switchgrass, following fermentation.

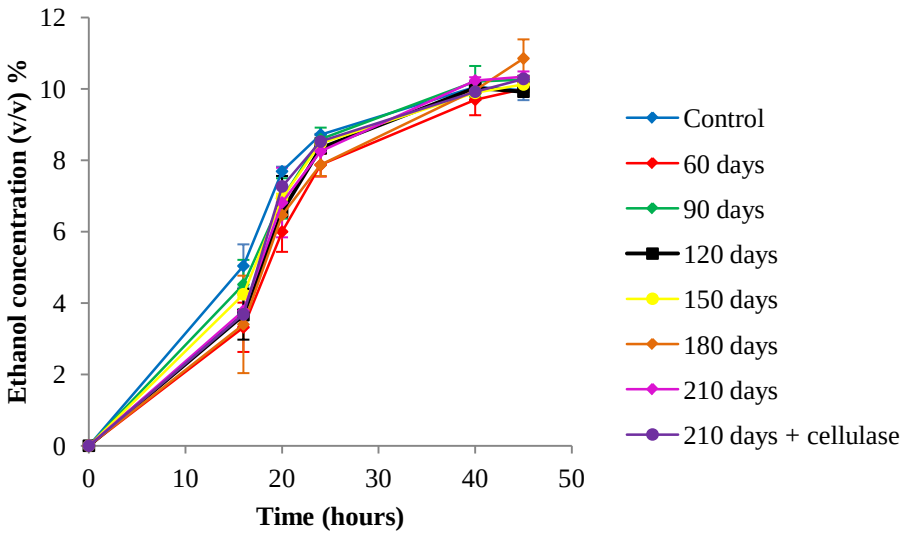


Figure 4.3 Comparison of the rate of ethanol production from corn mash fermentations supplemented with switchgrass pretreated with *Pleurotus ostreatus* Oyrm 1.

Results plotted are the mean of triplicate flasks with the standard deviation of each treatment represented by error bars. + cellulase indicates that exogenous cellulase was added to the fermentation.

Table 4.19 End point fermentation composition and efficiency of corn mash liquor supplemented with switchgrass pretreated with *Pleurotus ostreatus* Oyrm 1.

Pretreatment time (days)	Dextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Control	0.09	0.11	0.01	0.64	9.96±0.35	99.08
60	0.09	0.15	0.01	0.56	9.99±0.09	98.91
90	0.07	0.11	0.03	0.58	10.25±0.13	99.11
120	0.12	0.06	0.01	0.58	9.91±0.24	99.16
150	0.10	0.05	0.03	0.55	10.12±0.22	99.23
180	0.12	0.07	0.01	0.63	10.85±0.67	99.18
210	0.16	0.08	0.03	0.56	10.33±0.18	98.86
210 + cellulase	0.13	0.06	0.04	0.53*	10.27±0.10	99.03

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*). + cellulase indicates that exogenous cellulase was added to the fermentation.

The addition of pretreated switchgrass to the corn mash fermentation appeared to have minimal impact on the rate of ethanol production and the final ethanol yield obtained in the majority of fermentations (Figure 4.3). The highest concentration of ethanol was detected with the culture containing switchgrass pretreated for 180 days; however, when the standard deviations of the fermentations were taken into account, the difference between this fermentation and the control mash fermentation was relatively small. The efficiencies of each fermentation were also similar, with an

efficiency of over 98 % determined in each fermentation (Table 4.19). It is also worth noting that the fermentations containing switchgrass (Figure 4.3) had a higher initial rate of fermentation than those containing corncobs (Figure 4.2). It is not fully understood why this was although it could be due to a number of reasons. For instance, the initial lag phase of the yeast may have been longer in the corncobs fermentation which as a result would increase the incubation time. Furthermore, the oxygen, heat and mass transfer as well as CO_2 dissolution may have been different in the two fermentations due to the solids in the substrate, which may also have influenced the fermentation rate (Reisman, 1993; Barigou *et al.*, 1998; Littlejohns *et al.*, 2007). Littlejohns *et al.* (2007) investigated the effect of different polymers on the transfer of oxygen in the reactor. The authors determined that polymers such as silicone and rubber reduced the mass transfer coefficient by up to 63 %, whilst nylon increased the mass transfer by as much as 268 %.

It would appear that during the initial 18 hours of fermentation, the rate of ethanol production from the sample fermentations were marginally lower than that of the control. In some cases however, such as the fermentation containing switchgrass pretreated for 180 days the error bars were appreciably wide, with the difference between this and the control being minimal when the standard error was considered. As the fermentation proceeded the difference between the rate of ethanol production decreased, with the end point of the fermentations being the same. As a result, the initial increase in the rate of production would have minimal impact on the overall fermentation time.

It was apparent that the ethanol yield from the control was marginally lower than that of some of the other fermentations. This may be due in part to the yeast from the control utilising a higher portion of the carbohydrate for glycerol production, where it was apparent that the control broth contained a marginally higher glycerol content following fermentation. Furthermore, the yeast may have utilised more carbohydrate for cell maintenance and growth, thus reducing the sugars available for ethanol production, as mentioned previously in Section 4.2.1.

No increase in the ethanol yield was observed in the fermentation containing pretreated switchgrass and exogenous cellulase in comparison to the other unsupplemented fermentations (Table 4.19). This was surprising considering a significant portion of the lignin fraction (58 %) was removed from the switchgrass during pretreatment and it was thought that due to this substantial degradation, an

increase in the ethanol concentration would be obtained, as a consequence of the fermentation of the hydrolysate. This did not appear to be the case however, with no increase in ethanol yield obtained, which may have been for a number of reasons. Firstly, insufficient lignin may have been removed from the substrate with the remaining portion of the lignin interfering with the hydrolysis of the cellulose, as explained previously in Section 4.2.2.1.

Secondly, the formation of inhibitors such as soluble phenolic compounds and furan derivatives from the lignocellulosic biomass may have inhibited or deactivated the cellulase enzymes during fermentation (Ximenes *et al.*, 2010; Kim *et al.*, 2011c). Kim *et al.* (2011c) demonstrated that soluble inhibitors in the form of furan derivatives, organic acids and phenolic compounds which were formed during liquid hot water pretreatment inhibited the action of cellulase enzymes on solka-floc. These inhibitors may have been formed during the sterilisation process in the switchgrass fermentations, where the lignocellulose substrate and corn mash was autoclaved for 20 minutes at 121 °C and 17.5 psi. Furthermore, phenolic compounds may have been released from the lignocellulosic substrate during pretreatment which may inhibit the action of cellulase enzymes.

Thirdly, the hydrolysis of the cellulose may have been affected by the native structure of the cellulose. For instance, the crystallinity of the cellulose substrate is understood to be one of the major physical parameters which can influence its hydrolysis by cellulases (Fan *et al.*, 1980; Hoshino *et al.*, 1997). The cellulase enzyme added to the broth may not be effective against the crystalline cellulose from switchgrass, where a different set of enzymes may be required for efficient conversion. Furthermore, the hydrolysis of this crystalline cellulose may also have been affected by the ethanol concentration in the mash. Chen *et al.* (2006), determined that concentrations of between 1 and 7 % (w/v) ethanol inhibited the hydrolysis of crystalline cellulose by cellulase from *Penicillium decumbens*. Finally, the cellulase enzyme added to the fermentation may have been inhibited by the high concentrations of glucose present, as cellulase enzymes are known to be inhibited by high concentrations of glucose in the medium (Duff *et al.*, 1996; Balat, 2011). The release of the glucose units from the corn starch during the initial stages of fermentation may have impeded the hydrolytic activity of the exogenous cellulase enzymes which were added to the fermentation. During the initial stages of fermentation, up to 7 % (w/v) glucose was present in the medium which may be sufficient to inhibit cellulase action.

Takagi (1984) investigated the inhibition of cellulase enzyme from *Trichoderma reesei* by a number of different fermentation products at an inclusion rate of 5 %. They determined that glucose inhibited up to 82 % of the cellulase activity when a concentration of 5 % was present in the broth. This inhibition would have only been during the initial stages of fermentation; however, with the inhibition being reduced as the glucose was utilised. It is thus unlikely that this is the explanation for the reduction in the cellulase activity as cellulase inhibition would have occurred throughout fermentation.

Consequently, it was apparent that the pretreatment of switchgrass with the white rot basidiomycete *Pleurotus ostreatus* Oyrm 1 was not an effective method of increasing ethanol production from corn mash. However, this method of replacement may have a considerable impact on the economics of the fermentation as the cost of the substrate can be reduced, although the cost of pretreatment would need to be taken into account. By replacing the corn mash in the fermentation the same concentration of ethanol was achieved using a smaller amount of corn mash, thus reducing the cost of the substrate, as switchgrass can be obtained relatively cheaply. In order to improve the value of such biomass, the remaining lignin fraction may need to be degraded to release the cellulose fibres. Alternatively, a different cellulase cocktail may be required to hydrolyse the cellulase substrate to glucose, whilst any inhibitors found in the broth may also need to be removed providing it is economically viable.

4.2.2.3 Ethanol production from corn mash supplemented with cottonseed hulls

To obtain an indication of the hygroscopicity of the substrate, a titration of different volumes of pretreated cottonseed hulls was also carried out to find the optimal inclusion rate. The substitution of 4 % (w/w) cottonseed hulls to the mash appeared to be the optimal dosage to provide a sufficient portion of lignocellulose to the substrate, whilst also not impeding ethanol fermentation and sampling (data not shown). Cottonseed hulls can absorb a significant amount of moisture and thus impede the fermentation if added at too high a level. Results presented in Figure 4.4 and Table 4.20 represents the rate of ethanol production from *P. thermotolerans* 7012 and the composition of the broths containing pretreated cottonseed hulls following fermentation.

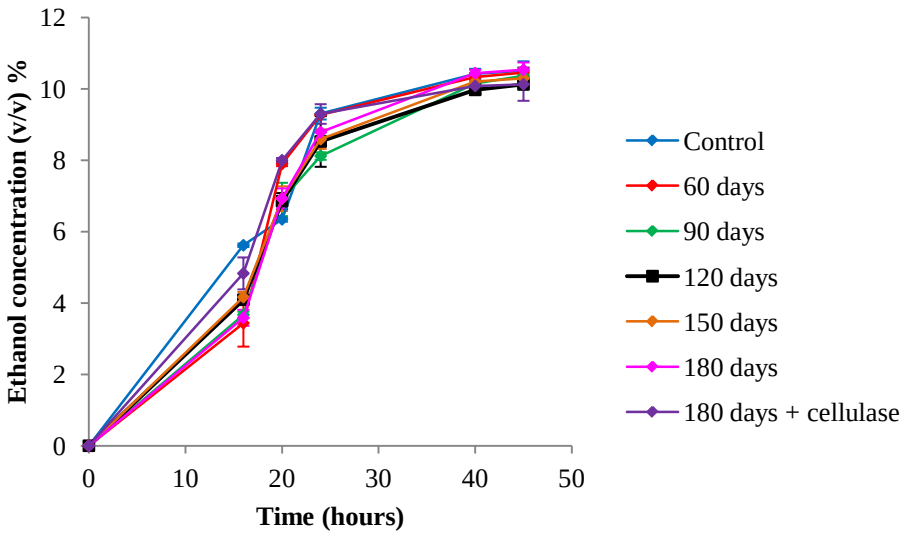


Figure 4.4 Comparison of the rate of ethanol production from corn mash fermentations supplemented with cottonseed hulls pretreated with *Pleurotus ostreatus* Oyrm 1.

Results plotted are the mean of triplicate flasks with the standard deviation of each treatment represented by error bars. + cellulase indicates that exogenous cellulase was added to the fermentation.

Table 4.20 End point fermentation composition and efficiency of corn mash liquor supplemented with cottonseed hulls pretreated with Pleurotus ostreatus Oyrm 1.

Pretreatment time (days)	Dextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Control	0.03	0.01	0.16	0.51	10.50±0.32	99.20
60	0.02	0.02	0.13	0.67	10.45±0.16	99.32
90	0.04	0.05	0.11	0.66	10.36±0.05	99.10
120	0.05	0.01	0.12	0.62	10.12±0.10	99.25
150	0.02	0.04	0.12	0.58*	10.29±0.27	99.26
180	0.04	0.03	0.11	0.57	10.53±0.25	99.07
180 + cellulase	0.03	0.02	0.11	0.57	10.12±0.58	99.33

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*). + cellulase indicates that exogenous cellulase was added to the fermentation.

Once again, the addition of lignocellulosic biomass pretreated with Oyrm 1 did not improve the rate of ethanol fermentation or the final ethanol yield from the fermentations. In addition, the final ethanol concentration was lower in all fermentations (Table 4.20) with the exception of the corn mash containing cottonseed hulls pretreated for 180 days. In this fermentation, the final ethanol concentration was marginally higher, although when the standard deviation was accounted for, little difference between this and the control was observed. Despite the final ethanol concentrations being relatively similar, 4 % less corn mash was used in the sample

fermentations to achieve the ethanol concentration, thus potentially significant savings can be made in terms of substrate cost. The efficiency of each fermentation were also very similar with an ethanol efficiency of over 99 % observed in all fermentations, suggesting that the differences in the final ethanol concentration are not due to the unutilised carbohydrates left following fermentations and may be due to the utilisation of the carbohydrates by the yeast, in which some fermentation were using a high portion for growth and maintenance, as mentioned previously in Section 4.2.2.1.

The rate of ethanol production appeared to be reduced during the initial 18 hours, with the concentration of ethanol produced being lower in the sample fermentations in comparison to the control. This decrease in the rate of fermentation may be due to a number of characteristics such as CO_2 dissolution, heat and mass transfer which may have been altered through the addition of cottonseed hulls to the substrate, as mentioned previously in Section 4.2.2.2. Additionally, inhibitors such as phenolic compounds may be slowing down the fermentation, with the production of ethanol subsequently increasing as the biomass density is increased. High cell densities in ethanol fermentations are known to help alleviate the effect of inhibitors during fermentation (Olsson *et al.*, 1996). As the fermentations proceeded however, the rate of ethanol production was altered with the control sample producing the lowest concentration of ethanol after 20 hours of fermentation. Despite this, the time in which each fermentation finished was similar, so any change in the rate of production before 40 hours will not have any effect on the length of fermentation.

A cellulolytic enzyme preparation was also added to fermentations containing cottonseed hulls pretreated for 180 days to determine whether this would impact on the final ethanol concentration as a result of the substrate hydrolysis. However, analysis of the data (Table 4.20) indicated that the addition of the enzyme did not improve the ethanol yield. This was likely as a result of the high lignin content (21.5 % w/v) still present in the cottonseed hulls substrate, where the lignin was protecting the cellulose microfibrils from hydrolysis by the cellulase enzymes. Additionally, the ethanol and glucose concentration in the fermentation may also have inhibited the hydrolysis of the cellulase, as explained previously in Section 4.2.2.2.

It was apparent that minimal changes in the overall rate of ethanol production and final ethanol yield were noted through the addition of pretreated cottonseed hulls to the mash. However, as with the previous fermentations, this replacement technique

could reduce the cost of the substrate considerably, as the amount of corn required is reduced.

4.2.2.4 Ethanol production from corn mash supplemented with cottonseed hulls and coconut fibre

To get an indication of the hygroscopicity of the cottonseed hull/coconut fibre material, a titration of different volumes of this pretreated biomass was used to replace an equal amount of the corn mash substrate. Findings indicated that the addition of 4 % (w/w) cottonseed hulls/coconut fibre to the mash was adequate to provide sufficient lignocellulosic material, whilst not impeding ethanol production and fermentation sampling as dried cottonseed hulls and coconut fibre were highly hygroscopic and the addition of too much made it difficult to sample the fermentation. Results presented in Figure 4.5 and Table 4.21 illustrate the rate of ethanol production from *P. thermotolerans* 7012 and the composition of the broth containing pretreated cottonseed hulls/coconut fibre following fermentation.

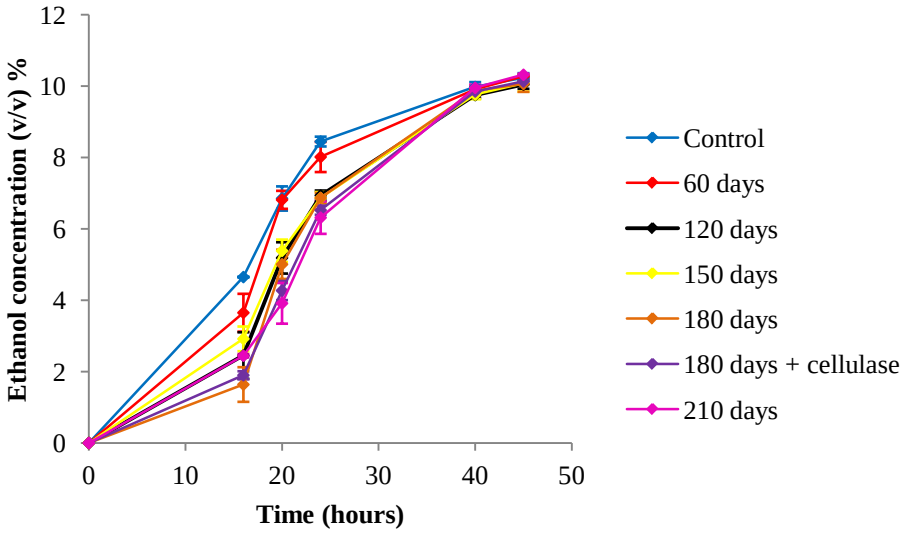


Figure 4.5 Comparison of the rate of ethanol production from corn mash fermentations supplemented with cottonseed hulls/coconut fibre pretreated with *Pleurotus ostreatus* Oyrm 1.

Results plotted are the mean of triplicate flasks with the standard deviation of each treatment represented by error bars. + cellulase indicates that exogenous cellulase was added to the fermentation.

It is apparent through the analysis of the ethanol production rate curves (Figure 4.5) that the rate of ethanol production from corn mash was decreased during the first 24 hours by the addition of pretreated cottonseed hulls and coconut fibre to the mash.

For instance, during the first 16 hours of fermentation, 4.6 % (v/v) ethanol was produced from of the control mash, whilst only 1.6 % (v/v) was produced during fermentation of the mash containing cottonseed hulls/coconut fibre pretreated for 180 days. Furthermore, it appeared that the fermentations containing biomass pretreated for longer periods of time (i.e. 180 days) tended to have the lowest rate of ethanol production. As the lignin was removed from the material during the pretreatment process, the hemicellulose may be more available, with furfural formation increased as a consequence of the substrate hydrolysis. Furfural, which is known as an inhibitor of ethanol production is thought to be produced during the liquid hot water extraction of the material (Alvira *et al.*, 2010), which can lead to inhibition of the microorganism during downstream processes (Boyer *et al.*, 1992). This may have been produced during the sterilisation process in which the free xylose sugars were converted to furfural. Alternatively, the increase in the soluble lignin concentration through pretreatment may have inhibited the rate of ethanol production. It was apparent that the soluble lignin fraction (Section 4.1.4), which is known to contain ρ-coumaric, ferulic acid and vanillin (Yasuda *et al.*, 1990), was increased with increasing pretreatment time. These soluble phenolic compounds are generally regarded as inhibitory towards yeast during ethanol production (Palmqvist *et al.*, 2000b). The reduction in the ethanol production rate may also be as a result of the change in the characteristics of the fermentation such as the heat and mass transfer, which may impact on the cultivation of the yeast in the sample fermentations. Despite this apparent decrease in the initial fermentation rate, as the fermentations approached 40 hours the amount of ethanol produced by each fermentation was relatively similar, thus any change in the fermentation time before this point would not impact on the length of fermentation.

Table 4.21 End point fermentation composition and efficiency of corn mash liquor supplemented with cottonseed hulls/coconut fibre pretreated with *Pleurotus ostreatus* Oyrm 1.

Pretreatment time	Dextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol %(v/v)	Efficiency %
Control	0	0.00	0.11	0.61	10.23±0.15	99.58
60 days	0	0.02	0.13	0.63	10.27±0.04	99.42
120 days	0	0.02	0.12	0.61	10.04±0.15	99.42
150 days	0	0.02	0.12	0.61	10.08±0.29	99.46
180 days	0	0.02	0.11	0.60	10.06±0.28	99.45
180 days + cellulase	0	0.00	0.11	0.56	10.13±0.09	99.56
210 days	0	0.01	0.12	0.58	10.32±0.04	99.48

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*). + cellulase indicates that exogenous cellulase was added to the fermentation.

The efficiency of each fermentation appeared to be good with over 99 % efficiency achieved during each fermentation and as a result, minimal carbohydrates were available in the mash following fermentation (Table 4.21). This indicated that any difference in the final ethanol yields between cultures was not due to incomplete fermentations or other factors such as the yeast utilising more carbohydrate for maintenance and growth. Additionally, it was evident that the addition of pretreated cottonseed hulls and coconut fibre did not lead to the production of additional ethanol, with the final ethanol concentrations from the control fermentations being relatively similar to that of the control fermentation when the standard error rate was taken it account.

Finally, the addition of exogenous cellulase enzyme to the fermentation containing cottonseed hulls and coconut fibre pretreated for 180 days did not appear to have a significant effect on the final ethanol concentration. This was likely due to one of a number of reasons which were outlined previously in Section 4.2.2.2, such as the insufficient removal of lignin from the material.

4.2.2.5 Ethanol production from corn mash supplemented with oat straw

In order to determine how hygroscopic the oat straw was, a titration of different volumes (0.2-4 %) of this pretreated biomass was used to replace an equal amount of the corn mash substrate. This indicated that the addition of 2 % (w/w) of oat straw to the mash was adequate to provide a portion of lignocellulosic material, whilst the sampling of the fermentation was not impeded. Dried oat straw was highly

hygroscopic, with it being able to soak up 4 times its weight in water, thus the addition of too much of this material would make it difficult to sample during fermentation. Figure 4.6 represents the rate of ethanol production during fermentation of the sample fermentations in comparison to the control, whilst Table 4.22 indicates the composition of the medium following fermentation with *P. thermotolerans* 7012.

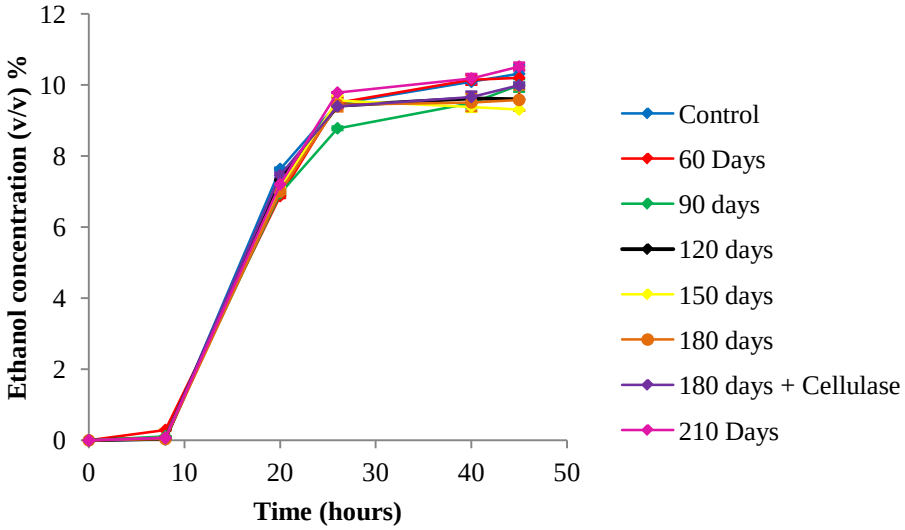


Figure 4.6 Comparison of the rate of ethanol production from corn mash fermentations supplemented with oat straw pretreated with *Pleurotus ostreatus* Oyrm 1 for different lengths of time.

Results plotted are the mean of triplicate flasks with the standard deviation of each treatment represented by error bars. + cellulase indicates that exogenous cellulase was added to the fermentation.

Table 4.22 End point fermentation composition and efficiency of corn mash liquor supplemented with oat straw pretreated with *Pleurotus ostreatus* Oyrm 1.

Pretreatment time	Dextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Control	0	0.04	0.06	0.73	10.31±0.32	99.61
60 days	0	0.06	0.04	0.62	10.20±0.28	99.59
90 days	0	0.05	0.06	0.71	9.99±0.22	99.57
120 days	0	0.12	0.14	0.66	9.59±0.09	98.89
150 days	0	0.09	0.06	0.63	9.30±0.03	99.30
180 days	0	0.19*	0.18*	0.69	9.58±0.37	98.40
180 days + cellulase	0	0.13*	0.15*	0.66	9.99±0.09	98.81
210 days	0	0.12	0.16	1.07	10.51±0.14	98.88

Results displayed are a representation of the mean of triplicate fermentations The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*). + cellulase indicates that exogenous cellulase was added to the fermentation.

In contrast to the fermentations containing cottonseed hulls/coconut fibre, the rate of ethanol production did not appear to be affected by the addition of the pretreated oat straw to the mash (Figure 4.5). The rate of ethanol production from each fermentation appeared to be relatively similar, although the final ethanol concentration was lower in the majority of fermentations in comparison to the control. For instance, the fermentation containing oat straw pretreated for 150 days contained over 1 % (v/v) less ethanol than the control, which equates to an almost 10 % reduction. This was also the case in a number of other fermentations, where the amount of ethanol produced from the fermentation was substantially reduced by the addition of pretreated oat straw. Only 2 % of corn mash was removed from the fermentations, thus it was surprising that the final ethanol concentration was reduced to such a degree. It is possible that addition of this material affected the growth of the yeast to such as degree where, a larger portion of the carbohydrate was utilised by the yeast for cell growth, repair and maintenance in comparison to the control, as the reduction in the final ethanol concentration did not appear to be due to the decrease in ethanol efficiency of the fermentations (Table 4.22). However, in saying this, the reduction noted in these fermentations was not statistically significant. It is also worth noting that the increase in glycerol concentration was relatively proportional to the increase in the ethanol concentration, where the higher the ethanol yield the greater the glycerol concentration. This indicated that the production of ethanol in this set of fermentations was not impacted by the concentration of fermentable sugars utilised for glycerol formation.

Although a decrease in the final ethanol concentration was observed through the addition of oat straw pretreated for 180 days, when exogenous cellulase was added to the broth, this reduction in the ethanol concentration did not appear to be as pronounced. It is likely that the addition of the cellulase to the mash may have hydrolysed a portion of the cellulose from straw, resulting in the release of glucose, which can subsequently be converted to ethanol.

Despite the reduction in the final yield not being statistically significant (Table 4.22) the decrease detected during fermentation could lead to a substantial reduction in the final ethanol yield. If the ethanol yield was reduced by 3 % this could lead to a reduction of up to 2.1 million litres of ethanol per crop season in a medium sized distillery (Basso *et al.*, 2008), indicating that the ethanol yield from this fermentation could be decreased by up to 0.7 million litres. However, straw pretreated for 210 days

did appear to increase the final ethanol concentration marginally, whilst a reduction in the amount of corn mash used could also be achieved, which would likely result in a reduction in the cost of the substrate for fermentation if this was scaled up to a production facility, whilst a higher yield of ethanol could also be obtained.

4.2.3 Ethanol production from corn mash supplemented with corncobs pretreated with white rot fungi and *Aspergillus* spp.

As mentioned previously in Section 1.3.1, the primary enzymes produced by white rot fungi are ligninolytic enzymes such as manganese and lignin peroxidase. Enzymes including cellulases and hemicellulases; however, have also been reported to be produced by white rot fungi. Despite this, research into production of these enzymes from white rot fungi is in the early stages of development (Levin *et al.*, 2008), as the vast majority of research groups focus on more well recognised families of fungi that are known producers of cellulase and hemicellulase enzymes. Within this group of fungi, Aspergillus and Trichoderma are the most extensively studied in terms of enzyme production, where well established methods have been developed to achieve high enzyme titres (Hu *et al.*, 2011).

Preliminary studies investigating the cellulase and hemicellulase production from both *Trametes versicolor* 3086 and *Pleurotus ostreatus* Oyrm 1 determined that relatively low cellulase and xylanase activities of just 5 U g^{-1} were achieved after 30 days of fermentation. These activities were relatively low in comparison to the titres achieved through fermentation of corncobs with *Aspergillus niger* 102.12 and *Aspergillus oryzae* 553.65 under optimal conditions (Section 3.2). It was thought that due to the low titres achieved with white rot fungi, insufficient hydrolysis of the cellulose fraction would occur yielding a relatively poor concentration of readily accessible sugars being produced for utilisation during ethanol fermentation. To address this, corncobs were initially pretreated for 120 days with a number of strains of white rot fungi to degrade the lignin fraction of the corncobs. Corncobs were then harvested, freeze dried to remove the moisture and fermented again with either *Aspergillus niger* 102.12 or *Aspergillus oryzae* 553.65. The optimal conditions for enzyme production (as determined in Section 3.2) were employed during these fermentations. These conditions were used to ensure maximal cellulase and hemicellulase production, thus ensuring optimal cellulose and hemicellulose conversion. This material was then used to replace 6 % of corn mash to determine

whether the addition of pretreated lignocellulosic material could improve the ethanol yield and/or fermentation rate. Fermentations that contained corncobs pretreated with *Aspergillus niger* 102.12 and *Aspergillus oryzae* 553.65 only were also included. Each fermentation was carried out using the conditions described previously in Section 2.2.6.5, with a control of corn mash used as a reference fermentation in each study.

A number of different strains of white rot fungi were analysed to determine whether there were any appreciable differences in ethanol production and fermentation rates between strains. These included *Pleurotus ostreatus* Oyrm 1, 1833 and 32783, *Pleurotus salmoneostramineus* 5338, *Trametes versicolor* 3086 and *Phanerochaete chrysosporium* 24725. The strains were selected during initial screening (results not displayed) due to their capacity to grow in solid state fermentation and to colonise the corncob substrate relatively quickly.

Due to the number of strains analysed in this study, three separate experiments using an identical fermentation procedure were carried out, with a control corn mash fermentation included in each separate experiment. The corn mash was freshly prepared prior to each experiment, therefore some differences in the carbohydrate composition of the control fermentations were noted. However, the overall carbohydrate content was similar as the same weight of ground corn was used in each batch. Samples were collected at various time points throughout the fermentation, with the ethanol concentration and carbohydrate utilisation monitored using a HPLC system, as described previously in Section 2.2.6.4. Each fermentation was completed after 41 hours with the exception of the fermentations displayed in Figure 4.7, which were allowed to continue for a further 9 hours, as ethanol production had not plateaued following 41 hours of fermentation.

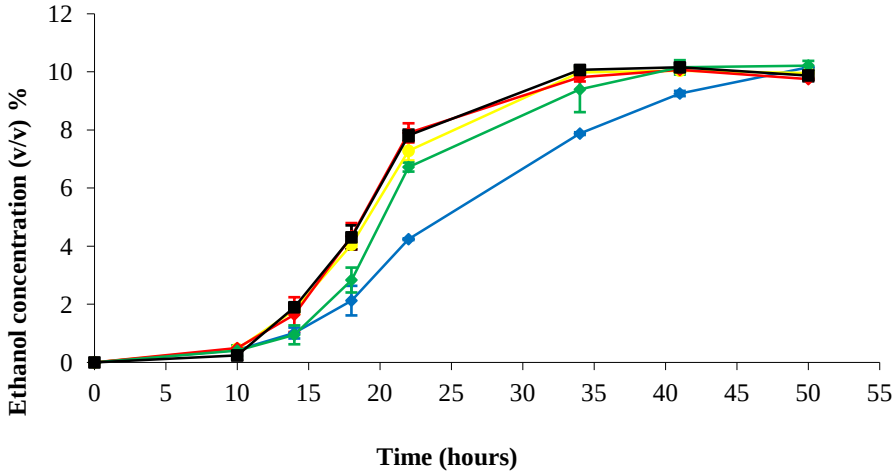


Figure 4.7 Comparison of the rate of ethanol production from corn mash fermentations supplemented with corncobs fermented with a selection of white rot and Aspergillus fungi.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars.
Legend: Control; (♦), *P. ostreatus* 32783 and *A. niger* 102.12; (♦), *P. ostreatus* 32783 and *A. oryzae* 553.65 (♦), *A. niger* 102.12 (♦), *A. oryzae* 553.65; (■).

Table 4.23 (a) Initial and final carbohydrate composition of corn mash fermentations supplemented with corncobs pretreated with a selection of filamentous fungi.

Pretreatment strains	Initial Glucose % (w/v)	Final Glucose % (w/v)	Initial Dextrin % (w/v)	Final Dextrin % (w/v)	Initial Maltose % (w/v)	Final Maltose % (w/v)
Control	0.1	0.04	14.2	0.06	0.26	0
P. ostreatus 32783/ *A. niger* 102.12	0.6*	0.12*	14.0	0.52*	0.41	0.11*
P. ostreatus 32783/ *A. oryzae* 553.65	0.65*	0.09*	13.9	0.53*	0.26	0.03*
A. niger 102.12	0.95*	0.18*	13.9	0.52*	0.53*	0.13*
A. oryzae 553.65	0.6*	0.02*	13.8	0.54*	0.50*	0.1*

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.23 (b) Fermentation product formation and efficiency of corn mash fermentations supplemented with corncobs pretreated with a selection of filamentous fungi.

Pretreatment strains	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency % (w/v)
Control	0.72	10.15±0.01	99.58
P. ostreatus 32783/ *A. niger* 102.12	0.75	9.96±0.16*	96.79
P. ostreatus 32783/ *A. oryzae* 553.65	0.88	9.75±0.04*	97.18
A. niger 102.12	0.71	10.22±0.20	96.57
A. oryzae 553.65	0.79*	9.87±0.25	97.12

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

It is evident through analysis of the data in Figure 4.7 and Table 4.24 that the rate of fermentation was significantly ($p<0.05$) increased through the addition of pretreated corncob to the mash. In comparison to the control, the time required to produce the same concentration of ethanol was significantly reduced when pretreated cobs were added. In some of the sample fermentations, like for example the *A. oryzae* 553.65 fermentation, the time to reach maximal ethanol production was 41 hours, whilst the control fermentation took 50 hours to obtain a similar concentration of ethanol. This is a reduction of 9 hours on the fermentation time, which could result in considerable savings if this method was to be utilised in a larger scale. For instance, the energy costs to run this fermentation could be reduced by 18 % as a consequence of this reduction in the ethanol production rate, whilst the turn over time would also be reduced, allowing for the productivity of the plant to be increased, although the pretreatment cost would need to be taken into account.

In the previous section (Section 4.2.2), which analysed the pretreatment of corncobs with white rot fungi, no difference in the ethanol production rate was noted. However, when this material was subsequently fermented with *Aspergillus* spp. the rate of ethanol production was substantially increased. This indicated that the change in the rate of ethanol production was due to secondary pretreatment phase. Alterations to the chemical composition by the Aspergillus fungi may have released soluble nutrients which may have impacted positively on the fermentation. An increased rate of ethanol production may be as a result of the notable increase in the initial glucose content of the experimental fermentations in comparison to the control (Table 4.23 a). Prior to fermentation of the control corn mash, a concentration of 0.1 % (w/v) glucose was detected. In contrast, the sample broths contained between 0.6–0.95 % (w/v)

glucose, with the *A. niger* 102.12 fermentation containing the highest initial glucose concentration at 0.95 % (w/v). This increase in the initial glucose concentration was likely as a result of residual glucose found in the corncobs, primarily due to hydrolysis by the cellulolytic enzymes produced from *Aspergillus* spp. As a consequence, this may result in greater cell growth during the initial stages of fermentation, which may have influenced the rate of ethanol production due to the higher cell density. Francesca *et al.* (2010) determined that the rate of biomass production from *Saccharomyces cerevisiae* was higher in a medium containing 2 % (w/v) glucose in comparison to flasks containing 0.5 % initial glucose. Furthermore, Matsushika *et al.* (2010) analysed the effect of the initial cell concentration on ethanol production from *Saccharomyces cerevisiae* and determined that a higher cell concentration increased both the substrate utilisation and the ethanol production rate during fermentation. Alternatively, the increase in fermentation rate detected may be due to a potential increase in the protein/nitrogen content, due to the addition of fermented corncob. Yeast fermentation requires carbohydrate, nitrogen and water, where if one is unavailable or present at sub optimal concentrations the fermentation can be affected. Yao *et al.* (2012) investigated the effect of fermentation conditions on the production of ethanol from a corn-soy mash. The authors determined that the addition of soy skim, which is a protein rich liquid increased, the rate of fermentation by between 18-27 %, depending on the amount of soy skim added to the broth.

Despite the increase noted in initial glucose concentrations, the final ethanol concentration (after 50 hours) was reduced in all fermentations, with the exception of the those containing cob pretreated with *A. niger* 102.12 (Table 4.23 b), in which a minor increase in the final ethanol yield was observed. Although, despite the decreases in final ethanol concentration measured after 50 hours in a number of sample fermentations, if the ethanol concentration of the 41 hour time point (Table 4.24) from the sample fermentation were compared to the control at 50 hours, minimal difference in the yields of ethanol were noted. This was as a result of a small decrease in ethanol yield which was detected during the final 9 hours in some of the sample fermentations. This decrease may be due to the yeast starting to consume ethanol by respiration as a result of the glucose levels in the broth falling below 0.2 % (Denis *et al.*, 1983; Westerbeek-Marres *et al.*, 1988).

Table 4.24 Ethanol production (% v/v) from fermentations containing corncobs pretreated with a range of fungi.

Time (hours)	Control	*A. niger* 102.12	*A oryzae* 553.65	*P. ostreatus* 32783 + *A. oryzae* 553.65	*P. ostreatus* 32783 + *A. niger* 102.12
0	0.00	0.00	0.00	0.00	0.00
10	0.43	0.41	0.24	0.49*	0.47
14	1.01	0.95	1.90*	1.65	1.80*
18	2.13	2.84	4.30*	4.34*	4.05*
22	4.24	6.72*	7.81*	7.90*	7.28*
34	7.87	9.39*	10.06*	9.81*	9.97*
41	9.25	10.15*	10.15*	10.06*	10.04*
50	10.15	10.22	9.87	9.75*	9.96*

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a one-way analysis of variance to evaluate the significance of the samples to the control where the confidence level was set at 95 %, as denoted by an asterisk (*).

During the first 10 hours of fermentation the rate of ethanol production was relatively low with less than 0.5 % (v/v) ethanol produced during this time (Table 4.24). In comparison to the other trials detailed in Figures 4.8 and 4.9 this rate of initial fermentation was considerably low. A number of factors are known to affect the production of ethanol during fermentation. These include yeast viability, sugar concentration, temperature, enzyme concentration, water activity and fermentation pH. Fermentation parameters were identical during each fermentation so it is unlikely that these process conditions were the inhibitory factors during the initial stages of ethanol production. It is more probable that the initial poor rate of ethanol production in comparison to the fermentations in Figures 4.8 and 4.9 was due to an increase in the lag phase. This increase in the initial lag phase can be attributed to a number of factors that are known to influence lag. The inoculum quality greatly influences the performance of the fermentation, with the inoculum size and age being critical factors in the length of the lag time. As the age of the inoculum is increased the lag time becomes longer, whilst the daughter cells also have longer lag phases than young parent cells (Ginovart *et al.*, 2011). Furthermore, it is thought that the inoculum size may have been too small due to a low yeast viability which may have influenced the initial lag phase during fermentation. Although the inoculum was prepared using the same procedure in each of the fermentations carried out in Section 4.2.3, the viability of the yeast may have been lower in this fermentation thus increasing fermentation time. Finally, the long lag phase may have been due to sub-lethal damage that

occurred during initial propagation, which reduced the cell viability and consequently the rate of ethanol production (Swinnen *et al.*, 2004).

It is clear through analysis of Table 4.23 (b) that the reduction in the final ethanol concentration is due in part to the decrease in the fermentation efficiency, where a higher concentration of sugars were left unfermented in the sample broth in comparison to the control mash (4.23 a). If this remaining carbohydrate was utilised during fermentation, the final ethanol concentration could be increased to a value similar to the control. For instance, if the carbohydrates that were left unutilised at the end of the fermentation containing corncobs pretreated with *Pleurotus ostreatus* 32783 and *Aspergillus niger* 102.12, were converted to ethanol a concentration of 10.08 % could be achieved, which was 0.1 % higher than the potential ethanol concentration that can be obtained with the control fermentation. In some cases, up to 0.54 % (w/v) dextrin and 0.1 % (w/v) maltose was left unutilised in the fermentation, which could lead an appreciable increase in the ethanol concentration if these carbohydrates were fermented. Furthermore, if the sample fermentation were harvested after 41 hours (the time after which maximal ethanol yield was achieved), the concentration of ethanol in the sample fermentations could be higher than that of the control if the efficiencies were improved.

A distinct difference was also observed between the fermentations which contained corncobs pretreated with the different fungal strains also. For instance, fermentations containing corncobs pretreated with *Pleurotus ostreatus* 32783 and *A. oryzae* 553.65 had the fastest fermentation rate, whilst the rate achieved with corncobs pretreated with *A. niger* 102.12 was marginally lower. This difference in fermentation rate may be as a result of different structural amino acids available in the different fermentations as the structural complexity of the nitrogen source is known to strongly influence the yeast metabolism and thus the fermentation rate (Júnior *et al.*, 2008; Yao *et al.*, 2011a).

It is also worth noting that the fermentations containing corncobs pretreated with individual *Aspergilli* spp. achieved the same ethanol yield and fermentation rate as the dual fermentations, further indicating that the increase in the fermentation rate was as a consequence of the secondary fermentation with Aspergilli. It also suggested that while a degree of lignin removal occurred during pretreatment, it did not appear to influence the fermentable sugars available following the secondary fermentation.

This method of pretreatment appears to be a viable method of increasing the fermentation rate and would significantly impact on the cost of fermentation, as the length of fermentation time can be dramatically reduced. Additionally, the final ethanol concentration, after 50 hours, was significantly reduced in the fermentations containing the corncobs pretreated with *P. ostreatus* 32783, which could drastically reduce the yield of the fermentation. However, it would appear that the maximal ethanol production from these fermentations which occurred after 41 hours of fermentation was closer to the maximal yield of ethanol from the control fermentation after 50 hours. It was evident that the fermentation containing corncobs pretreated with *A. niger* 102.12 performed the best, with a significant increase in the rate of ethanol production as well as a marginal increase in the final ethanol yield measured, which could result in a substantial reduction in the cost of fermentation.

Corncobs pretreated with selected other white rot fungi were also investigated with the rate of ethanol production monitored during fermentation (Figure 4.8). Moreover, the carbohydrate and glycerol concentration as well as fermentation efficiencies were also monitored throughout the process (Table 4.25).

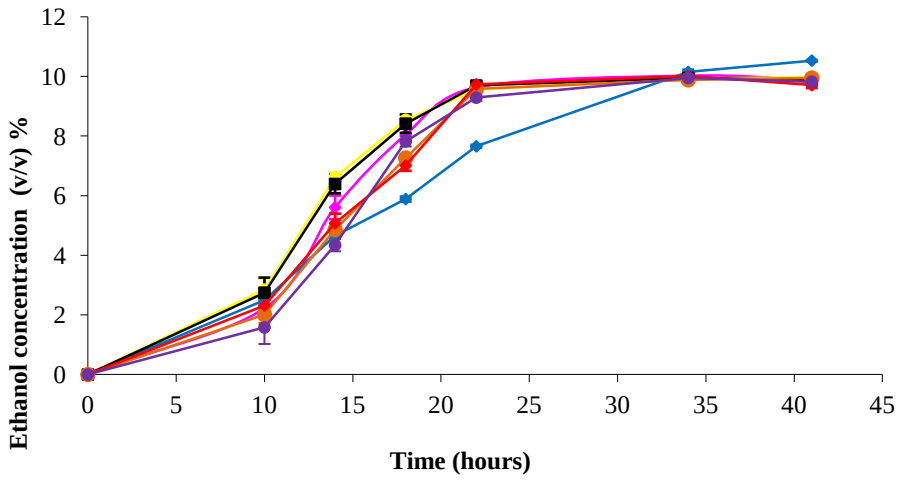


Figure 4.8 Comparison of the rate of ethanol production from corn mash fermentations supplemented with corncobs pretreated with a selection of white rot and Aspergillus fungi.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars.
Legend: Control; (♦), *T. versicolor* 3086 and *A. oryzae* 553.65; (♦), *T. versicolor* 3086 and *A. niger* 102.12; (●), *P. salmoneostramineus* 5338 and *A. oryzae* 553.65; (■), *P. salmoneostramineus* 5338 and *A. niger* 102.12; (●), *P. ostreatus* Oyrm 1 and *A. oryzae* 553.65; (♦), *P. ostreatus* Oyrm 1 and *A. niger* 102.12; (●).

Table 4.25 (a) Initial and final carbohydrate composition of corn mash fermentations supplemented with corncobs pretreated with a selection of filamentous fungi.

Pretreatment strains	Initial Glucose % (w/v)	Final Glucose % (w/v)	Initial Dextrin % (w/v)	Final Dextrin % (w/v)	Initial Maltose % (w/v)	Final Maltose % (w/v)
Control	0.5	0.02	14.2	0.12	2.02	0.12
P. ostreatus Oyrm 1/ *A. niger* 102.12	1.5*	0.07*	13.8	0.88*	2.06	0.39*
P. ostreatus Oyrm 1/ *A. oryzae* 553.65	1.25*	0.06*	13.8	0.76	2.03	0.25*
P. salmoneostramineus 5338/ *A. niger* 102.12	1*	0.04*	14.0	0.79*	2.11*	0.24*
P. salmoneostramineus 5338/ *A. oryzae* 553.65	1.05*	0.06*	14.1	0.77*	2.06	0.26*
T. versicolor 3086/*A. niger* 102.12	1.1*	0.06	13.9	0.73	2.06	0.12
T. versicolor 3086/*A. oryzae* 553.65	0.93*	0.07*	14.1	0.81*	1.94	0.25*

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.25 (b) Fermentation product formation and efficiency of corn mash fermentations supplemented with corncobs pretreated with a selection of filamentous fungi.

Pretreatment strains	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency % (w/v)
Control	0.76	10.53±0.02	98.90
P. ostreatus Oyrm 1/*A. niger* 102.12	0.62*	9.82±0.05*	94.40
P. ostreatus Oyrm 1/*A. oryzae* 553.65	0.70	9.71±0.12*	95.35
P. salmoneostramineus 5338/*A. niger* 102.12	0.72*	9.97±0.14*	95.47
P. salmoneostramineus 5338/ *A. oryzae* 553.65	0.73*	9.87±0.02*	95.35
T. versicolor 3086/*A. niger* 102.12	0.76	9.95±0.01*	96.10
T. versicolor 3086/*A. oryzae* 553.65	0.86*	9.89±0.12*	95.19

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.26 Ethanol production (% v/v) from fermentations containing corncobs pretreated with a range of fungi.

Time (hours)	Control	*T. versicolor* 3086/ *A. niger* 102.12	*T. versicolor* 3086/*A. oryzae* 553.65	*P. salmoneo-stramieus* 5338/ *A. niger* 102.12	*P. salmoneo-stramieus* 5338/ *A. oryzae* 553.65	*P. ostreatus* Oyrm 1/ *A. niger* 102.12	*P. ostreatus* Oyrm 1/ *A. oryzae* 553.65
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	2.49	2.01	2.23	2.85	2.75	1.58	2.32
14	4.66	4.86	5.61*	6.62*	6.39*	4.34	5.08
18	5.89	7.27*	8.05*	8.54*	8.42*	7.82*	7.01*
22	7.66	9.58*	9.63*	9.56*	9.70*	9.29*	9.73*
34	10.15	9.89*	10.03	9.92*	9.95	9.95*	10.01
41	10.53	9.95*	9.89*	9.97*	9.87*	9.82*	9.71*

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a one-way analysis of variance to evaluate the significance of the samples to the control where the confidence level was set at 95 %, as denoted by an asterisk (*).

Similar to the results obtained in Figure 4.7, the rate of ethanol production was also increased through the addition of pretreated corncobs to the fermentation (Figure 4.8). In each fermentation, the rate of ethanol production started to increase after ~10 hours of fermentation, which reduced the fermentation time required significantly ($p<0.05$) in comparison to the control fermentation. In general, maximal ethanol production from the sample fermentations occurred after 34 hours of fermentation, with the exception of the fermentation containing corncobs pretreated with TV 3086 and *A. niger* 102.12. In the other sample fermentations in which this was not the case minimal ethanol was produced in the final 7 hours of fermentation and it is likely that the fermentations would be stopped at 34 hours due to the extremely low productivity. By contrast, the control fermentation took 7 hours longer for complete fermentation, with the maximal ethanol yield also being higher in the control. Although the addition of the pretreated corncobs increased the fermentation rate, if the control fermentation was completed after 34 hours the ethanol concentration would still be higher than that of the sample fermentations. In saying this however, near maximal ethanol production occurred after 22 hours in the sample fermentations which was not the case in the control fermentation, where the yield was reduced by 2.49 % (v/v). This reduction of 12 hours may be more economical than continuing the fermentation further and achieving an increase in ethanol concentration of less than 1 % (v/v). This reduction in fermentation time observed is likely as a result of the increase in the initial glucose concentration (Table 4.25 a). In turn, this may have influenced the cell biomass, thus

increasing the fermentation rate through reduction of the lag phase, which increased the rate of ethanol production. However, the additional nitrogen which may be available in the broth as a result of the pretreated corncob addition may also have been a factor. Furthermore, the changes in the fermentation conditions such as the heat and mass transfer and the available moisture which may have been altered through the addition of the pretreated corncobs can also influence the fermentation rate, as explained previously in Section 4.2.2.2.

A significant ($p<0.05$) increase in the initial glucose concentration was observed (Table 4.25 a) in most fermentations in comparison to the control fermentation. For example, in the fermentations containing corncobs pretreated with Oyrm 1, an additional 1 and 0.75 % of glucose was added to the broth through the addition of the corncobs pretreated with *A. niger* 102.12 and *A. oryzae* 553.65, respectively. In spite of this, a decrease in the final ethanol yield was measured in each sample fermentation, with the most significant reduction of 0.82 % (v/v) noted between the Oyrm 1/*A. oryzae* 553.65 fermentation and the control. It is possible that this decrease is as a result of the yeast utilising a larger portion of the carbohydrate content for growth and cell repair in the sample fermentation in comparison to the control. However, lower fermentation efficiencies were also observed in the sample fermentations in comparison to the control indicating that the fermentation may have become stuck, thus leaving a quantity of carbohydrate unutilised and reducing the final ethanol concentration. It is possible that the starch from the corn which was unutilised has become retrograded during fermentation, which can result in a slower rate of degradation due to crystallisation (Tako, 1996; Yoon *et al.*, 2009). Another point worth noting is that the initial carbohydrate content of the control fermentation (0.4 % w/v) (Table 4.25 a) is distinctly different to that of the first set of fermentations (Table 4.23 a). This difference in carbohydrate content is likely due to difference in the rate of hydrolysis between corn mash batches as the mash was freshly prepared prior to each set of fermentations.

As mentioned previously the final ethanol yield of the sample fermentation was also affected, with a reduction in the ethanol concentration by as much as 0.82 % (v/v) observed in comparison to the control fermentation (Table 4.25 b). It is worth noting however, that the maximal ethanol production occurred after 34 hours in a number of the sample fermentations (Table 4.26), with the difference between these values and the maximal ethanol yield from the control fermentation being lower. This reduction

in the ethanol concentration noted in some of the sample fermentations during the final 7 hours of fermentation, was as mentioned previously in this section, likely due to the consumption of ethanol by yeast due to a deficiency of available carbohydrates.

The final set of fermentations investigated the pretreatment of corncobs with the white rot fungi *P. chrysosporium* 24725 and *P. ostreatus* 1833. These corncobs were subsequently fermented with Aspergilli prior to them being added to the mash. The rate of ethanol production (Figure 4.9) and the composition of the fermentation broth (Table 4.27) were monitored during fermentation to analyse the influence of the corncobs on the production of ethanol from corn.

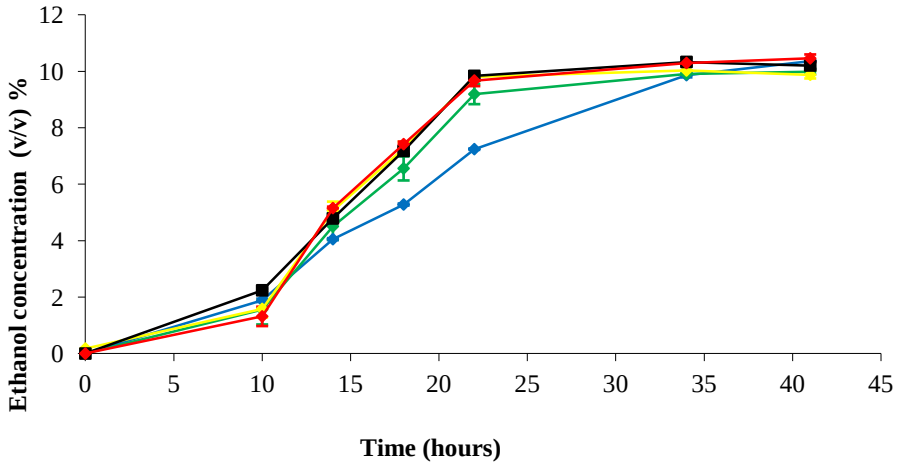


Figure 4.9 Comparison of the rate of ethanol production from corn mash fermentations supplemented with corncobs pretreated with a selection of white rot and Aspergillus fungi.

Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars.
Legend: Control; (♦), *P. ostreatus* 1833 and *A. oryzae* 553.65; (♦), *P. ostreatus* 1833 and *A. niger* 102.12; (♦), *P. chrysosporium* 24725 and *A. oryzae* 553.65; (♦), *P. chrysosporium* 24725 and *A. niger* 102.12 (■).

Table 4.27 (a) Initial and final carbohydrate composition of corn mash fermentations supplemented with corncobs pretreated with a selection of filamentous fungi.

Pretreatment strains	Initial Glucose % (w/v)	Final Glucose % (w/v)	Initial Dextrin % (w/v)	Final Dextrin % (w/v)	Initial Maltose % (w/v)	Final Maltose % (w/v)
Control	0.75	0.06	13.9	0.24	1.82	0.29
P. ostreatus 1833/ *A. niger* 102.12	1.06*	0.07	13.7	0.75*	1.59	0.18*
P. ostreatus 1833/ *A. oryzae* 553.65	0.72	0.11*	13.6	0.66*	1.93	0.26*
P. chrysosporium 24725/ *A. niger* 102.12	1.11*	0.04*	13.7	0.52*	1.93	0.15*
P. chrysosporium 24725/ *A. oryzae* 553.65	1.14*	0.04*	13.6	0.31*	1.86	0.12*

Results displayed are a representation of the mean of triplicate fermentations. The probability values were determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.27 (b) Fermentation product formation and efficiency of corn mash fermentations supplemented with corncobs pretreated with a selection of filamentous fungi.

Pretreatment strains	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency % (w/v)
Control	0.71	10.35±0.02	97.57
P. ostreatus 1833/ *A. niger* 102.12	0.83	9.99±0.12*	95.77
P. ostreatus 1833/ *A. oryzae* 553.65	0.86*	9.87±0.16*	95.57
P. chrysosporium 24725/ *A. niger* 102.12	0.87*	10.20±0.16	97.02
P. chrysosporium 24725/ *A. oryzae* 553.65	0.92*	10.46±0.17	98.06

Results displayed are a representation of the mean of triplicate fermentations. The probability values determined using a T- Test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %, as denoted by an asterisk (*).

Table 4.28 Ethanol production (% v/v) from fermentations containing corncobs pretreated with a range of fungi.

Time (hours)	Control	*P. ostreatus* 1833/*A. niger* 102.12	*P. ostreatus* 1833/ *A. oryzae* 553.65	*P. chrysosporium* 24725/ *A. oryzae* 553.65	*P. chrysosporium* 24725/ *A. niger* 102.12
0	0.00	0.00	0.00	0.00	0.00
10	1.89	1.56	1.58	1.32	2.24*
14	4.05	4.51	5.06*	5.15*	4.78*
18	5.28	6.56*	7.27*	7.42*	7.18*
22	7.24	9.19*	9.80*	9.67*	9.84*
34	9.86	9.90	10.03*	10.29*	10.33*
41	10.35	9.99*	9.87*	10.46	10.20

Results displayed are a representation of the mean of triplicate fermentation. The probability values were determined using a one-way analysis of variance to evaluate the significance of the samples to the control where the confidence level was set at 95 %, as denoted by an asterisk (*).

Results from these fermentations (Figures 4.9 & Table 4.27) were similar to the two previous set of fermentations in which the rate of ethanol production was substantially increased through the replacement of a portion of the corn mash with pretreated corncobs (Figure 4.9). This increased rate of fermentation was mostly likely due to the increase in the initial glucose concentration noted, as explained previously, although it is possible that the other nutrients (nitrogen/protein) found in the pretreated corncob may have also influenced the rate of fermentation. In addition, the change in the fermentation conditions such as heat and mass transfer rate may also have impacted on the fermentation rate. This reduction in fermentation time (7 hours) could lead to significant reductions in the fermentation cost due to a lower energy requirement during fermentation.

It is possible that the fermentation time in the sample fermentation could be reduced even further. Despite the ethanol concentrations of the sample fermentations after 22 hours being marginally lower than if the fermentations were continued, the cost associated with running these fermentations for a further 19 hours may possibly outweigh the increase in the final ethanol concentration observed. This may be not the case with the control fermentation; however, as reduction of over 2 % (v/v) was noted if the fermentation time was decreased by 19 hours. By contrast, a reduction of less than 0.79 % (v/v) was observed in the fermentation containing corncobs pretreated with *P. chrysosporium* 24725 and *A. oryzae* 553.65 after a reduction of 19 hours in the fermentation time.

The initial glucose concentration was also increased in comparison to that of the control fermentation, with the exception of the fermentation containing corncobs pretreated with *Pleurotus ostreatus* 1833/*A. oryzae* 553.65 (Table 4.27 a). It was thought that this increase would result in a higher final ethanol concentration as more glucose was available for fermentation. However, this was not the case in the majority of fermentations, where a decrease in the final ethanol concentration was noted. This is likely due in part to the decreased fermentation efficiencies observed as well as the yeast using more carbohydrate for maintenance and growth. Furthermore, in this case it appears that a greater concentration of the substrate was used for glycerol formation as the sample fermentation tended to have a higher concentration than that of the control (Table 4.27 b). In the fermentation containing corncobs pretreated with *Phanerochaete chrysosporium* 24725/ *A. oryzae* 553.65, where the efficiency of the fermentation was similar to that of the control sample, the final ethanol yield was

numerically higher (0.11 % v/v) than that of the control. If the efficiencies from the other fermentations were also increased the final ethanol yields may also be increased to close to that of the control fermentation.

It is evident that the rate of ethanol fermentation was significantly ($p<0.05$) increased through the addition of pretreated corncobs to the substrate (Table 4.28). Furthermore, an increase in the final ethanol concentration from the fermentation containing corncobs pretreated with *P. chrysosporium* 24725 and *A. oryzae* 553.65 was noted. Thus it can be concluded that this fermentation would be the optimal fermentation, as a significant reduction in the rate of ethanol production can be achieved without a decrease in the yield. In addition, the final ethanol concentration from the fermentations containing corncobs pretreated with *Pleurotus ostreatus* 1833 was also significantly ($p<0.05$) decreased, although as explained previously this decrease is due in part to the decreased efficiencies of the fermentations, where a sizable fraction of the maltodextrin remained unutilised in the fermentation.

4.2.4 Scale up of supplemented corn mash fermentations

Laboratory fermentations are generally good indicators of what is likely to occur in large-scale processes; however, they do not mimic the exact conditions that can occur. For instance, in larger volumes the fermentation conditions are often poorly controlled, whilst the hydrodynamic conditions can also differ from laboratory scale processes (Casalta *et al.*, 2010). Erlenmeyer flasks are widely used in preliminary studies, although the major disadvantage with this system is that they do not allow the precise control of oxygenation, thus running the risk of deviations in the yeast metabolic activity (Zimmermann *et al.*, 2006). Additionally, in larger volume fermentations some process parameters can also be more easily controlled, like for example pH and dissolved oxygen. This in turn can allow better manipulation of the reactor conditions (Junker, 2004), which may increase the productivity of the fermentation in some cases. Furthermore, aeration and mixing in larger scale vessels tends to differ from that of laboratory Erlenmeyer flasks, which can result in the change of certain characteristics in the fermentation. These characteristics include mixing/turnover time, foaming characteristics, shear, CO_2 dissolution and heat and mass transfer, which can impact on the growth rate and ethanol production (Reisman, 1993; Littlejohns *et al.*, 2007).

As a consequence, the production of ethanol in pilot-scale reactors was investigated as the process parameters would be more indicative of a large-scale

production facility. A 30 L jacketed vessel at 30 °C and 200 rpm, with a working volume of 20 L, was utilised to investigate the difference between the results obtained through laboratory and pilot scale fermentations. A total of five different fermentations were carried out using scaled up versions of the methods described previously in Sections 2.2.6.3 & 2.2.6.5. Firstly, a control corn mash fermentation was carried out to use as a reference for each fermentation. Subsequent fermentations included 6 % (w/v) of corncobs pretreated with either Oyrm 1 or TV 3086, which replaced the same weight of corn mash. Additionally, a fermentation of corncob which was first pretreated with Oyrm 1 and secondly pretreated with *Aspergillus niger* 102.12, as described previously in Section 2.2.5.3 was also used to replace 6 % (w/v) of the mash. Finally, a fermentation was undertaken using oat straw pretreated with Oyrm 1 at a replacement level of 2 % (w/v) of the corn mash. A lower volume of straw was used as preliminary tests revealed that straw absorbed significantly more water from the fermentation than corncob. Samples were taken intermittently throughout each fermentation, with carbohydrate utilisation and product formation analysed using the HPLC method described previously in 2.2.6.4. Figure 4.10 displays the rate of ethanol production from each fermentation over time, whilst Table 4.29 indicates the composition of the final broth of each mash following fermentation.

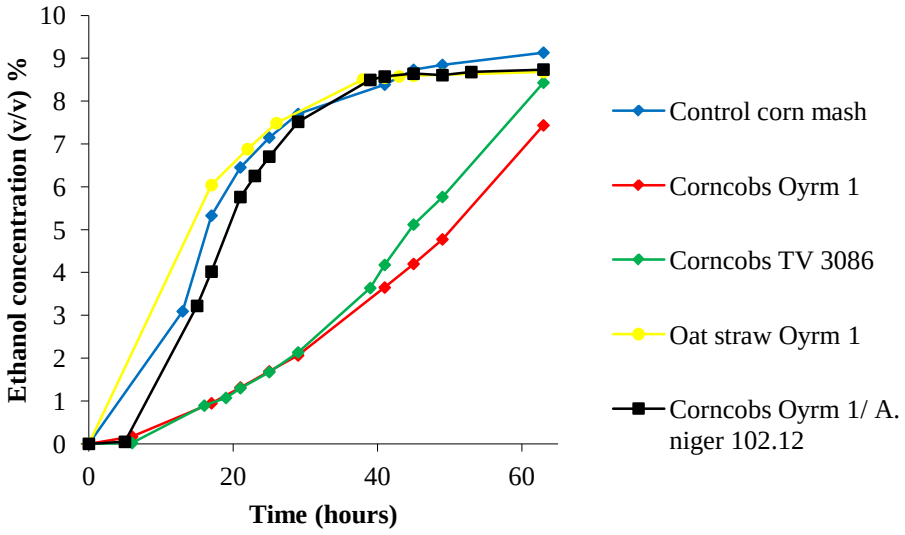


Figure 4.10 Comparison of ethanol production from corn mash fermentations supplemented with varying volumes of pretreated corncobs and oat straw.

Table 4.29 Comparison of the final carbohydrate concentration, product formation and ethanol efficiency of corn mash fermentations supplemented with corncob and oat straw pretreated with a selection of different fungi.

Pretreatment	Dextrin % (w/v)	Maltose % (w/v)	Glucose % (w/v)	Lactic acid % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Ethanol Efficiency %
Control corn mash	0.21	0.14	0.05	0.11	0.65	9.13	98.04
Corncobs Oyrm 1	0.35	0.17	1.02	0.06	0.49	7.44	91.82
Corncobs TV 3086	0.47	0.13	0.32	0.07	0.57	8.43	95.27
Oat straw Oyrm 1	0.23	0.13	0.04	0.13	0.65	8.68	97.97
Corncobs Oyrm 1/ *A. niger* 102.12	0.29	0.11	0.04	0.01	0.6	8.74	97.74

Results displayed are the means of triplicate samples.

In previous fermentations, where the mash was supplemented with corncob pretreated with Oyrm 1 and *A. niger* 102.12, an increase in the rate of fermentation was noted in comparison to the control (Section 4.2.3). Furthermore, an increase in the initial glucose concentration was observed in the fermentation broth, although this did not lead to an increase in the final ethanol concentration of the broth. This did not appear to be case in pilot scale fermentations; however, with no apparent increase in fermentation rate observed (Figure 4.10).

Analysing the data from Figure 4.10, it can be determined that the rate of ethanol production was considerably less when corncobs pretreated with either Oyrm 1 or TV 3086 were added to the mash in comparison to the control. Furthermore, the final ethanol concentration in the broth was also reduced, with a decrease of 8 and 19 % in the final ethanol concentration determined, respectively. Additionally, a discernible difference between the final ethanol yields of the fermentation containing corncobs pretreated with Oyrm 1 was noted in comparison to the fermentation supplemented with corncobs pretreated with TV 3086. When the composition of the final mash (Table 4.29) was analysed, it was evident that appreciably more glucose remained in the Oyrm 1 fermentation, with the difference in the yield being attributed in part to the variation in fermentation rate between the two cultures.

In contrast to the two other corncob fermentations discussed previously in this section, the rate of ethanol production from the mash containing corncobs pretreated

with Oyrm 1 and *Aspergillus niger* 102.12 was not as considerably affected, with the fermentation rate curve being markedly similar to that of the control. This was also true for the fermentation supplemented with oat straw in which the ethanol rate curve was also comparable. The difference between the fermentation rates of the samples and the control could be due to a number of issues which could have affected the fermentation. The change in the composition of the corncob substrate through pretreatment with TV 3086 and Oyrm 1 may have resulted in the formation of products that may inhibit yeast fermentation. For instance, furfural has been shown to reduce the specific growth rate of the cell mass yields on ATP, as well as the volumetric and specific ethanol productivities of yeast (Palmqvist *et al.*, 1999; Taherzadeh *et al.*, 1999a; Palmqvist *et al.*, 2000b). Furfural can be produced through the decomposition of the xylose substrate in water at high temperatures (Jing *et al.*, 2007), with the liberation of formic and acetic acid from lignocellulosic substrates producing a small amount of self catalysis to aid in the production of furfural (Ibbett *et al.*, 2011). Consequently, it is conceivable that during sterilisation of the fermentation medium furfural may have been produced leading to an inhibition of the fermentation. Yang *et al.* (2011) investigated methods of pretreatment of corn stover to improve the production of pyrolysis products. They determined that the biological pretreatment of the corn stover increased the amount of furfural recovered by 4.68-fold. They attributed the increase in the furfural content of the substrate to the degradation of the lignin, leading to the decomposition of the linkages between the lignin and hemicellulose fibres. Alternatively, the production of phenolic compounds during fermentation may also have resulted in the inhibition of the yeast growth. Phenolic compounds have been suggested to exert considerable inhibitory effects during the fermentation of lignocellulosic hydrolysates, with the low molecular weight compounds being the most toxic (Büchert *et al.*, 1989; Palmqvist *et al.*, 2000b). Phenolic compounds are known to be produced in white rot fungi (Vaz *et al.*, 2011a), whilst they also making up a considerable portion of lignin, with a small fraction of this being soluble in water and can thus inhibit yeast growth and ethanol production.

It was not understood why the rate of ethanol production from the other two fermentations of oat straw and corncobs were not affected, although it could be due to a number of issues. For instance, it may be due to the higher concentration of xylose present in corncobs in comparison to the oat straw, resulting in a higher concentration of furfural produced. Furthermore, the secondary fermentation in the other corncob

fermentation with *Aspergillus niger* 102.12 may have utilised any available xylose in the fermentation which would result in a reduction in furfural production as furfural in produced during xylose dehydration.

Maximal ethanol production was achieved during fermentation of the control mash, with 9.13 % (v/v) of ethanol produced after 63 hours. The greatest ethanol yield from the fermentation supplemented with lignocellulosic biomass was from that which contained corncob pretreated with Oyrm 1 and *Aspergillus niger* 102.12, yielding 8.74 % (v/v) ethanol. This equated to a 5 % reduction in the ethanol yield of the fermentation in comparison to the control, whilst the efficiency of the fermentation was also marginally reduced. During the initial laboratory-scale fermentations, an increase in the initial glucose concentration was noted in the fermentation containing cob pretreated with two fungal strains. This did not appear to be the case here as a concentration of 0.5 % (w/v) glucose was noted in the control, whilst a concentration of 0.37 % (w/v) was measured in the fermentation supplemented with corncob pretreated with Oyrm 1 and *Aspergillus niger* 102.12 (data not shown).

It was apparent that the addition of pretreated corncobs or oat straw did not have a positive effect on the rate of ethanol production, as the final ethanol concentration from each fermentation decreased when compared to the control. While the reduction in the yield of ethanol in some fermentations does not appear to be considerable, if this was scaled up to a production scale facility it could amount to a reduction in potentially millions of litres of ethanol every season, reducing the profitability of the plant, as discussed previously in Section 4.2.1. In saying this however, whilst a decrease in the ethanol yield (4-5 %) was noted during fermentation, there is little incentive to continue production beyond 41 hours in the control fermentation, as the volumetric productivity of the fermentation is relatively low in comparison to the first 30 hours. Thus, it would not be cost effective to run the fermentation beyond this point as the amount of ethanol produced with respect to the cost to run the fermentation would be relatively low. If the control fermentation was stopped after 41 hours of fermentation the final ethanol concentration for the control would be similar to that of the oat straw fermentation and the fermentation containing corncobs pretreated with Oyrm 1 and *A. niger* 102.12 at the same time point. In addition, the two sample fermentations would be more economical as less corn mash was utilised to produce the same ethanol yield, although the cost of pretreating the corncobs would also have to be taken into account. However, further work, may be

required to confirm these results as due to time constraints in the pilot facility only single fermentations were carried out. Additional analysis may give a clearer picture which could establish why the differences between the laboratory pilot scale fermentations existed.

4.2.5 Ethanol production from lignocellulosic residues in a simultaneous saccharification and fermentation system

A number of methods exist for the fermentation of lignocellulosic hydrolysates to ethanol. The classic configuration is the separate hydrolysis and subsequent fermentation of this hydrolysate (Sánchez *et al.*, 2008) in a process known as separate hydrolysis and fermentation (SHF), which has been used for a number of years. This method is advantageous due to the operator being able to a carry out both the hydrolysis and fermentation under the optimal conditions for each process. However, it suffers from a number of inherent drawbacks, one of which is, the inhibition of the cellulase and β-glucosidase enzymes by the glucose, which is released from the substrate, resulting in the need for higher enzyme loadings (Balat, 2011). This inhibition can be reduced however, through use of a simultaneous saccharification and fermentation (SimSF) technique. The glucose produced during hydrolysis is fermented immediately after production using this technique, reducing the inhibition and the need for higher enzyme loads. A drawback of this method however, is that the hydrolysis and fermentation are carried out under the same conditions and typically neither reaction is carried out under its optimal conditions. For instance, enzymatic hydrolysis of cellulose is typically carried out at 44-50 °C, whilst yeast usually operates at lower temperatures of between 27-37 °C. Consequently a compromise has to be found between the two different operating temperatures (Tengborg *et al.*, 2001).

In this study experiments were carried out to determine whether it was possible to produce ethanol from biologically pretreated lignocellulosic residues. A SimSF technique was employed in which an aliquot of dried lignocellulosic biomass pretreated for 150 days (6-30 g) was made up to 150 mL in the flask with dH_2O, as described in Section 2.2.6.6. These materials included the lower and upper stem of corn (6 g), switchgrass (8 g), straw (6 g), cottonseed hulls (10 g), cottonseed hulls/coconut fibre (10 g) and corncob (30 g). Each substrate was sterilised and a solution of filter sterilised crude enzyme extract (100 mL) was added to the fermentation, to give a final volume of 250 mL. This enzyme cocktail was added after

the sterilisation of the lignocellulosic material so as to minimise the denaturation of the enzyme due to heating. The crude enzyme extract was obtained through fermentation of corncobs using the optimal conditions for fermentation with *Aspergillus niger* 102.12 and *Aspergillus oryzae* 553.65, determined in Section 3.2. The two cocktails from *Aspergillus oryzae* 553.65 and *Aspergillus niger* 102.12 contained a cellulase concentration of 5.2 and 6.5 CMCu mL^{-1} and a xylanase activity of 85.3 and 65.1 XU mL^{-1}, respectively. Each fermentation was inoculated with a yeast cream, with samples taken intermittently throughout the fermentation, where the samples were analysed for carbohydrate, fermentation acid and ethanol concentration using a HPLC system, previously described in Section 2.2.6.4.

4.2.5.1 Ethanol production from pretreated cottonseed hulls

Ethanol production from pretreated cottonseed hulls was assessed with the rate of ethanol and glycerol production as well as carbohydrate utilisation determined during fermentation (Figure 4.11 and Table 4.30).

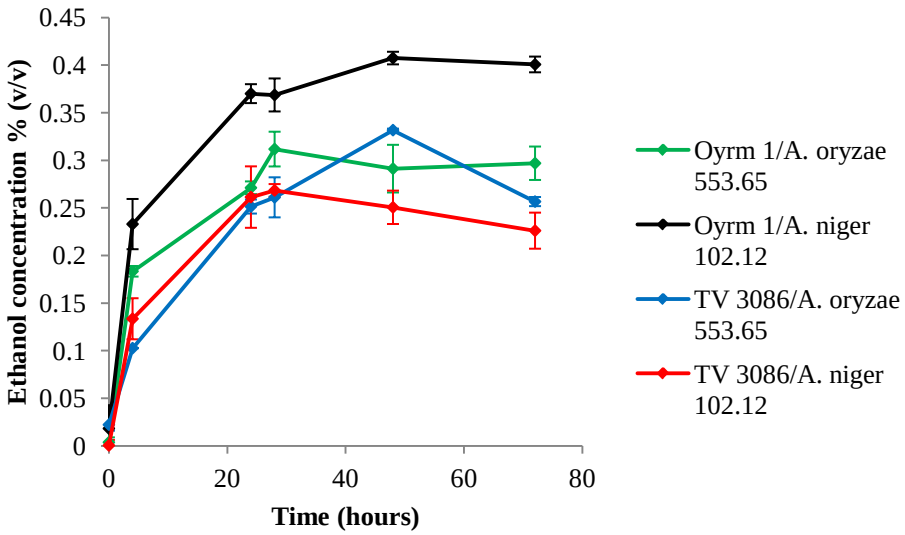


Figure 4.11 Rate of ethanol production from cottonseed hulls pretreated with *Pleurotus ostreatus* Oyrm 1 or *Trametes versicolor* 3086.

Results plotted are the mean of triplicates with the standard deviation represented by error bars.

Table 4.30 Composition and fermentation efficiency of pretreated cottonseed hull hydrolysates during fermentation.

Fermentation	Time (hours)	Dextrin % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Oyrm 1/*A. oryzae*	0	0	0.41	0	0	0
553.65	28	0.000	0.186	0.000	0.31	77.78
	48	0.039	0.178	0.000	0.29	76.24
	72	0.000	0.223	0.000	0.30	76.35
Oyrm 1/*A. niger*						
102.12	0	0.022	0.39	0	0	0
	28	0.060	0.141	0.000	0.37	81.27
	48	0.020	0.109	0.000	0.41	88.30
	72	0.018	0.260	0.000	0.40	77.64
TV 3086/*A. oryzae*						
553.65	0	0	0.27	0	0	0
	28	0.000	0.043	0.000	0.26	93.62
	48	0.000	0.115	0.000	0.33	87.53
	72	0.000	0.122	0.000	0.26	83.62
TV 3086/*A. niger*						
102.12	0	0	0.35	0	0	0
	28	0.000	0.104	0.031	0.27	86.28
	48	0.000	0.016	0.039	0.25	93.00
	72	0.000	0.159	0.024	0.23	77.51

Results displayed are the means of triplicate flasks with the standard deviation of each sample determined to be less than 5 % of the sample.

Maximal ethanol production was achieved through the fermentation of cottonseed hulls pretreated with Oyrm 1 and hydrolysed with an enzyme cocktail produced from corncobs fermented by *Aspergillus niger* 102.12 (Figure 4.11). An ethanol concentration of 0.4 % (v/v) was recorded during fermentation, which was relatively low in comparison to the ethanol concentrations typically achieved through fermentation of corn mash, although in saying this a lower substrate concentration was used. An initial glucose level of 0.39 % (w/v) from this fermentation was noted prior to fermentation, with 75 % of this being utilised during the initial 48 hours of fermentation (Table 4.30). This initial glucose concentration was as a result of the 0.1 % (w/v) solution of glucose being supplemented to each broth prior to fermentation as mentioned earlier in Section 2.2.6.6. The remaining glucose present at the start of fermentation is likely due to the residual sugars from the enzyme cocktail, which were extracted from corncobs fermented by *A. niger* 102.12 and *A. oryzae* 553.65.

The glucose concentration from the cottonseed hulls pretreated with Oyrm 1 and supplemented with an enzyme cocktail from *A. niger* 102.12 was decreased during

the first 48 hours of fermentation, with a subsequent increase of 0.15 % (w/v) measured thereafter. It was evident that hydrolysis of the cellulose fraction in the lignocellulosic occurred during fermentation, with this glucose being subsequently converted to ethanol. It would not be possible to produce 0.41 % (v/v) of ethanol from a 0.39 % (w/v) solution of glucose without glucose being produced through hydrolysis. The maximal theoretical ethanol yield yeast can produce is 0.51 grams of ethanol per gram of glucose consumed, thus the maximal yield from 0.39 % (w/v) glucose would be 0.25 % (v/v). In reality however, the yeast will produce less as some of the glucose will be utilised for cell repair, growth and other essential functions.

The rate of cellulose hydrolysis appeared to be relatively poor during fermentation, with the increase in glucose concentration found in the fermentations being low. The cottonseed hulls (10 g) which were estimated to contain 38 % (w/v) glucose (in the form of cellulose) (Table 4.10) were added to the fermentation, which would equate to 1.52 % (w/v) of glucose being present in the 250 mL broth if it was completely hydrolysed. Analysis of the results (Table 4.30) however, indicated that complete hydrolysis did not occur with a maximal glucose concentration of 0.26 % (w/v) being observed, although some of the glucose may have been directly utilised during fermentation to produce ethanol. It is not known why the rate of enzyme hydrolysis was low, although it could be due to a number of reasons. Cellulose hydrolysis may have been affected by the crystallinity of the cellulose substrate as this is thought to interfere with the degradation of the substrate. Enzymatic degradation of crystalline cellulose occurs through the action of two enzymes, namely endoglucanase and cellobiohydrolase (Henrissat *et al.*, 1988). The enzyme cocktail from the Aspergillus fungi may lack one of these enzymes or alternatively the enzyme may be present in low concentrations, thus affecting the rate of hydrolysis. Additionally, the lignin remaining from the pretreatment may be protecting the cellulose fibre from hydrolysis, where the cellulase enzyme could not access the cellulose microfibers. This was likely to be the case as a significant portion of the lignin (89 %) (Table 4.10) was still remaining in the biomass following pretreatment with *Pleurotus ostreatus* Oyrm 1.

Despite a quantity of glucose remaining in the fermentation after 48 hours no further ethanol was produced during the final 24 hours. This may be as a result of the yeast becoming dormant during fermentation due to the low levels of carbohydrate in

the fermentation resulting in the fermentation becoming stuck, which would also have impacted on the efficiency.

Fermentation of the other pretreated cottonseed hull substrates resulted in a lower final ethanol yield than that of the cottonseed hulls pretreated with Oyrm 1 and hydrolysed with a cocktail from *A. niger* 102.12. In these three fermentations, a final ethanol concentration of between 0.23 and 0.30 % (v/v) was determined after 72 hours of fermentation. It appeared that after 28 hours of fermentation, maximal ethanol was produced in the fermentation containing cottonseed hulls pretreated with *Trametes versicolor* 3086 and *A. niger* 102.12, with a slight reduction in the ethanol concentration following this. With respect to the Oyrm 1 and *A. oryzae* 553.65 pretreatment, the ethanol concentration was found to increase during the first 48 hours of fermentation followed by a sharp decrease in the ethanol concentration during the final 24 hours. This decrease in the ethanol concentration noted during the final 24 hours may have been due to the consumption of ethanol by the yeast, as explained previously in Section 4.2.3. An increase in the glucose concentration was observed during the final 24 hours in all four fermentations due to the hydrolysis of the cellulose substrate. However, none of this was utilised during the fermentation, with concentrations of up to 0.26 % (w/v) glucose remaining after 72 hours fermentation. Consequently, this has negatively impacted on the efficiency of the fermentation, with a maximal fermentation efficiency of 84 % detected. This failure to utilise the remaining glucose may be due to the yeast becoming dormant, as explained previously in Section 4.2.3, resulting in the fermentation becoming stuck.

Hydrolysis of cellulose by the enzyme cocktail appeared to be slow, with very little glucose produced during fermentation. This as mentioned previously, was possibly due to inhibition of the cellulase enzyme by inhibitors such as phenolic compounds, glucose and cellobiose, whilst the crystallinity of the substrate may also be a factor. However, it was likely as a result of the lignin in the biomass protecting the cellulose fibres from hydrolysis by the cellulase enzymes.

4.2.5.2 Ethanol production from pretreated oat straw and switchgrass

The production of ethanol from oat straw and switchgrass residues was also investigated. The rate of ethanol and glycerol formation was monitored during fermentation, whilst the carbohydrate concentration during fermentation was also determined (Figure 4.12 & Table 4.31).

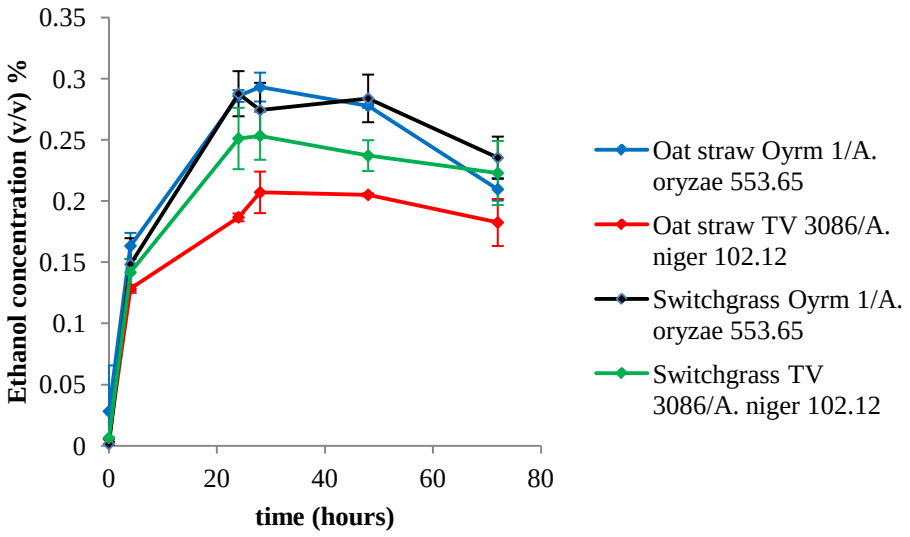


Figure 4.12 Rate of ethanol production from oat straw and switchgrass pretreated with *Pleurotus ostreatus* Oyrm 1 or *Trametes versicolor* 3086.

Results plotted are the mean of triplicates with the standard deviation represented by error bars.

Table 4.31 Composition and fermentation efficiency of pretreated oat straw and switchgrass hydrolysates during fermentation.

Fermentation	Time (hours)	Dextrin % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Switchgrass Oyrm	0	0.054	0.318	0	0	0
1/*A. oryzae* 553.65	28	0.042	0.055	0.046	0.27	86.90
	48	0.046	0.057	0.041	0.28	86.62
	72	0.085	0.052	0.021	0.24	79.90
Switchgrass TV						
3086/*A. niger*	0	0.062	0.447	0	0	0
102.12	28	0.112	0.079	0.053	0.25	75.43
	48	0.103	0.073	0.029	0.24	75.75
	72	0.059	0.065	0.041	0.22	80.68
Oat straw Oyrm						
1/*A. oryzae* 553.65	0	0.075	0.466	0	0	0
	28	0.120	0.078	0.019	0.29	77.37
	48	0.126	0.077	0.029	0.28	75.90
	72	0.062	0.066	0.019	0.21	79.22
Oat straw TV						
3086/*A. niger*	0	0.048	0.240	0	0	0
102.12	28	0.066	0.044	0.041	0.21	81.31
	48	0.039	0.039	0.016	0.21	85.89
	72	0.166	0.051	0.032	0.18	65.81

Results displayed are the means of triplicate flasks with the standard deviation of each sample determined to be less than 5 % of the sample.

Maximal ethanol production from oat straw and switchgrass occurred when they were both pretreated with Oyrm 1 and hydrolysed with an enzyme cocktail from

A. oryzae 553.65. An ethanol concentration of 0.29 % (v/v) was observed after 28 hours of fermentation of the oat straw, with the ethanol concentration being marginally reduced thereafter in both fermentations (Figure 4.12). Oat straw that was pretreated with *Trametes versicolor* 3086 produced the least amount of ethanol, with a concentration of 0.21 % (v/v) achieved after 28 hours of fermentation, where a slight decrease in the ethanol concentration was also noted at the end of this fermentation. Correlating these results with the degradation patterns from Section 4.1, it was apparent that the rate of ethanol production from the lignocellulosic material corresponded to the amount of lignin and cellulose removed from the substrate. The two Oyrm 1 pretreated fermentations, which produced the highest ethanol yields, also incurred the most significant reduction in the lignin fraction during pretreatment. Additionally, the cellulose concentration (Sections 4.1.5 & 4.1.6) was increased during both of these fermentations as a result of the pretreatment process. By contrast, the oat straw pretreated with TV 3086 incurred the least amount of lignin loss and also resulted in the lowest amount of ethanol being produced. Furthermore, a lower degree of lignin degradation occurred in the switchgrass pretreated with TV 3086 in comparison to the Oyrm 1 fermentation, resulting in a lower concentration of ethanol being produced.

Unlike the fermentations containing cottonseed hulls, no increase in the glucose concentration was observed during the final stages of fermentation, with glucose concentrations being extremely low following 72 hours of fermentation. Another point worth noting was the increase in maltodextrin content determined indicating that starch residues are being released during the hydrolysis of the biomass, with some of these being subsequently utilised during fermentation.

Analysis of the glucose data (Table 4.31) suggested that a small amount of glucose was released from the substrate through hydrolysis. In each fermentation, the amount of ethanol produced in comparison to the amount of glucose present at the start of fermentation was high considering the maximal theoretical ethanol yield for yeast is 0.51 g g^{-1} (Gulati *et al.*, 1996). The maximal glucose which can be released from each fermentation is dependent on the amount of glucose available from the lignocellulose substrate (Section 4.1.5 & 4.1.6), as well as the amount of material added to the fermentation (Section 4.2.5). For example, 6 g of pretreated oat straw containing 47 % glucose (in the form of cellulose) (Section 4.1.6) was present in the fermentation broth of the oat straw pretreated with Oyrm 1, which corresponded to a concentration of 1.12

% (w/v) glucose in the substrate. It was clear that 1.12 % (w/v) of the glucose was not released from the substrate during fermentation (Table 4.31), although this would not be unexpected considering that 42 % of the insoluble lignin was still remaining in the substrate after pretreatment. In any case, the increase in the ethanol concentration suggested that a low rate of glucose was released, considering the amount of lignin removed from the fermentation and as well as the length of fermentation. This relatively low rate of hydrolysis could be due to a number of issues described previously in Section 4.2.2.2, such as the inhibition due to glucose and phenolic compounds, the lignin concentration remaining in the biomass or the degree of crystallinity of the cellulose. Furthermore, the cellulase concentration may have been too low to sufficiently hydrolyse the fermentation where a higher cellulase concentration may be required. Typically 15 FPU g^{-1} of glucan is added to lignocellulosic ethanol fermentations (Sathitsuksanoh *et al.*, 2010). In these fermentations a dosage of 5.2 and 6 CMCU per ml of fermentation broth was added, which would not be considered low as only between 6-30 g of lignocellulosic biomass containing between 27-46 % of glucan was found in these fermentations. It can be difficult to correlate the activity between different cellulase assays, although the cellulase activity from the filter paper assay is generally lower than that obtained from the carboxymethylcellulase (CMC) assay. Martins *et al.* (2008) determined that the filter paper activity from *Trichoderma reesei* cellulase was 7 times lower than the CMCU activity obtained, whilst the filter paper activity from *Penicillium echinulatum* was 6 times lower than the corresponding CMC activity. This indicated that 5.2 and 6 CMCU mL^{-1} would over 2 times that which would typically required for hydrolysis of the cellulose in the material.

4.2.5.3 Ethanol production from pretreated corncobs and corn stems

Different pretreated corn wastes were fermented to determine whether ethanol could be produced from the pretreated lignocellulosic residues. The rate of ethanol production (Table 4.13) as well as the composition of the fermentation broth (Table 4.32) were monitored throughout to determine the rate of conversion of the lignocellulosic substrate to ethanol.

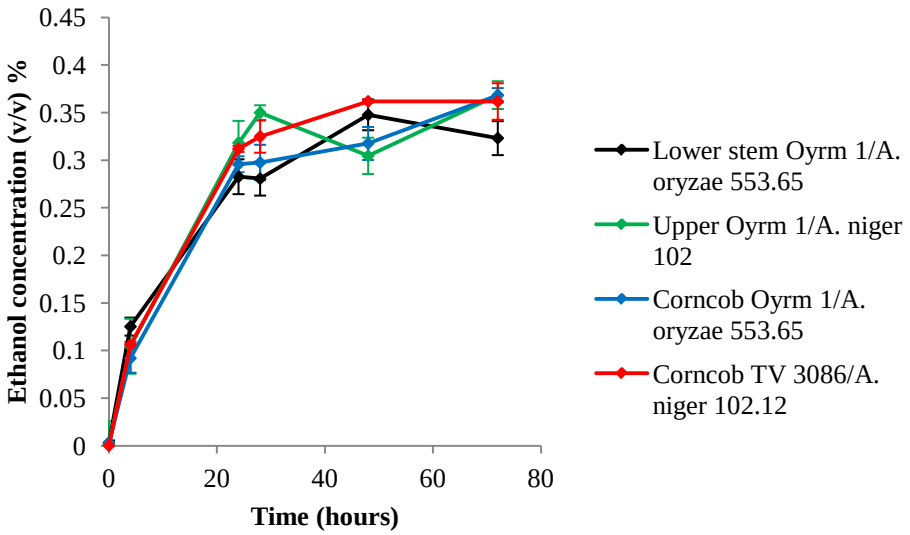


Figure 4.13 Rate of ethanol production from corncobs and corn stems pretreated with *Pleurotus ostreatus* Oyrm 1 or *Trametes versicolor* 3086.

Results plotted are the mean of triplicates with the standard deviation represented by error bars.

Maximal ethanol production was achieved after 72 hours fermentation from the upper stem and corncob fermentations, with the highest rate of ethanol production occurring during the first 24 hours of fermentation (Figure 4.13). Optimal production from the lower stem occurred after 48 hours with a slight decrease in the ethanol concentration thereafter. Ethanol production from the corncob and upper stem residues appeared to be higher than that from the oat straw and switchgrass, with the final concentration of 0.37 % (v/v) being higher than the maximal yield of 0.29 % (v/v) obtained with switchgrass and oat straw.

Table 4.32 Composition and fermentation efficiency of pretreated corncobs and corn stems hydrolysates during fermentation.

Fermentation	Time (hours)	Dextrin % (w/v)	Glucose % (w/v)	Glycerol % (w/v)	Ethanol % (v/v)	Efficiency %
Corncob Oyrm	0	0.140	0.340	0	0	0
1/*A. oryzae* 553.65	28	0.218	0.009	0.035	0.30	74.64
	48	0.318	0.006	0.035	0.32	68.69
	72	0.247	0.003	0.049	0.37	76.71
Corncob TV						
3086/*A. niger*	0	0.299	0.344	0	0	0
102.12	28	0.191	0.010	0.018	0.32	78.43
	48	0.186	0.007	0.034	0.36	80.78
	72	0.157	0.003	0.028	0.36	83.51
Corn lower stem						
Oyrm 1/*A. oryzae*	0	0	0.44	0	0	0
553.65	28	0.000	0.073	0.024	0.28	87.14
	48	0.074	0.083	0.051	0.35	83.77
	72	0.000	0.125	0.064	0.32	83.35
Corn upper stem						
Oyrm 1/*A. niger*	0	0.018	0.548	0	0	0
102.12	28	0.052	0.085	0.051	0.35	85.36
	48	0.000	0.120	0.064	0.30	83.51
	72	0.063	0.088	0.030	0.37	85.13

Results displayed are the means of triplicate flasks with the standard deviation of each sample determined to be less than 5 % of the sample.

It appears that any available glucose was almost completely utilised during fermentation of the pretreated corncob substrate (Table 4.32). In fact minimal levels of glucose remained following fermentation, which was in contrast to the fermentation of cottonseed hulls and oat straw (Tables 4.30 & 4.31). A relatively large amount of maltodextrin remained unutilised in the broth following fermentation of the corncob. During fermentation of the corncob pretreated with Oyrm 1 the maltodextrin content increased by over 125 %, with a portion of this being subsequently utilised by the yeast. In contrast no increase in the maltodextrin content was noted during the fermentation with TV 3086, where a steady decrease was observed during fermentation.

Fermentation of the two stem portions resulted in 83 and 85 % reduction in the glucose portion of the Oyrm 1 and TV 3086 pretreated substrate, respectively during the first 24 hours. A small increase was determined thereafter, which was likely due to the hydrolysis of the cellulose residues during fermentation. It was apparent that glucose was produced during these fermentations, as the amount of ethanol produced

during fermentation (0.32 and 0.37 % w/v) cannot be solely attributed to the fermentation of the initial glucose, as this would result in an ethanol yield which is beyond what is theoretically possible.

Similar to the fermentation of the other residues in Sections 4.2.5.1 & 4.2.5.2, the hydrolysis of the cellulose fraction was extremely low, which could be due to a number of factors that have been outlined previously in Section 4.2.2.2. However, in the case of the corncob substrate it was likely as a result of the significant amount of lignin still bound to the cellulose microfibers, which has inhibited the action of the cellulolytic enzymes. This may also be the case with the stem portions, although substantially more of the lignin was removed during pretreatment of these residues than was the case with the corncob substrate (Section 4.1.2). Additionally, inhibition due to phenolic compounds produced during pretreatment may also have been a factor, whilst enzyme dosage and crystallinity could also have played a role.

It appears that this method of ethanol production is a promising alternative to ethanol production from traditional substrates such as corn mash and molasses provided a number of issues are resolved. The rate of hydrolysis of the fermentation substrate was appreciably low during all fermentations, which may have been due to a number of issues, outlined previously is Section 4.2.2.2. Despite being beyond the scope of this current project, further investigation may shed light on the slow rate of hydrolysis observed. If this was to be determined, the rate of hydrolysis could be vastly improved, thus improving the economic viability of such fermentations.

The highest concentration of ethanol was achieved during fermentation of cottonseed hulls pretreated with Oyrm 1 (Table 4.30), which was surprising considering the low degree of lignin degradation which occurred during pretreatment (Table 4.10). In any case, the concentration of ethanol produced was considerably lower compared to that which could be produced from a corn mash fermentation with an equivalent carbohydrate content, as in the lignocellulosic substrate fermentations 100 % of the carbohydrate was not available. When glucose concentrations of the cottonseed hulls fermentations is considered (1.52 % w/v) it is apparent that a larger amount of lignocellulosic substrate needed to be added to the fermentation in order to achieve ethanol yields on the same scale as those from corn mash fermentations.

4.2.6 General discussion

In general, the addition of lignocellulosic material pretreated with a single fungal strain of either Oyrm 1 or TV 3086 did not have a discernible effect on the rate of ethanol production or the final ethanol concentration (Section 4.2.2). However, the addition of this material resulted in the reduction of the amount of corn mash required in the fermentation to produce a similar ethanol concentration as the control sample, which could lead to a potential decrease in the cost of the fermentation substrate. In a number of fermentations, specifically the corn mash fermentations containing switchgrass pretreated for 180 days, an increase in the final ethanol yield was observed, which could substantially increase the total amount of ethanol produced in a production scale facility. However, in other fermentations such as oat straw pretreated for 150 days, the final ethanol concentration was reduced considerably, negatively influencing the final ethanol yield and economic viability.

The addition of exogenous cellulase to a number of the fermentations containing pretreated lignocellulosic biomass was also investigated (Section 4.2.2). Exogenous cellulase appeared to have minimal impact on the hydrolysis of the pretreated lignocellulosic substrates, with the final ethanol yield being relatively similar to that of the unsupplemented fermentation. By contrast, supplementation of the fermentation containing oat straw resulted in an increase in the final ethanol yield (0.41 % v/v) in comparison to the mash fermentation without exogenous cellulase. During pretreatment of the lignocellulosic substrates, oat straw saw the highest degree of lignin removal which would have resulted in a greater availability of the cellulose for hydrolysis during bioethanol production.

Corncobs pretreated with strains of white rot fungi and Aspergilli were also used to replace a portion of the corn mash and resulted in a significant increase in the rate of ethanol production during fermentation (Section 4.2.3). As a consequence, the time required for fermentation could be substantially reduced by up to 19 hours, which would significantly reduce the cost of the process. Additionally, if this fermentation was scaled up to a production facility, the lower process time may result in a higher productivity in the plant. However, in these fermentations, it is likely that a cost analysis would need to be carried out to determine whether the reduction in fermentation time would result in a higher saving than the revenue lost due to the reduction in the final ethanol yield observed. In the majority of fermentations in which

the final ethanol concentration was reduced a lower efficiency was also noted. If these fermentations efficiencies could be increased to values similar to that of the control the reduction in yield could be reduced, which would make the fermentation more economically viable. In a number of other fermentations however, namely the *P. chrysosporium* 24725 + *A. oryzae* 553.65 fermentation, an increase in the final ethanol yield was detected as well as an increase in the fermentation rate, which would substantially increase the profitability of the process in comparison to the control.

During pilot-scale fermentation (Section 4.2.4), the two fermentations containing corncobs pretreated with Oyrm 1 and TV 3086 resulted in a substantial decrease in the fermentation rate when compared to the control. Furthermore, the efficiency of these fermentations was reduced considerably in comparison to the control. This was not the case in the laboratory-scale fermentations however, where a similar rate of fermentation coinciding with a similar final ethanol yield, was observed. Consequently, it is possible that the fermentations may not be a viable method of bioethanol production, as the rate of ethanol production tended to be reduced in a number of fermentations, whilst the final ethanol yield was also reduced. In contrast to the results measured during laboratory-scale fermentation containing corncobs pretreated with Oyrm 1 and *A. niger* 102.12, in which an increase in the ethanol fermentation rate was found, no increase in the fermentation rate was observed during pilot scale fermentation. This was attributed to the difference in reactor configuration between laboratory- and pilot-scale fermentations in which a higher degree of mixing occurred during pilot scale which may have affected a number of fermentation parameters such as heat and mass transfer.

In the fermentations containing pretreated lignocellulosic material as the sole substrate (Section 4.2.5), the highest rate of ethanol production was observed during fermentation of the cottonseed hulls pretreated with Oyrm 1. This was surprising considering one of the lowest degrees of lignin hydrolysis was noted in this substrate in comparison to other materials such as the oat straw, in which a higher degree of delignification was observed. In saying this, it is worth noting that the cottonseed hull fermentation contained a higher substrate concentration than that of oat straw. This method of ethanol production shows a degree of promise, although a number of issues such as the low degree of hydrolysis during the SimSF would need to be resolved to achieve an ethanol yield closer to the maximum theoretical yield of 0.51 grams of ethanol per gram of glucose available.

5. Enhancement of forages through biological pretreatment

5.1 Forage feed analysis

In response to changes in soil, climatic conditions, competition for land as well as the increased cost and availability of grain, an intensification of the development of high quality pasture and conserved forage feeds to replace a further portion of the grain in the ruminant diet has developed (Reid *et al.*, 1983). Furthermore, the availability and cost of grain has increased even further due to the advent of the bioethanol industry. This has put corn ethanol production in direct competition with the feed industry (Binod *et al.*, 2010), which has lead many researchers to investigate the manipulation of unconventional feeds to extract potential (Abdel-Azim *et al.*, 2011).

Forage quality is usually determined as a function of the nutrient concentration, the digestibility of the plant, the partitioning of metabolised products within the animal and the amount of forage taken in by the animal (Buxton, 1996). The single biggest factor that influences the quality of forage is herbage maturity. As plants revert from the vegetative to the reproductive stage of growth, the quality of the forage is usually decreased due to increased fibre content. This is as a result of the increased cell wall concentration in the feed, which decreases digestibility and reduces the intake by the ruminant (Moharrery *et al.*, 2009). Up until recently, forage breeders did not focus on the improvement of forage quality, due to the knowledge that harvesting immature herbages can obtain forage of higher quality than that which can be obtained through genetic manipulation. However, the reliance on harvesting immature herbages adds to processing costs due to the requirement for more harvests and a reduction in the stand life of the majority of perennial species (Buxton, 1996). As a consequence, breeders have refocused their efforts into improving the quality of the forage at advanced stages of cultivation (Buxton *et al.*, 1993).

Typically, ruminants extract less than one third of the energy present in the cell wall, whereas the contents within these walls are almost completely digestible (Buxton, 1996). Therefore, methods to disrupt the cell wall to extract maximal value from its components are needed to realise the full potential of lignocellulosic biomass. In this section, the use of biological pretreatment as a method of altering the nutritional

value of forage feed will be investigated. In particular, the feasibility of using this method to enhance the nutritional value in terms of digestibility and protein content will be examined.

Although the value of the forage is dependent on the animal, the product that is being produced (i.e. dairy or meat) and the type of forage, each treatment investigated in the following sections were ranked based on the general requirements for a beef livestock diet, with the improvement of each feed monitored over time. However, the effect of forage quality on animal production is difficult to assess as forages that differ in quality require different supplementation strategies to obtain maximal animal product and as such are difficult to compare (Allen, 1996).

To determine the cell wall components and digestibility of the forage the detergent system of feed analysis was used. This was first introduced by Van Soest (1965) and is still widely used today, although modifications have been made to the original method. In the detergent system, neutral detergent fibre (NDF) is used to measure the cell wall components which are only partially degradable and is negatively correlated to potential intake. The acid detergent fibre (ADF) is used to measure the cellulose and lignin fraction and is an indication of the digestibility of the forage, where the higher the ADF content, the lower the digestibility (Buxton, 1996; Udén *et al.*, 2005). The *in vitro* digestibility was also used to assess the digestibility of the forages, where each material was incubated in rumen fluid to determine how digestible the material would be in the rumen.

To monitor the influence of biological pretreatment on the nutritional value of common agricultural waste products, samples were taken every 30 days from fermentations of corn stover, corncobs, oat straw, switchgrass, cottonseed hulls and coconut fibre with selected white rot fungi. The cell wall content, digestibility, crude protein (CP), ethanol soluble carbohydrates (ESC), *in-vitro* true digestibility (IVTD), non-fibre carbohydrates (NFC) and total digestible nutrients (TDN) were determined at each time point throughout the fermentation, using the methods described in Section 2.2.9. All results were expressed on a dry matter basis, with the dry matter being determined prior to calculation.

5.1.1 Assessment of the forage value of corn waste fermented with white rot fungi

Corn waste was separated into fractions as each component can vary in composition (Wilman *et al.*, 1998). The corn waste was divided into the corncob (consisting of the inner portion of the ear with the kernels removed), the leaf blades (separated at the point of the blade meeting the stem), the upper stem (all herbage above the 5th node, excluding the leaf blade, husks, silks, rachis and grain) and the lower stem (all herbage above 5cm and below the 5th node excluding the leaf blades with the husks, silks, rachis and grains removed).

5.1.1.1 Corncobs

Corncob has long been established as a component in animal feed with, data showing corncob to be a satisfactory roughage for beef cattle diets as far back as 1955 (Fries, 1955; Harshbarger, 1955). Cattle cannot survive on corncobs alone; however, when it is supplemented with other nutrients it can serve as a valuable source of energy (Burroughs *et al.*, 1945; Burroughs *et al.*, 1946). Generally, the addition of corncobs to the forage can lead to a reduction in gains, less finish and a lower dressing potential (Geurin *et al.*, 1955). Conversely, there have been reports of steers getting started easier and staying on feed better when corncob was included in the diet (Gerlaugh *et al.*, 1935; Snapp, 1952). In addition, others have reported corncobs adding sufficient nutrients to the ration to result in greater cattle feed profits (Gerlaugh *et al.*, 1935; Gerlaugh *et al.*, 1948). In this section, the structural modification of corncobs by white rot fungi will be analysed through fermentation of corncob, with special attention paid to the potential use of this technology in the improvement of animal feed quality (in particular the digestibility and protein content).

Table 5.1 Compositional analysis of corncobs pretreated for 210 days with the fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Fungi	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% ESC	% IVTD
Oyrm 1	0	1.6	49.8	91	57	4.3	8.1	8	48
	60	2.3*	52.8*	88.9	50*	8.4*	7.3	7.2*	58*
	90	1.7	61.9*	90.9	59	3.4	6.1*	1.7*	59*
	120	2.2*	59*	90.8	56.5	4.3	5.75*	1.3*	62*
	150	2	57.8*	86.2*	59.5	4.75	9.9*	3.45*	58*
	180	2.3*	60.85*	89.15	55	5.2	7.25	1.85*	54*
	210	2	59.4*	88.95	55.5	5.1	7.75	2.6*	66*
TV 3086	0	1.6	49.8	91	57	4.3	8.1	8	48
	60	1.95	58.65*	89.75	61.5*	3.5	7	1.8*	63*
	90	2	55.6*	86.5*	58.5	3.7	10.2*	1.9*	65*
	120	1.95	55.2*	84.8*	59	3.75	11.95*	1.3*	71*
	150	1.95	54.8*	87.35*	59.5	3.45	9.45*	2.3*	73*
	180	2.3*	51.2	78.1*	59.5	4.55	18.3*	1.25*	70*
	210	2.2*	50.4	79.65*	52.5*	4.5	17.1*	2.4*	76*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility

Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

Typically, the corncobs utilised in these fermentations would be considered a poor quality forage due in part to the high percentage of NDF present in the material, as well as the extremely low crude protein content observed (Table 5.1). As a consequence, this material would generally be used as a low quality roughage to dilute the energy concentration of the feed in finishing diets and to reduce the incidence of acidosis (Rush *et al.*, 1997). Despite this, small portions (<5 %) of roughage from such material have been shown to increase gains and feed efficiencies in certain diets (Stock *et al.*, 1990).

NDF is a measure of the cell wall components including cellulose, hemicellulose and lignin and is generally used to estimate how much forage a ruminant will eat. The higher the NDF value, the less the animal can consume, resulting in inefficient nutrients being supplied to the animal. For example, a high producing dairy cow can eat about 1.2 % of its body weight in NDF meaning that a 1000 lb cow could eat 24 lb of forage containing 50 % NDF (Mertens, 1992). Likewise, if untreated corn cob containing 91 % NDF was fed, the animal would only be able to consume 10 lb,

meaning the quality and digestibility of the cob would need to be substantially greater to impart the same level of nutrients. The initial NDF content of the untreated corncob was extremely high (91 %); however, through pretreatment with TV 3086, this was reduced significantly ($p<0.05$) by over 10 %, which is a notable improvement on the quality of the corncob as a forage feed. By contrast, pretreatment with Oyrm 1 did not have as considerable an impact on the NDF content of the material. This may be due to the increase in the cellulose content observed during fermentation (Section 4.1.1), which coincided with a decrease in the xylan and lignin content, thus minimising any change in NDF during pretreatment. Despite the NDF having no bearing on the digestibility of the forage, values of below 40 % are usually observed in prime quality forage, while values over 65 % are typically found in poor quality forages (Rivera *et al.*, 2010). NDF normally affects the intake which in turn influences the quality of the ADF needed to promote gain, thus indirectly affecting forage quality. Researchers have shown that in poor quality forages, such as corncob, the consumption is controlled by the physical capacity of the animal whereas with higher quality forages, consumption is generally influenced by other factors, such as metabolic size and productive energy (Conrad *et al.*, 1964; Nutt *et al.*, 1980).

Acid detergent fibre (ADF) measures the indigestible portion of the plant whereby the lower the ADF, the higher the digestibility of the forage. In general, a forage with an ADF of below 31 % would be considered of prime quality, whereas above 45 % would be regarded as a poor ADF content (Rivera *et al.*, 2010). From Table 5.1 it can be determined that the ADF of the untreated corncob was substantially higher than 45 % and thus considered poorly digestible. Through pretreatment with Oyrm1, the digestibility of the material was decreased, with the ADF concentration being increased from ~50 to 60 %. This coincided with an apparent increase in the lignin content of the material which would have affected the forage digestibility. Analysing the fermentation with TV 3086, it appears that the ADF content was initially increased during the first 60 days and subsequently decreased over the next 150 days, with the final ADF content being determined as 0.6 % higher than that of the initial value observed in the untreated material, indicating that the substrate was marginally less digestible than the unfermented cob.

Crude protein content of the corncob was broadly in agreement with Thornton *et al*. (1969), who quantified the crude protein of mid-dent and mature corncobs to be between 2-2.1 %. However, in contrast to other forages such as alfalfa hay, which

contained a crude protein content of between 10 and 20 % (Meyer *et al.*, 1959) and corn silage, which contained a crude protein concentration of between 7-9 % (Pinkerton *et al.*, 1991), the crude protein content of the untreated corncobs was decidedly low. During pretreatment with white rot fungi, the crude protein content was increased by up to 43 %, although the final concentration was still substantially less than the other well known forage feeds. Bowman *et al.* (1995) established that when cattle are fed forage with a crude protein content of below 5.6 % weight loss was observed; however, when the crude protein was above 5.6 % weight gain was detected. Feeds containing CP below 6-7 % can typically be supplemented with non-protein nitrogen to stimulate intake (Minson, 1990). However, supplementing with nitrogen or crude protein adds to the cost of feeding thus biological pretreatment methods for improving crude protein content may be more sustainable.

Total digestible nutrients (TDN), which is the percentage of digestible material in the forage, is determined using the sum of the digestible protein, digestible non structural carbohydrate, digestible NDF and fat and is an indication of the total energy of the feed. This term is typically used to describe ration for beef or sheep rather than ration for dairy cows. Common TDN values range from between 38 to 71 % depending on the feed stuff, with the average TDN for corn silage being 67 %. Furthermore, a TDN of 71 % would be considered excellent quality and 38 % would be recognised as a poor forage (Lemus, 2009). Untreated cob would be thought of as a poor to fair forage based on its TDN content (Table 5.1). Following pretreatment for 60 days with TV 3086 the quality of the material was marginally improved, with an increase of 4.5 % in the TDN content observed. However, after 210 days the TDN value was reduced to a final content of 4.5 % less than the control sample, leading to a very poor quality forage, with regard to the TDN content. The total digestible nutrients of the Oyrm 1 treated material were increased slightly after 90 days pretreatment, although the digestibility was decreased again as the fermentation was continued. Accordingly, the optimal time for harvesting the fermentations would appear to be after 60 days of fermentation as the nutrient digestibility was decreased thereafter. The subsequent decrease in the TDN content noted following the initial increase in the TDN content is likely as a result of the decreased digestibility of the forage, which was observed through an increase in the ADF of the substrate.

In vitro true digestibility (IVTD) is a measure of the actual digestibility of the forage in the rumen and is determined by incubating the sample in rumen fluid for 30

hours, with forage digestibility calculated thereafter. From analysis of the results (Table 5.1), it was concluded that the true digestibility of the forage was increased significantly ($p<0.05$) during both fermentations. Untreated cob had a digestibility of 48 % which would be considered as fair for forages; however, as the cob was pretreated the true digestibility of the cob was dramatically increased to 66 and 76 % by Oyrm 1 and TV 3086, respectively, bringing it in line with prime quality forages, with respect to its digestible dry matter (Coppock, 1997). Yulistiani *et al.* (2012) investigated the pretreatment of ground corncob with *Trichoderma viride*, *Aspergillus niger* supplemented with urea. The author determined that the *in vitro* digestibility of the material was only substantially increased through pretreatment when urea was present, with both fungi having minimal impact on the forage digestibility. This was likely due to the inability of these fungi to sufficiently degrade the lignin during fermentation. Klopfenstein *et al.* (1972) examined the effect of sodium hydroxide pretreatment on a corncob substrate and concluded that pretreatment with 4 % NaOH increased the digestibility of the cob by 14.8 %, which was 13.2 % less than the increase in digestibility observed with TV 3086 pretreatment. However, 17 % dry matter loss (Table 4.1) was documented through fermentation with TV 3086, which did not appear to be the case with sodium hydroxide pretreatment.

Analysis of the results indicated that corncob was a poor forage for the animal diet with high NDF and ADF content as well as a poor crude protein and TDN content. However, it was evident that through pretreatment of the material with white rot fungi, the rumen digestibility of the material can be substantially enhanced. When analysing the IVTD results, it was apparent that after biological pretreatment the cob was highly digestible (Rivera *et al.*, 2010), which is in contention with the results obtained in the detergent analysis in this study. However, Oba *et al.* (2005) analysed the results from a number of IVTD and chemical digestibility studies and concluded that the IVTD was poorly related to the NDF, ADF and CP of corn silage and legumes and that it was an independent measurement of forage quality. It would appear that pretreated cob may be a suitable forage for high yielding dairy cows, as there have been a number of reports indicating that forage with enhanced IVTD increases milk yields from high yielding dairy cows (Oba *et al.*, 1999). However, the crude protein content of the corncob would be too low without supplementation to achieve sufficient milk yields, with dairy cattle requiring up to 21 % protein for sufficient milk yields. Typically, in

ruminant nutrition the higher the protein content in the diet the higher the milk yield that can be achieved.

The ethanol soluble carbohydrate (ESC) content is a measure of the soluble carbohydrates that dissolve in 80 % (v/v) ethanol and consists mainly of mono- and disaccharide sugars. During fermentation with TV 3086, the majority of the ethanol soluble carbohydrates were utilised in the first 60 days of fermentation, with 1.8 % remaining after this time. Throughout the rest of the fermentation the ESC content of the fermentation fluctuated, with values of between 1.25 and 2.4 % noted. This was likely as a result of the formation of soluble sugars during fermentation through hydrolysis of the cellulose and hemicellulose fractions of the biomass. This occurred through the action of enzymes from white fungi which hydrolyse the substrate to simple sugars that are required for growth and metabolism. In contrast, less than 1 % of the ESC was utilised during the first 60 days of fermentation with Oyrm 1, with the majority of the soluble sugars used after this. It was anticipated that the soluble sugars would be rapidly utilised during fermentation as fungi typically use readily available sugars first (as was the case with TV 3086). This may be attributed to a difference in colonisation rate during fermentation, in which the mycelia density of the TV 3086 fermentation was visibly greater than that of the Oyrm 1 fermentation after 60 days.

The non-fibre carbohydrates (NFC) are a measure of the non-structural carbohydrates available in the feed and consist of starch, sugars, pectins and fermentation acids. These are usually utilised in the rumen, where the microbial population breaks them down to be used as an energy source. During fermentation with TV 3086, the NFC was increased by 9 %. This increase may be due to the formation of fermentation acids during fermentation of the material. Alternatively, the non-fibre carbohydrate may have been condensed through the loss of celluloses, hemicelluloses and lignin, resulting in loss of weight from the material. Furthermore, this increase may also be due in part to the growth of the fungus and the soluble carbohydrates present in the fungal mycelia. This increase in soluble sugars from fungal mycelia may also influence the ESC concentration as it is also known to contain soluble carbohydrates such as mono- and disaccharides. Pramanik *et al.* (2007) determined that water soluble glucans were present in the fruiting bodies of *Pleurotus sajor-caju*, whilst Vaz *et al.* (2011b) also found water soluble polysaccharides in the fruiting bodies of 4 wild edible mushrooms.

5.1.1.2 Upper stem

When corn is harvested, approximately 50 % of the plant is considered waste, equating to about one third of the total digestive nutrients of the plant (Coppock *et al.*, 1968). As a consequence, with the increase in corn production due in part to the bioethanol industry, corn stover has become of increasing interest as forage feed. However, corn stover, which includes the leaves and stems, is not considered to be a ruminant feed of high nutritive value, with it generally offering similar levels of animal performance as a poor to average quality grass silage (O' Kiely *et al.*, 1995). Consequently, animals especially those in third world countries can suffer from underfeeding as a result of insufficient nutrient supplied by this forage. Accordingly, pretreatment studies to improve the quality and digestibility of the stover have been undertaken to enhance the intake, gain and yield from ruminants (Akinfemi *et al.*, 2009).

In this study, the stover portion of corn was separated into the lower and upper portion of the stem, as well as the leaf fraction. Previous studies have indicated that the leaf and stalk portion contain different biochemical compositions. Tolera *et al.* (1999) reported that the leaf portion of stover contained higher crude protein and organic matter digestion than the stem, while other reports have indicated that the crude protein content in leaves was higher but the ADF portion was lower than in corn stalks (Tang *et al.*, 2006; Tang *et al.*, 2008).

Table 5.2 Compositional analysis of corn upper stem pretreated for 240 days with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Fungi	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% ESC	% IVTD
Oyrm 1	0	4.6	39.9	57.9	57	5.9	31.1	14.6	64
	60	5.6*	48.1*	59.5	64*	2.5*	27.1*	5.5*	76*
	120	6.6*	48*	59.2	65*	1.6*	25.3*	11*	74*
	150	8.3*	50.8*	63.8*	63*	1.5*	19.5*	4.3*	76*
	180	8.9*	46.6*	53.7*	64*	2.2*	27.4*	3.9*	61*
	210	10.3*	45*	52.6*	64*	2.2*	26.7*	3.4*	57*
	240	10.8*	46.1*	57.5	64*	1.1*	20.9*	4.8*	73*
TV 3086	0	4.6	39.9	57.9	57	5.9	31.1	14.6	64
	60	7.2*	42.5*	63.3*	63*	3*	22.8*	2.6*	54*
	120	8.8*	37.3*	56.5*	63*	2.4*	25.2*	5.8*	56*
	150	10.5*	32.9*	52.9*	64*	1.1*	24.9*	2.5*	58*
	180	9.8*	33.8*	45.9*	61*	3.1*	30*	2.4*	55*
	210	13.5*	24.4*	43.8*	61*	2*	27.3*	2.4*	54*
	240	13.9*	24.7*	47.3*	59*	1.6*	22*	2.2*	50*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility

Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

It was evident when analysing the results (Table 5.2) that the upper stem was considerably more digestible than corncob, which was analysed previously (Section 5.1.1.1). The crude protein content (4.6 %) was notably higher in the upper stem, although it is still considerably lower than other forages such as legumes, which typically have a crude protein content of around 17 %, whilst cool season grasses contain about 13 % crude protein (Minson, 1990).

Biological pretreatment with both TV 3086 and Oyrm 1 (Table 5.2) increased the crude protein content during fermentation by 200 % and 134 %, respectively. In terms of quality, both these corn forages would now be considered good quality for corn stover with respect to crude protein content and would be suitable for most ruminant animal diets, with the exception of lactating dairy cows. These cows would generally require a crude protein content of over 15 % from forage which could be supplied through additional urea supplementation (Hristov *et al.*, 2004). This increase in crude protein is likely due to the presence the fungal mycelia that are known to contain substantial amounts of protein. Bauer Petrovska (2001), analysed the protein

content of 52 common mushrooms found in Macedonia and determined that the crude protein content can vary from 14-51 % on a dry matter basis and thus could make up a significant portion of the dry weight of the colonised substrate. The loss of structural carbohydrates and lignin may also have influenced the crude protein content. This reduction may have resulted in a concentration of the crude protein which has caused an apparent increase in the protein content during fermentation.

The lignin content of the upper stalk was also significantly ($p<0.05$) reduced by both fungi during fermentation, with over 80 % of the lignin removed through pretreatment with Oyrm 1, whilst 73 % was degraded by TV 3086. This should result in a substantial increase in the digestibility of the substrate as the ADF (which is an indication of digestibility) is a measure of the cellulose and lignin content in the forage. However, this did not appear to be the case with Oyrm 1, where the ADF was increased during the initial 150 days of fermentation and subsequently decreased thereafter, although not below the value of the control T0 sample. This increase in the ADF fraction may be due to the chitin content of the fungal cell wall which can interfere in the ADF determination. Chitin is structurally very similar to cellulose and as such can interfere in the ADF determination. Vetter (2007) analysed the chitin content of several commonly cultivated mushrooms and determined that *Pleurotus ostreatus* contained between 2.16-5.46 % chitin on a dry matter basis. Furthermore, various authors have suggested that the fibre found in insects using the detergent method from Van Soest (1967) was in fact chitin (Barker *et al.*, 1998; Finke, 2002; Finke, 2007). Alternatively, this increase in the ADF content may be as a consequence of the increasing cellulose content due to selective degradation of the hemicellulose and lignin, which was observed previously (Section 4.1.2.2) during pretreatment with Oyrm 1 (Table 4.5). In contrast to Oyrm 1 the digestibility (ADF content) of the upper stem was reduced by 15 %, through pretreatment with TV 3086, which resulted in a considerable increase in the forage quality. As a consequence of pretreatment with TV 3086, this forage would now be considered a prime forage feed, with regard to its digestibility (Coppock, 1997).

Initially, the NDF content of this feed was considered to be fair; however, pretreatment with TV 3086 reduced the NDF by 10 %, which improved the upper stem NDF content so as it was similar to a Class 2 forage based on Coppock's (1997) forage classifications. A Class 1 forage would be considered of excellent quality where as a Class 5 fodder would be considered to be poor. In the case of Oyrm 1, NDF was

increased during the first 150 days of fermentation and subsequently reduced thereafter to the same value as that of the initial value, indicating that no improvement is quality was achieved.

Analysing the ethanol soluble carbohydrate (ESC) fraction, which is a measure of soluble mono- and disaccharides, it was noted that the majority of the soluble carbohydrates were utilised during the first 60 days of fermentation with Oyrm 1. Nonetheless, it was surprising to discover that after 240 days of fermentation with Oyrm 1, 4.8 % of readily fermentable carbohydrates were left unutilised in the fermentation. Despite up to 67 % of the soluble carbohydrates being used in the fermentation, it was anticipated that the fungus would have completely utilised the easily fermentable sugars during fermentation. Cellulases and hemicellulases are constitutively produced by the fungus during fermentation and is induced by increased glucose levels which accounts in part, with liberated glucose, to higher than expected sugar level. Pretreatment with TV 3086 resulted in a significant ($p<0.05$) decrease in the ESC concentration during the initial 60 days of fermentation. The soluble carbohydrates were subsequently increased during the next 30 days, with over a two-fold increase in the ethanol soluble carbohydrate concentration observed. These soluble carbohydrates were then decreased during the next 120 days with a ESC concentration of 2.2 % noted following fermentation. The apparent increase in ESC concentration may be as a result of variations in the rate of substrate degradation by the fungi in the different fermentations bags, as explained previously in Section 4.1.1.

The NFC of the material was reduced through pretreatment with both fungi, where a decrease of 10 and 9 % was detected during fermentation with Oyrm 1 and TV 3086, respectively. This reduction in the non-fibre carbohydrate was expected as the sugars and starches which comprise the NFC are generally utilised during the initial stages of fermentation. However, a large portion of the NFC still remained in both fermentations after 240 days. This likely consists of fermentation acids and unutilised polysaccharides such as pectin which is a constituent of plant cell walls.

Analysing the TDN (total digestible nutrient) content of the untreated material, the upper stem appears to be a good quality forage, with pretreatment further improving the quality of the forage. Surprisingly the most dramatic increase in total digestible nutrients was observed with Oyrm 1, which was unexpected considering the improvement in ADF, NDF and crude protein achieved through pretreatment with TV 3086. Biological pretreatment with Oyrm 1 improved the upper stem *in vitro*

digestibility (IVTD) significantly ($p<0.05$) to a point where the material would be regarded as a prime quality forage feed, with respect to it dry matter digestibility (Rivera *et al.*, 2010). By contrast, the digestibility of the upper stem was reduced by 14 % through pretreatment with TV 3086, where the forage would be considered as poorly digestible in the rumen. It is probable that a significant ($p<0.05$) reduction in the lignin content may have had a substantial impact on the digestibility of the material, with 81 and 73 % degraded by Oyrm 1 and TV 3086, respectively.

The IVTD method for determining the digestibility is thought to be the most accurate method of analysing the digestibility of the forage. IVTD tends to more accurately predict the digestibility of the forage as rumen fluid from the cows is used in comparison to the acid digestibility technique.

5.1.1.3 Lower stem

Table 5.3 Compositional analysis of corn lower stem pretreated for 240 days with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Fungi	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% ESC	% IVTD
Oyrm 1	0	4.7	41.5	59.7	56	6.8	30	12.9	68
	60	6.1*	50.4*	66*	64*	2.1*	21.8*	1.9*	52*
	120	8.7*	48.9*	61	66*	1.3*	23.3*	3.9*	59*
	150	7.5*	48.6*	60.1	62*	4.2*	26.4*	4.6*	61*
	180	9*	50.2*	59.1	64*	2.3*	23.7*	3.7*	58*
	210	8.8*	46.1*	52.7*	66*	1.6*	28.8*	3.4*	63*
	240	9.4*	44.8*	52.3*	67*	0.7*	27.3*	4.8*	64*
TV 3086	0	4.7	41.5	59.7	56	6.8	30	12.9	68
	60	6.4*	50.2*	68.1*	60*	4.6*	21.2*	1.4*	49*
	120	7.3*	42.2*	58.7	64*	2.6*	25.8*	1.7*	51*
	150	8.4*	39.8*	57.4*	61*	3.8*	25.3*	2.6*	55*
	180	8.6*	36*	51.9*	63*	2.4*	28.6*	2.8*	56*
	210	9.4*	31.5*	49.8*	65*	1.7*	29.3	3.3*	46*
	240	10.6*	33.3*	53*	62*	1.3*	23.2*	1.3*	45*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility

Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

Comparing the composition of the lower and upper stalk, it was noted that the ADF and NDF from the untreated lower stems were ~2 % higher than the equivalent

untreated material from the upper portion. Similarly, the lignin fraction from the lower stem was 1 % higher than that of the upper stem portion, whilst the ESC from the lower stem was less than that of the upper portion.

Similar to the pretreatment of the upper stem, the crude protein content of the lower stems (Table 5.3) was also increased with biological pretreatment. The increase in crude protein observed through pretreatment with TV 3086 (125 %) resulted in slightly higher protein content than through pretreatment with Oyrm 1 (100 %). This difference in the crude protein increase between the two fungal species is likely due to the differences in protein composition between the fungi. For instance, the protein content of a strain of *Pleurotus ostreatus* from Macedonia has been shown to be 24.69 %, whilst *Agaricus Bisporus* contained 38.62 % protein (Bauer-Petrovska, 2001). With such variance in the protein content of mushrooms it is conceivable that the difference in crude protein observed in this study may be due to these differences. However, the loss of structural carbohydrates and lignin during pretreatment may also have resulted in a concentration of the protein fraction during fermentation, as explained previously in Section 5.1.1.2. In addition the mycelial density of the fungi in the fermentation may also influence the protein content of the material.

Pretreatment of the lower stalk with Oyrm 1 and TV 3086 resulted in an overall reduction in the NDF content of the stover. During the initial 60 days of fermentation an increase in NDF of 6 and 8 % by Oyrm 1 and TV 3086, respectively was observed. This increase is possibly due to the substantial loss of ethanol soluble carbohydrates, which has resulted in an apparent increase in the structural carbohydrates and lignin content of the material. However, as the fermentation proceeded, the NDF concentration of both fermentations was reduced to between 52-53 %, which was notably lower than the NDF content of the untreated material, thus significantly ($p<0.05$) improving the potential intake of these feeds. Analysing these fermentations using Coppock's (1997) forage rating system for livestock diets it was evident that both these pretreated forages were upgraded from category 3 to category 2 forages which would equate to these now being considered as good forages in respect of their NDF content.

The ADF content of the lower stem followed the same trend as the upper stem. ADF levels were reduced following pretreatment with TV 3086 but were increased by Oyrm 1 fermentation. Pretreatment with TV 3086 resulted in an overall reduction in the ADF content to 33 %, making this an excellent forage feed for a livestock diet. On

the other hand however, pretreatment of the lower stem with Oyrm 1 downgraded the fodder from a fair to poor forage as a result of an increase of up to ~9 % in the ADF content. The reduction in the ADF content of the TV 3086 fermentation was most likely due to the lignin loss noted during fermentation, with over 80 % of the initial lignin being removed during the 240 day fermentation. Furthermore, 90 % of the lignin was removed through pretreatment with Oyrm 1 which was not reflected in the ADF content, where a reduction in digestibility was expected. This was most likely due in part to an increase in the cellulose fraction, where the cellulose was concentrated in the substrate due to selective degradation of the lignin and hemicellulose fraction, as observed previously in Section 4.1.2.1 (Table 4.3).

Analysing the TDN of both fermentations, it was clear that the forage was upgraded following biological pretreatment, with an 8 and 6 % increase in the total digestible nutrients detected with Oyrm 1 and TV 3086, respectively. A TDN content of above 56 % is usually regarded as a good forage feed, with a value of above 60 % considered as excellent (Coppock, 1997). At 67 and 62 %, pretreatment with Oyrm 1 and TV 3086 was particularly successful at improving the potential energy of the lower stem of corn stover, with both forages being considered of prime quality with respect to their TDN content.

The ethanol soluble carbohydrates from both fermentations were almost completely utilised during the first 60 days of fermentation; however, the soluble carbohydrate content tended to be increased thereafter. This was likely due to release of soluble sugars from the substrate by cellulase and xylanase enzymes, as mentioned previously in Section 5.1.1.1. Furthermore, soluble sugars from the fungal mycelia may also influence the ESC concentration resulting in an increased ESC content.

The NFC content was reduced by 28 and 29 % during the first 60 days of fermentation with Oyrm 1 and TV 3086, respectively. This coincided with the decrease in the ESC which would account for a significant portion of the NFC. After this point, the non-fibre carbohydrate concentration was increased, although the final concentration was still lower than that of the control (T0) sample. This may have been due to the production of acids during the fermentation or alternatively, as a result of the soluble carbohydrates in the white rot fungi, which can influence the NFC content. In addition, it was apparent that the increase in the NFC content of the material coincided with the increase in the ethanol soluble carbohydrates which are produced

during fermentation as the soluble sugars determined in the ESC would also make up a substantial part of the NFC.

In vitro digestibility of the lower stem was reduced over time (Table 5.3) through pretreatment with both fungi. *Trametes versicolor* 3086 decreased the digestibility by 34 % which was a substantially larger reduction than with Oyrm 1, which decreased the IVTD by 6 % during fermentation. In the case of TV 3086, pretreatment significantly ($p<0.05$) reduced the digestibility of the feed, with it now being considered a poorly digestible forage. By contrast, despite the digestibility of the Oyrm 1 fermentation being decreased, the forage would still be regarded as highly digestible (Rivera *et al.*, 2010). These results were in contrast to the results obtained from pretreatment of the corncob and upper stem which improved the digestibility of the forage in a number of the fermentations (Table 5.1 & 5.2). When the loss of matter during fermentation (Tables 4.3 & 4.4) (Section 4.1.2.1) was accounted for, it was clear to see that the decrease in the *in vitro* digestibility of the forage as well as the amount of weight loss that occurred during fermentation (~50 %) may make this method of upgrading the feed uneconomical. However, pretreatment of the substrate with TV 3086 appears to be the most promising as a higher final crude protein content was observed as well as a decrease in the NDF and ADF content. In saying this however, the Oyrm 1 treated material was still highly digestible with respect to its *in vitro* digestibility and also contained an appreciable crude protein content.

Biological pretreatment of corn stover appeared to compare favourably with other techniques such as chemical pretreatment. For instance, Chang *et al.* (2012) investigated the pretreatment of corn stover using steam explosion. It was determined that this technique reduced the ADF content by 14 %, which was lower than the reduction in ADF observed during pretreatment with TV 3086 (20 %). However, a greater reduction in the NDF was noted during stream explosion indicating that a higher degree of hemicellulose removal occurred.

5.1.1.4 Corn leaves

Table 5.4 Compositional analysis of corn leaves pretreated for 240 days with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Fungi	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% ESC	% IVTD
Oyrm 1	0	11.9	33.8	63.2	59	4.4	18.6	5.1	83
	60	15*	34.6	51.1*	70*	1.1*	27.7*	4.1*	83
	120	17*	33.4	45.6*	72*	1*	30.6*	4.3*	84
	150	21.9*	31.7*	38.4*	74*	0.9*	34*	4.9	85
	180	32.3*	25*	33.7*	75*	1.1*	31*	5.4	85
	210	28.5*	17.8*	20.8*	75*	2.6*	44.6*	5.1	86
	240	32*	14.1*	19.5*	76*	2.4*	43.4*	3.6*	91*
TV 3086	0	11.9	33.8	63.2	59	4.4	18.6	5.1	83
	60	22.2*	33.3	40.3*	74*	0.8*	32.1*	4.7	83
	120	26*	18*	27.5*	75*	1.6*	40.5*	5.5	87*
	150	30.1*	8.5*	15.*	77*	2.4*	48.5*	4.7	85
	180	24.6*	18.1*	25*	76*	1.3*	43.6*	4.7	78*
	210	29.8*	16.6*	23.9*	79*	0.1*	41.1*	13.1*	84
	240	26.1*	13.3*	20.2*	80*	0.1*	46.7*	4.4*	83

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility

Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

Comparing the leaves to the other stover fractions it was apparent that the initial crude protein content from corn leaves (Table 5.4) was substantially higher than that obtained from the stem fractions, whilst the NDF content was also higher. Additionally, the ADF in the corn leaves was 7.7 % lower than the ADF from the lower stem, while the lignin content of the leaves was also less. The digestibility (ADF content) of the corn leaves was considerably greater than the stalks, which is likely due to the higher amount of protein and cell solubles as well as a lower cell wall content (Karunanandaa *et al.*, 1995). In general these results were broadly in line with other authors who similarly concluded that the digestibility of corn leaves was greater than that of corn stalks (Tolera *et al.*, 1999; Tang *et al.*, 2008).

The crude protein content of the corn leaves (Table 5.4) was significantly ($p<0.05$) increased following biological pretreatment, with an increase of 268 and 252 % observed with Oyrm 1 and TV 3086, respectively. As explained previously, this

was mostly likely due to protein formation by the fungi, although the loss of structural carbohydrates and dry matter (Section 4.1.2.3) may have also result in an apparent increase in crude protein. A crude protein content above 19 % is generally considered of prime quality for forages (Rivera *et al.*, 2010). In these fermentations, both forages could supply crude protein far in excess of that and would be suitable for dairy cattle, where forage with high crude protein concentrations is required. Typically, corn silage would contain 10 % crude protein, which is similar to the results obtained in this study; however, through pretreatment the CP content was increased almost 3-fold. The crude protein required for livestock generally ranges from 7 % for mature beef cows to 19 % for high producing dairy cows (NRC, 1984), with this modified forage capable of providing sufficient crude protein for both dairy and livestock diets.

ADF content was decreased by over 50 % during both fermentations. The most dramatic reduction occurred during the third and fourth month of fermentation with TV 3086, whilst the majority of degradation occurred following 150 days of fermentation with Oyrm 1. This reduction in ADF content has significantly ($p<0.05$) increased the digestibility of the forage with both fodders now considered of prime quality with respect to their ADF content. Similarly, the NDF content of the feed was also significantly ($p<0.05$) reduced, thus increasing the intake potential. The NDF value of both fermentations was reduced by 40 %, which is most likely due to the substantial loss of lignin and cellulose from the forage, reflected in Tables 4.7 and 4.8 (Section 4.1.2.3).

Overall, both forages would be considered excellent quality with respect to the TDN and IVTD of the feed. The total digestible nutrient contents were increased by Oyrm 1 and TV 3086 to 75 and 80 % respectively, with a TDN above 60 % being considered an excellent quality forage (Coppock, 1997). When the IVTD was considered, it was apparent that the forage pretreated with Oyrm 1 was more digestible than corn leaves pretreated with TV 3086, with an increase of 8 % observed in the Oyrm 1 fermentation. Pretreatment with TV 3086 resulted in no improvement in the IVTD despite a notable decrease in the ADF of the material; however, as explained previously in Section 5.1.1.1, there is a poor relationship between the ADF of the forage and the *in vitro* digestibility. In spite of the IVTD not being improved during pretreatment with TV 3086 the digestibility of the forage was still excellent, whilst the crude protein content was also dramatically increased.

The non-fibre carbohydrate concentration was significantly ($p<0.05$) increased by 139 and 161 % with Oyrm 1 and TV 3086, respectively during pretreatment. This increase was likely due to the production of soluble carbohydrates such as glucans during fermentation of the corn leaves as outlined previously in section 5.1.1.1. The pronounced increase in the non-fibre carbohydrate content coincided with a similarly significant ($p<0.05$) increase in the crude protein content of the fermentation. The increase in crude protein content was likely the result of the production of fungal mycelia, indicating that the increased NFC content may also be as a result of the soluble carbohydrates from the mycelia.

It would appear that the leaf fraction underwent more extensive degradation than the stem samples with a more considerable change in the NDF and ADF fractions observed. It is worth noting that the corn leaves contained a lower lignin content in comparison to the other stover fractions, which would have influenced the initial digestibility of the forage and subsequently the degradation of the substrate. Wilman *et al.* (1998) investigated the *in vitro* digestibility of several forage species and determined that rapeseed and corn leaves contained the highest *in vitro* digestibility, whilst the stems from *Desmodium intortium* (tick clover) were the least digestible of the forages examined. Furthermore, Karunanandaa *et al.* (1995) similarly observed that the degree of improvement in the quality of rice leaf was higher than that of the rice stalk when fermented with three strains of white rot fungi (*Pleurotus sajor-caju*, *Phanerochaete chrysosporium* and *Cyathus stercoreus*).

The results obtained in this study from pretreatment of corn stover are predominantly in line with other authors, such as Karunanandaa *et al.* (1992), who determined that the digestibility of the stover was increased through pretreatment with *Cyrathus stercoreus*. In contrast, the author also observed that *Phanerochaete chrysosporium* reduced the *in vitro* digestibility by indiscriminately removing the hemicellulose and cellulose fractions, a trait which was also noted by TV 3086 in the present study. In both cases, the dry matter loss was substantially greater with Oyrm 1 and TV 3086 in comparison to the fungal strains tested by Karunanandaa *et al.* (1992), albeit with an appreciably longer fermentation time. While biological pretreatment has generated an excellent forage in terms of digestibility and crude protein content, from an economic stand point, this fermentative approach may not be suitable due to the degree of weight loss (50 %) observed (Tables 4.7 & 4.8).

5.1.2 Assessment of the forage value of cottonseed hulls pretreated with white rot fungi

Cottonseed hulls are a major by-product of the cottonseed oil and cotton production industry and are available in ample quantities for livestock feed in large parts of the world (Tillman *et al.*, 1954). However, cottonseed hulls are known as a poor quality forage in comparison to traditional grass and hay forages, due in part to their high quantity of indigestible lignin. Bartle *et al.* (1994) fed alfalfa hay and cottonseed hulls at various different percentages to finishing beef cattle and found that the dry matter intake (DMI) was less and the efficiency of gain was greater for cattle fed alfalfa than for cattle fed cottonseed hulls. Additionally, across the forage range, DMI increased at a faster rate for cottonseed hull diets in comparison to alfalfa diets. Despite the high NDF and ADF content of the cottonseed hulls (traits which are usually associated with decreased digestibility), they have been shown to be very palatable and cattle tend to increase their feed intake when supplemented to the diet. Consequently, they could potentially make a more attractive forage feed if the digestibility could be improved through either physical, chemical or biological pretreatment methods (Hall *et al.*, 2000).

Table 5.5 Compositional analyses of cottonseed hulls pretreated for 210 days with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% ESC	% IVTD
Oyrm 1	0	4.9	65.5	86.5	38	18.1	7.8*	1.3	30
	60	7.7*	62.8*	73.3*	76*	13.3*	2.6*	2.5*	36*
	90	8.1*	61*	74.4*	80*	11.4*	2.7*	2.5*	39*
	120	7*	60*	71.6*	80*	8.9*	4.9*	4.4*	44*
	150	9.5*	59.9*	69.4*	82*	8.2*	3.3*	2.9*	48*
	180	7.8*	61.3*	72.6*	83*	7.8*	2.5*	1.9*	41*
	210	8.2*	60.9*	71.4*	85*	6.9*	1.4*	1.1	40*
TV 3086	0	4.9	65.5	86.5	38	18.1	7.8	1.3	30
	60	8.4*	56.9*	64.8*	73*	12.8*	3.4*	3.4*	48*
	90	11.9*	54.5*	62.6*	76*	11.4*	2*	2*	51*
	120	10.3*	62.5*	69.2*	77*	12.9*	1*	0.8*	48*
	150	12.7*	47.9*	53.1*	76*	14.1*	6.8*	2.2*	44*
	180	13.9*	53.7*	60*	77*	11.3*	2.7*	0.9*	41*
	210	13.3*	64.8	78.9*	83*	10.8*	3.5*	3.3*	43*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility

Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

The crude protein content of the cottonseed hulls (CSH) (Table 5.5) was substantially lower than that observed in corn leaves and was more in line with that of corn stalks. Furthermore, the lignin content noted in the cottonseed hulls was considerably higher than any of the other forages tested, with the lignin content found to be nearly four times that of the corn leaves. As a consequence, this affected the NDF which was appreciably higher than the corn stover components and more in accordance with that of corncob. The ADF of the cottonseed hulls was also substantially higher, with it being nearly twice that of the corn leaves. As a result, the IVTD of the cottonseed hulls was extremely low, with them being less than half as digestible as corn leaves.

The crude protein concentration of cottonseed hulls (Table 5.5) was significantly ($p<0.05$) increased during the course of the fermentation, with the content being increased by 93 and 180 % through pretreatment with Oyrm 1 and TV 3086, respectively. Optimal crude protein content of 13.9 % from TV 3086 fermentation was observed after 180 days, with a decrease detected thereafter. The crude protein content

of the TV 3086 fermentation was far in excess of the Oyrm 1 fermentations, where it was found to be 45 % higher than the maximal crude protein content detected from the Oyrm 1 fermentations. The highest rate of increase in crude protein content was observed during the first 60 days fermentation, which was likely due to colonisation of the substrate by the fungus. This increase in crude protein to between 8-13 % resulted in an upgrading of the protein content to the point where it could be considered an average to good forage, with respect to their crude protein content (Lemus, 2009).

The NDF content of the cottonseed hulls was significantly ($p<0.05$) reduced by both fungi, with TV 3086 being the most efficient at reducing the NDF. Nonetheless, these values were still above 65 % after 210 days pretreatment, with an NDF content of above 65 % typically acknowledged to be an extremely poor forage feed, as it limits the intake by the animal thus potentially limiting performance (Coppock, 1997). The NDF levels are very important in dairy cows as it is closely related to chewing activity which can determine the pH of the rumen and as a consequence, dairy diets are usually formulated to a specific NDF value (Oba *et al.*, 1999). Although NDF contents below 40 % are considered ideal for a prime quality forage, NDF in the form of roughage is still an important part of the diet as it helps prevent digestive upset and maximises energy intake by feedlot cattle (Galyean *et al.*, 2003). Despite pretreatment with Oyrm 1, the NDF content of the CSH was still very high and the forage quality was still considered to be poor. By contrast, pretreatment with TV 3086 improved the quality substantially more, with the NDF content being reduced to 53.1 % and now being classed as a good quality forage. Similarly, the ADF content of the CSH was dramatically reduced (18 %) after 150 days through pretreatment with TV 3086, whilst a 5 % reduction was noted by Oyrm 1 pretreatment. However, according to Coppock (1997) both these forages would still be considered poor forages on the basis of their ADF content, despite the removal of a significant ($p<0.05$) portion of ADF content.

Lignin was substantially degraded during both fermentations, with 62 and 40 % removed by Oyrm 1 and TV 3086, respectively. It would appear that the decrease in the ADF content by Oyrm 1 was mostly due to selective removal of the lignin, as minimal cellulose degradation occurred, as previously observed in Table 4.10 (Section 4.1.3), in comparison to the amount of lignin removed. The decrease in ADF by TV 3086 was conceivably due to the degradation of cellulose and lignin, with TV 3086 having previously been shown to non-selectively degrade biomass.

Pretreatment with Oyrm 1 resulted in a reduction in the NFC concentration during the initial 60 days of fermentation. The NFC content was subsequently increased thereafter, which coincided with an increase in the ESC of the fermentation. Similarly, the NFC content of the TV 3086 fermentation was gradually reduced during the first 120 days of fermentation. The NFC content was subsequently increased during the next 30 days, with about half of these soluble carbohydrates being utilised during the next 60 days. It is not fully understood why such an increase occurred during these fermentations, although it coincided with an increase in the ESC, which as noted previously, was due to soluble sugars released from the material as well as soluble carbohydrates from the fungi that would also make of part of the NFC content.

It would appear that the TDN (a measure of digestible energy) was significantly ($p<0.05$) increased during fermentation, to a point where both forages would be considered excellent (Coppock, 1997). The TDN content of the fermented materials was increased by 123 and 118 % through the use of Oyrm 1 and TV 3086, respectively. To put these results into context, alfalfa hay which is regarded as an excellent forage feed, typically has a TDN of 64-71 %, while grass contains between 45-65 % TDN (Ball *et al.*, 2002). In this study a TDN content of 83 % was observed, which is substantially greater than both alfalfa hay and grass.

Optimal IVTD of 48 % was detected after 150 days of fermentation with Oyrm 1, whereas a maximal *in vitro* digestibility of 51 % was observed following 120 days pretreatment with TV 3086. The digestibility of these forages was significantly ($p<0.05$) increased during pretreatment with both fungi, which was conceivably due to the substantial lignin degradation observed during fermentation (Table 5.5). Despite this, these forage would still be regarded as Class 5 forages with respect to their true digestibility following pretreatment (Rivera *et al.*, 2010). A higher *in vitro* digestibility was detected in the forage pretreated with TV 3086, which was surprising considering a higher degree of lignin removal occurred in the Oyrm 1 fermentation. This may be as a consequence of the larger ethanol soluble carbohydrate content available following 210 days of fermentation with TV 3086, which would have been appreciably digestible in the rumen.

It would appear that biological pretreatment would be a viable option to improve the quality of the forage, with the *in vitro* digestibility being increased by up to 70 %, with relatively little loss of dry matter (20.47 % and 14.68 % by TV 3086 and Oyrm 1, respectively) noted during fermentation (Tables 4.9 & 4.10) (Section 4.1.3),

in comparison to other substrates tested. Furthermore, the crude protein content of the forages was significantly increased during fermentation, which would also increase the desirability of the forage as a feed. Yu-Feng *et al.* (2012) investigated the change in composition of cottonseed hulls using a range of edible fungi and determined that the CP was increased by up to 40 %, with NDF being decreased by between 3 and 40 % depending on the fungal strain utilised, whilst the ADF was also reduced by between 17 - 45 %. This was generally in agreement with the data displayed in this present study, where increased crude protein and decreased ADF and NDF contents were observed.

5.1.3 Assessment of the forage value of cottonseed hulls/coconut fibre fermented with white rot fungi

In tropical regions around the world coconut copra and coconut meal have often been used as forage feed in livestock diets (Creswell *et al.*, 1971). However, coconut fibre or coir as it is also known, has not been commonly used as a forage due in part to its high lignin content (~48 %), making it extremely indigestible (Shashirekha *et al.*, 2007). Coconut coir is a vast renewable source in many countries, with an estimated 500,000 metric tonnes being produced every year (Murthy, 2011). Due to the indigestibility of the coir pith, pretreatment techniques are required to produce a more digestible substrate. Although, due to its high lignin content and amorphous powdery nature it supports poor fungal growth and poor biological conversion (Shashirekha *et al.*, 2007). Accordingly, the coir was mixed with cottonseed hulls to promote growth and colonisation of the substrate by the fungi. Cottonseed hulls were used as they had previously been shown to support excellent fungal growth. A ratio of 1 part coconut fibre to 1.4 parts cottonseed hulls was used in each fermentation, with samples being taken every 30 days to determine the compositional change of the substrate through biological pretreatment of the material.

Table 5.6 Compositional analysis of cottonseed hulls/coconut fibre fermentations pretreated with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086 for 210 days.

Fungi	Time (Days)	% CP	% ADF	% NDF	% TDN	% Lignin	% ESC	% IVTD
Oyrm 1	0	6	77.7	81.9	23	32.5	1.2	32
	60	5.9	69.1*	80.1*	40*	19.5*	4.5*	41*
	90	6.8*	69*	79.7*	45*	15*	4.1*	37*
	120	7.4*	68.7*	72.7*	49*	13.7*	4*	43*
	150	7.6*	66.5*	71.2*	55*	9.3*	4.3*	40*
	180	8.7*	65.9*	68.7*	57*	8.6*	5*	48*
	210	8.8*	67.9*	72.3*	51*	12.3*	4.7*	43*
TV 3086	0	6	77.7	81.9	23	32.5	1.2	32
	60	6	72.5*	78.7*	39*	21.2*	3.9*	40*
	90	7.8*	62.8*	65.4*	44*	21*	2.4*	37*
	120	8*	54.8*	56.5*	45*	25*	1.9*	40*
	150	8.1*	68.7*	72.1*	38*	25.5*	3.7*	32
	180	8.2*	53.98*	55.4*	50*	19*	3.1*	33
	210	8.5*	59.3*	60.9*	44*	24.2*	6.8*	40*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility
Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

As expected the lignin content of the cottonseed hull/coconut fibre mixture (Table 5.6) was extremely high at 32 %, equating to over twice the lignin content of the cottonseed hulls alone. This indicates that the lignin content of the coconut coir was far in excess of the cottonseed, at over 40 % of the total weight of the coconut fibre. Similarly, the NDF and ADF content of the mixture were also extremely high due in part to the high lignin content of the mixture.

Removal of a portion of the lignin was achieved during both fermentations. Following 180 days of pretreatment 74 % of lignin was removed with Oyrm 1, whilst 42 % was degraded by TV 3086 over the same time period. As envisaged, the removal of lignin by Oyrm 1 and TV 3086 resulted in a sizable reduction in the NDF content of the material, culminating in a 13 and 25 % decrease in NDF, respectively. The difference in the amount of NDF removed during fermentation was likely as a result of the non-selective removal of cellulose, hemicellulose and lignin by TV 3086 (which was discussed in Chapter 4), whereas Oyrm 1 selectively removed the hemicellulose and lignin fractions leaving the cellulose portion relatively undegraded. Furthermore, the difference in the rate of degradation of the substrate between the two strains may

also have been a factor. Despite a substantial portion of the NDF content being degraded through fermentation by both fungi, the NDF content in the Oyrm 1 fermentation was still indicative of a poor quality forage. Conversely, pretreatment with TV 3086 upgraded the forage to an average classification with respect to the NDF content (Coppock, 1997). The ADF content was also significantly ($p<0.05$) reduced, with 10 and 18 % degraded after 210 days of fermentation by Oyrm 1 and TV 3086, respectively. Despite a notable portion of the ADF was removed from the material, it was still classified as a poor forage.

The crude protein content of the material was marginally increased during both fermentations, although the rate of increase in the protein content was not as dramatic as the increases in protein content noted during fermentation of other substrates i.e. corn leaves and cottonseed hulls (Table 5.4 & 5.5). This may be due to a decrease in the mycelial density due to the presence of coconut fibre in the fermentation. Kanmani *et al.* (2009), investigated the growth of six fungal strains on coir waste and discovered that only two strains (*Rhizopus stolonifer* & *Phanerochaete chrysosporium*) colonised the substrate completely with the other fermentations exhibiting low mycelial density. In this present study it was apparent, through visual examination, that the mycelial density in the mixed substrate fermentation was lower than that of the other fermentations such as corn stover and cottonseed hulls.

The TDN of both fermentations was increased by 32 and 27 % through biological pretreatment with Oyrm 1 and TV 3086, respectively. Consequently, the forage pretreated with Oyrm 1 was upgraded to a good quality forage. On the other hand, despite an increase in the TDN, the forage pretreated with TV 3086 was still classified as a poor fodder. Feeding poor quality forages with lower than 8 % crude protein and below 52 % TDN limits the feed intake of the animal, reducing the overall feed performance (Rivera *et al.*, 2010). Nonetheless, the relatively low TDN content of the two forages still contains sufficient nutrients for a 900 lb dry pregnant mature cow or a 1600 lb bull for maintenance, indicating that it would still be a viable forage feed (Pinkerton *et al.*, 1991).

The ESC content was relatively low during the initial stages of fermentation, which was also the case in the cottonseed hulls fermentation. During fermentation the ESC was increased which is likely, as explained previously in Section 5.1.1.1, due to the release of soluble sugars from the substrate or as a result of the soluble carbohydrates present in fungal mycelia.

The IVTD data was in agreement with the TDN, with maximal *in vitro* digestibility of 48 and 40 % observed after 180 and 60 days of fermentation with Oyrm 1 and TV 3086, respectively. There was a notable increase in the digestibly of the material, with the cottonseed and coconut fibre being considerably more digestible than the untreated material following fermentation. However, these forages would still be regarded as poor despite the increase in digestibility. Typically forages with a digestibility of below 53 %, such as these two pretreated forages, would have a quality ranking of Class 5 (Coppock, 1997).

Shashirekha and Rajarathnam (2007) monitored the bioconversion of coir pith by the white rot fungus *Pleurotus florida* during mushroom growth and determined that the cellulose, hemicellulose and lignin content were reduced during fermentation, results which are in contrast with those displayed in Table 5.6. In the present study, an alternate strain of *Pleurotus spp*. selectively degraded the lignin and hemicellulose in the substrate, which is thought to be more typical of the Pleurotus species.

5.1.4 Assessment of the forage value of switchgrass fermented with white rot fungi

Switchgrass, a perennial C_4 warm season grass native to North America, has been used widely as a forage prior to it being established as an energy crop (Keshwani *et al.*, 2009). Studies examining the nutritive quality of warm season grasses in feeding and grazing determined that despite the relatively low crude protein, dry matter digestibility and high fibre content, they supported high feed intake and good average daily gains (Reid *et al.*, 1992). However, due to the higher fibre content switchgrass would need to be pretreated to extract the maximal potential from the forage. As a consequence, switchgrass was biologically pretreated with two selected strains of white rot fungi to determine the influence that this process would have on the nutritional value of the forage (Table 5.7).

Table 5.7 Compositional analysis of switchgrass pretreated for 210 days with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Fungi	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% IVTD
Oyrm 1	0	6.6	43.0	73.2	56.0	5.9	16.8	46.0
	60	6.5	45.0	73.7	56.0	3.7*	12.6*	62.0*
	90	6.5	48.3*	75.0*	56.0	3.2*	10.5*	57.0*
	120	8.6*	43.3	66.9*	56.0	3.0*	14.3*	66.0*
	150	10.0*	44.4	64.9*	55.0	3.5*	13.8*	70.0*
	180	10.0*	44.1	66.1*	53.5	3.9*	11.6*	64.0*
	210	10.7*	43.2	64.9*	55.5	3.2*	13.0*	66.0*
TV 3086	0	6.6	43.0	73.2	56.0	5.9	16.8	46.0
	60	10.3*	35.9*	67.1*	57.0	4.4*	14.8*	60.0*
	90	8.7*	39.5	67.4*	57.0	4.5*	16.7	65.0*
	120	11.3*	31.3*	64.0*	60.0*	3.7*	16.9	49.0
	150	12.0*	28.9*	62.38	60.5*	3.7*	16.7	51.0*
	180	13.8*	26.4*	58.8*	60.0*	4.2*	17.7	52.0*
	210	16.3*	24.6*	47.9*	57.5	5.5*	22.5*	59.0*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; IVTD: *in vitro* true digestibility
Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

The switchgrass used in the present study contained relatively low crude protein and high cell wall content (Table 5.7), which was consistent with results reported from other authors (Luginbuhl *et al.*, 2000; Lemus *et al.*, 2002). Crude protein content of the switchgrass increased during pretreatment with both fungal genera. The rate of crude protein increase was more pronounced with TV 3086 (Table 5.7), where a 158 % gain was noted after 210 days fermentation. In contrast, the rate of increase in CP through Oyrm 1 fermentation was substantially less, with a 62 % rise observed following 210 days pretreatment, which was equivalent to the increase in crude protein detected after 60 days of fermentation with TV 3086. Switchgrass pretreated with TV 3086 would now be regarded as a Class 2 forage with respect to its crude protein content due to the increase in protein, whilst switchgrass pretreated with Oyrm 1 would be a class 4 forage (Coppock, 1997). The difference in crude protein formation is likely due to protein synthesis by the fungi, whilst the concentration of the protein fraction due to loss of structural carbohydrates may also be a factor. In fungi, the protein content can be affected by a number of factors, namely; the strain of fungi,

the stage of development, the part sampled, the level of nitrogen and the substrate on which it was grown (Longvah *et al.*, 1998). It would appear in this case that the difference in protein content was due to genetic differences between the fungi or the different growth rates of each fungus, as each of the other parameters were constant. Analysis of the complete data set in this chapter indicated that TV 3086 contains a higher protein content than Oyrm 1. This was true in all TV 3086 fermentations with the exception of corn leaves, where the composition of the biomass and the growth rate of the fungi may have impacted on the protein content of the fungal strains.

The NDF content of the switchgrass (Table 5.7) was reduced through pretreatment with both fungi. The greater degree of NDF removal occurred during pretreatment with TV 3086, where 20 % of NDF was removed compared to 9 % by Oyrm 1. Similarly, 18 % of the ADF was degraded in the switchgrass by TV 3086, whilst the ADF content in the Oyrm 1 fermentation remained unchanged at 43 %. This was surprising as the lignin content of the forage was reduced by 50 % during fermentation with Oyrm 1. It is possible that the limited change in ADF content was due in part to an increase in the cellulose content of the substrate during fermentation with Oyrm 1, which was noted previously in Section 4.1.5 (Table 4.13).

The non-fibre carbohydrate concentration was significantly ($p<0.05$) reduced through pretreatment with Oyrm 1, with the lowest NFC content of 10.5 % observed after 90 days of fermentation. Decreases in the NFC content was likely as a result of the utilisation of the readily fermentable sugars by the white rot fungi. In contrast to the Oyrm 1 fermentation, NFC content was increased by 33 % during TV 3086 pretreatment. This increase was possibly due to the soluble carbohydrates such as glucans which are produced during synthesis of the fungal mycelia, as mentioned previously in Section 5.1.1.1. This increase in NFC content also coincides with an increase in the crude protein content, with maximal crude protein production and NFC content observed after 210 days of fermentation. This indicates the soluble carbohydrate from the fungi may have influenced the NFC content.

The TDN of the switchgrass remained relatively unchanged during the fermentation with Oyrm 1, whereas the TDN of switchgrass pretreated with TV 3086 was marginally improved (Table 5.7). Despite the lack of change in composition following pretreatment, the TDN of the forage was still relatively good, with a TDN of over 58 % considered excellent and forages with a TDN of 55-57 % considered good (Lemus, 2009). The *in vitro* digestibility of the material was significantly ($p<0.05$)

improved by both fungal strains, with increases of up to 52 and 41 % observed with Oyrm 1 and TV 3086, respectively. Consequently, the switchgrass pretreated with Oyrm 1 and TV 3086 would be regarded as prime quality, with respect to their digestibility (Rivera *et al.*, 2010). When the dry matter loss of between 28-34 % from the switchgrass (Section 4.1.5) is considered in relation to the improvement in switchgrass digestibility, it was evident that pretreatment of the switchgrass with these fungi may not be a viable candidate for upgrading the material. Alternatively, due to the excellent colonisation of the substrate by *Pleurotus ostreatus Oyrm 1*, oyster mushrooms could be produced from switchgrass and the waste grass fed to cattle as an upgraded animal feed, with the sale of the mushrooms offering an alternate revenue stream to combat the losses due to dry matter degradation (33.59 & 27.53 % by TV 3086 and Oyrm 1, respectively) (Kim *et al.*, 2011a).

5.1.5 Assessment of the forage value of oat straw fermented with white rot fungi

Cereal straws are one of the most abundant agricultural wastes in nature and contribute a major part of ruminant diets in the developing world (Karunanandaa *et al.*, 1995). Similarly, grain straws have become popular in dairy diets as a means of dilution of the nutrient density and assuring adequate fibre in the diet (Anderson *et al.*, 2006). However, due to recalcitrant lignin, silica and phenolic contents, the utilisation of the energy rich cell walls is severely restricted (Karunanandaa *et al.*, 1995). Straws are generally of a low quality, with high fibre and low nitrogen and crude protein contents. Therefore, pretreatment of this substrate could potentially improve the quality of the forage, thus improving animal production.

Table 5.8 Compositional analysis of oat straw pretreated for 210 days with *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Fungi	Time (days)	% CP	% ADF	% NDF	% TDN	% Lignin	% NFC	% ESC	% IVTD
Oyrm 1	0	3.9	48.8	76	55	4.9	14.6	4	31
	60	7.2*	50.4*	71.7*	63*	3.2*	19.7*	1.9*	53*
	90	6*	49.9*	72.3*	60*	5.1	20.3*	2.6*	50*
	120	8*	52.4*	66.3*	60*	3.9*	21.4*	4.8*	54*
	150	7.7*	52.1*	64.3*	60*	5.6	25.6*	2.1*	69*
	180	8.4*	50.6*	61.5*	66*	2.5*	26.6*	4.6*	67*
	210	11.9*	47.7*	62.7*	65*	0.9*	18.8*	4	70*
TV 3086	0	3.9	48.8	76	55	4.9	14.6	4	31
	60	6*	47.6*	78.9	64*	2.7*	15.6	2.2*	34*
	90	6.8*	47.8	75	57	6.1*	17.6*	3.5	49*
	120	6.8*	43*	68.6*	56	7.9*	23.4*	1.7*	56*
	150	6.3*	47.6*	75.1	59*	5.1	17.9*	4.5*	37*
	180	10.6*	38.3*	65.8*	68*	1*	21.7*	2.5*	47*
	210	7.5*	43.9*	72.1	62*	3.9*	19.7*	1.9*	50*

Abbreviations: CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; TDN: total digestible nutrients; NFC: non-fibre carbohydrates; ESC: ethanol soluble carbohydrates; IVTD: *in vitro* true digestibility

Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk (*) denotes a significant change in the composition of the particular fraction in comparison to the control sample.

The composition of oat straw (Table 5.8) was consistent with other authors, who reported the crude protein content to be low and the NDF concentration to be high. However, the TDN of this straw was higher than other oat straws previously evaluated (Anderson *et al.*, 2006). The energy content (TDN) of straws can range from 25 to 55 %, whilst the digestibility of the NDF can be as low as 20 % (Hoffman *et al.*, 2001).

As was noted with other lignocellulosic wastes the CP content was increased during pretreatment, with a maximal crude protein content of 11.9 % observed following 210 days of fermentation with Oyrm 1. This was equivalent to a 200 % increase in the crude protein content in comparison to the control (T0) sample. The increase in crude protein by TV 3086 was moderately lower at 171 %, which was likely as a consequence of the poorer mycelial density of the fermentation. According to Coppock (1997), these hays would carry a quality standard of 3 out of 5, where 1 would be considered an excellent forage, whereas in terms of straw forage it would be considered very high.

Following fermentation with both fungal species, the ADF content was only marginally reduced, with the largest decrease in ADF observed with TV 3086. Despite the minimal change in ADF with Oyrm 1, over 80 % of the lignin was removed from the fermentation, which should have improved the digestibility of the forage. The lack of change in the ADF content was likely due to the apparent increase in the cellulose content noted during fermentation (Section 4.1.6), which would impact on the ADF content. By contrast, the NDF of the oat straw was reduced in both fermentations, suggesting a higher potential intake in animal diets. However, despite the decrease in the NDF fractions, the quality of the forage would still be regarded as relatively fair (Lemus, 2009).

The NFC content of the material was improved through pretreatment, with increases of 82 and 60 % noted by Oyrm 1 and TV 3086, respectively. This increase in the non-fibre carbohydrate content was most likely due to the soluble carbohydrates that are present in the fungal mycelia as well as sugars released during substrate hydrolysis, as explained previously in Section 5.1.1.1.

The TDN of both forages was also increased through pretreatment, with a maximal increase of between 11-13 % detected, depending on the fungal strain utilised. As mentioned previously, the TDN of the untreated straw was in the upper limits of typical TDN contents observed in oat straw; however, pretreatment improved this status to give an excellent quality forage. The IVTD of the Oyrm 1 fermentation was also dramatically improved, with the forage being over twice (70 %) as digestible as untreated material (31 %). Similarly, higher IVTD levels were achieved with TV 3086, although the rate of increase was not as pronounced as with Oyrm 1, which was likely due to the poorer colonisation of the substrate. According to Pinkerton *et al.* (1991), these forages would be sufficient to provide enough energy for steers to gain 1.5 lb per day or pregnant yearling heifers in the last third of pregnancy gaining 1.4 lb per day.

Jung *et al.* (1992) investigated changes in the chemical composition of oat straw after pretreatment with a range of white rot fungi for 30 days. They determined that the total fibre present in the oat straw was reduced by 12 % through pretreatment, whilst the NDF fraction was also decreased. Furthermore, the reductions in lignin were not as substantial as that noted in the present study. This is most likely due to the differences in incubation time between the fermentations. Generally, the results are in agreement with those obtained in this study, where the NDF content of the material

was reduced with significant dry matter loss (53.12 & 57.25 % by TV 3086 and Oyrm 1 respectively) determined throughout the fermentation. The results in this present study also compared favourably with the use of chemical agents such as ammonia and sodium hydroxide which are other common methods used to treat straw to improve the digestibility. Castrillo *et al.* (1995) investigated the pretreatment of barley straw with 30 g kg^{-1} of ammonia as a method of upgrading the forage. The author determined that treatment with ammonia reduced the NDF, ADF and lignin content, although the level of degradation of the straw was lower than that observed in the present study. Similarly, the degree of degradation noted by Madrid *et al.* (1997) through pretreatment with NaOH was substantially less than using biological pretreatment, although losses in dry matter was less considerable using chemical treatment methods.

5.2 General discussion

It is apparent that fermentation with the selected strains of white rot fungi was an effective method for upgrading the nutritional value of a number of the lignocellulosic forages tested. For instance, the IVTD of the oat straw was significantly increased during fermentation, making it a far more attractive forage feed. In fact, in almost all cases through pretreatment with these fungi the *in vitro* true digestibility of the substrate was increased during fermentation. Furthermore, the crude protein content of the material was also improved during fermentation, with TV 3086 typically displaying the most substantial increases in protein content. The difference in the crude protein content of the residues was attributed to the difference in the protein content that can be detected between different fungi during mycelia formation. Maximal crude protein content was observed during pretreatment of the corn leaves with Oyrm 1, where a protein content of 32 % (w/v) was observed following fermentation. In contrast the lowest crude protein levels were noted in the corncob fermentations, in which the maximal crude protein content determined was as low as 2.3 % (w/v) following pretreatment.

Corn leaves were the most digestible substrate prior to biological pretreatment. However, fermentation of the corn leaves resulted in a significant increase in the digestibility of the biomass, with the corn leaves now upgraded to a premium quality forage, following pretreatment. By contrast, corncobs displayed the least significant change in composition, with minimal reduction in the NDF fraction, whilst the ADF and lignin contents were increased. The cottonseed hulls/coconut fibre material was

one of the most indigestible substrates tested, with extremely high NDF (81.9 %) and ADF (78 %) contents noted, whilst the IVTD was also appreciably low. Pretreatment of this material resulted in an increase in the digestibility of the substrate, with both the NDF and ADF contents being significantly decreased during fermentation. Furthermore, the lignin content was also substantially reduced, which would have been one of the major factors leading to the poor digestibility in the untreated forage. Despite this however, the overall digestibility of this forage was still relatively poor, with fungal pretreatment not being considered a viable method for improving the material sufficiently to be considered an appropriate forage feed. By contrast, pretreatment of the oat straw improved the digestibility significantly, where an increase in the IVTD of over 2-fold was noted during pretreatment with Oyrm 1, thus significantly improving the quality of the forage.

In general, TV 3086 tended to have most influence over on the ADF and NDF content of the forage in comparison to the Oyrm 1, with a greater reduction in both fractions determined. The opposite was true for the IVTD however, in which pretreatment with Oyrm 1 had the most significant impact on the true digestibility of the majority of forages tested. In terms of digestibility of the forage, the IVTD method tends to be a more accurate method of digestibility evaluation as it is determined using rumen fluid and closely mimics what occurs in the rumen. It can therefore be concluded that pretreatment with Oyrm 1 would be a more effective method of increasing the digestibility of the substrate compared to TV 3086. However, pretreatment of the biomass with TV 3086 would be the most suitable method of increasing the crude protein content of the feed, with dairy cattle requiring a substantial crude protein content, as the higher the crude protein of the feed, the greater the milk yield. In conclusion, it was determined that biological pretreatment was an effective pretreatment method for improving the digestibility and crude protein content of a number of these forage samples, although the rate improvement was strongly dependent on the composition of the forage as well as the fungal strain utilised for pretreatment.

6. Antioxidant production

6.1 Antioxidant production by solid state fermentation

Oxidation is an essential function in many living organisms that provides energy for numerous fundamental processes (Yang *et al.*, 2002). Despite humans possessing antioxidant defence systems themselves, these systems are often inefficient and do not prevent damage entirely (Simic, 1988). Consequently, antioxidant supplements from fruits, vegetables, grain crops and beverages are required to reduce the damage caused by oxidation which can lead to aging, atherosclerosis, diabetes and cancer (Halliwell *et al.*, 1990). At present, the majority of antioxidants produced for use in foods are chemically synthesised due to the cost and ease of production. However, due to perceived carcinogenic effects of these chemically produced antioxidants, their use is being limited by legislation (Sultana *et al.*, 2007; Li *et al.*, 2012a). As such, research has shifted to producing antioxidants in large scale via more natural methods (Cheung *et al.*, 2003).

Antioxidants produced through solid state fermentation of low cost lignocellulosic waste materials are an attractive prospect due to the lower cost and relative ease of production. The most commonly used fungi for the production of antioxidants are white rot fungi which have been reported to produce antioxidants, mainly in the form of phenolic compounds (Vaz *et al.*, 2011a).

Agricultural waste residues have also been demonstrated to contain antioxidant properties, with phenolic compounds being measured in corn stover (Dong *et al.*, 2011), cotton (Lege *et al.*, 1995), corncob, rice bran, wheat husk (Sultana *et al.*, 2008) and wheat straw (Arora *et al.*, 2011). Lignocellulosic waste materials such as corn stover and switchgrass offer enormous potential as a sustainable energy source; however, the production of this fuel may hinge on the potential to extract high value co-products from these materials due to the high costs associated with lignocellulosic ethanol production. The extraction of antioxidants following biological pretreatment offers an alternate revenue stream to the bioethanol industry to achieve a more economically feasible process without the need for Government subsidies. Currently, production of lignocellulosic ethanol has struggled to be competitive with fossil fuels due in part to the cost of the fermentation substrate and recovery process (Lau *et al.*, 2004). A non-hazardous,

non-toxic, inexpensive solvent, which would not interfere in the production process, would be most suitable for the extraction process. To address this, water was used as the extraction solvent which would allow a straight forward extraction process prior to the hydrolysis of the biologically pretreated material. Additionally, this would avoid the use of harsher pretreatments such as hot water extraction which are typically more costly. One drawback of this method would be the loss of residual sugars from the fermentation medium through the extraction process which may impact on the ethanol production from the biomass. However, due to the relatively low cellulase and hemicellulase titres produced from white rot fungi, the residual sugar loss may not be significant.

The antioxidant potential of biologically pretreated residues was determined using a number of assays, including assays to measure the total phenolic content, reducing power, β-carotene bleaching and radical scavenging activity (using the DPPH and $ABTS^{+}$ assays). Particular attention was paid to the total phenolic content of the samples, which was thought to make up the majority of the antioxidant potential of these fermentations (Eraso *et al.*, 1990). Antioxidant activity was monitored during solid state fermentation of the lignocellulosic residues, with samples taken every 30 days and analysed for antioxidant activity using the methods described previously in Section 2.2.7. The samples in this study were assessed on a weight equivalent basis to determine the antioxidant capacity of each fermentation time point.

The β-carotene bleaching assay described previously in Section 2.2.7.2, examined the ability of an antioxidant to inhibit bleaching of a β-carotene substrate in an emulsion through donation of hydrogen atoms, where the higher the percentage activity obtained, the greater the bleaching inhibition. The reducing power (Section 2.2.7.3), measures the capacity of an antioxidant to reduce a molecule from a higher to a lower valency state, with the higher the percentage activity achieved the greater the reducing potential of the sample.

Positive controls of 1 mg mL^{-1} β-carotene and α-tocopherol were included in three assays (β-carotene bleaching, reducing power and DPPH radical scavenging assay) to compare the samples to known concentrations of common antioxidants. In these three assays, the activity of the sample was expressed as a percentage of a 1 mg mL^{-1} solution of BHT. The total phenol content (Section 2.2.7.4), which measured the phenolic antioxidants in the solution, was determined using a standard curve of

gallic acid and was expressed as gallic acid equivalents (GAE), where the gallic acid standard curve was prepared using the method described in Section 2.2.7.4. The concentration of phenolic compounds in the sample was expressed in milligrams of phenol per gram of dry matter of each sample. The $ABTS^+$ radical scavenging activity (Section 2.2.7.6) was expressed as the trolox equivalent antioxidant capacity (TEAC), where a standard curve of trolox was constructed, as described in Section 2.2.7.6. The DPPH (Section 2.2.7.5) and $ABTS^+$ assays monitor the ability of the antioxidant to scavenge free radicals from the liquid, where the higher the percentage activity achieved, the greater the antioxidant's ability to scavenge radicals. No positive controls of BHT and α-tocopherol were included in the $ABTS^+$ scavenging and phenol assay. Standards curves of gallic acid and trolox, which are known as potent antioxidants, were sufficient as reference standards for the $ABTS^+$ scavenging assay and the total phenol assay.

There is no one test to determine the capacity of an antioxidant as each substance may operate differently during each set of test conditions. For instance, α-tocopherol is an excellent radical scavenger but a poor antioxidant against lipid peroxidation (Niki *et al.*, 2000). Therefore, to determine the antioxidant activity it is best to measure the capacity of the antioxidant under a number of different conditions to fully understand the antioxidant potential of the sample.

6.1.1 Antioxidant production through fermentation of corncobs with white rot fungi

Due to the vast quantity of corncobs available after harvesting of corn kernels, researchers have investigated the potential for producing high value compounds from this waste material. As a result of the high concentration of lignin and carbohydrates found in corncob, antioxidants are typically found in abundance, with phenolic compounds being the predominant form (Buxton *et al.*; Eraso *et al.*, 1990). The two most common phenolic compounds found in the cell walls of plants are ferulic and coumaric acids, which are covalently linked to polysaccharides by ester bonds and to lignin by either ester or ether bonds (Scalbert *et al.*, 1985). Topakas *et al.* (2004) investigated the antioxidant potential of corncob and determined that it was one of the most promising residues for the production of ferulic acid, containing significantly more than other agricultural residues such as wheat straw, maize bran and wheat bran, while high concentrations of coumaric acids were also determined.

Consequently, corncobs were biologically pretreated with white rot fungi to evaluate whether the antioxidant potential of the corncob residues could be improved through solid state fermentation (Table 6.1).

Table 6.1 Antioxidant activities produced from corncobs fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	16.65	20.93	49.63	41.58	1.24
Oyrm 1	60	18.29	23.54	25.29*	6.69*	1.30*
	90	40.04*	22.36	24.21*	8.21*	1.36*
	120	59.82*	28.00	30.11*	6.25*	1.43*
	150	22.06	27.32	28.70*	8.63*	1.38*
	180	17.96	26.63	29.58*	10.69*	1.49*
	210	23.83	32.72*	32.37*	11.91*	1.51*
Control	0	16.65	20.93	49.63	41.58	1.24
TV 3086	60	34.49*	21.65	53.24*	3.88*	1.41
	90	49.24*	21.58	55.77*	3.66*	1.48*
	120	73.30*	32.20*	71.96*	5.46*	1.56*
	150	77.77*	18.32	76.93*	8.78*	1.56*
	180	66.22*	26.69	82.62*	10.34*	1.78*
	210	61.01*	23.59	77.56*	12.01*	1.81*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: RA; relative activity

Analysis of the data from Table 6.1 indicated that the untreated corncob material contained moderate quantities of antioxidants prior to fermentation with white rot fungi. Through pretreatment the total phenolic content of both fermentation substrates was significantly ($p<0.05$) increased with both fungal strains, where the maximal increase in phenol content was observed following 210 days of fermentation. Pretreatment with TV 3086 resulted in a more pronounced increase in the phenol content in comparison to fermentation with Oyrm 1, with an increase of 0.57 mg g^{-1} (45 %) observed. The difference in phenolic content detected between the fermentation of Oyrm 1 and TV 3086 may be due to increased fungal density,

where the fungi colonised the substrate at a higher rate. Alternatively, this difference may be as a result of a higher concentration of phenolic compounds produced by TV 3086 in comparison to Oyrm 1. Palacios *et al.* (2011) investigated the phenolic content of a number of different edible mushrooms and determined that *Boletus edulis* contained significantly more phenolic compounds than the other strains tested, whilst it also contained over twice the amount of phenolics compared to *Pleurotus ostreatus*. Alternatively, the increase in phenolic content may be as a result of the mobilization of phenolic antioxidants through the hydrolysis of the lignocellulosic material by ligninolytic enzymes from white rot fungi (McCue *et al.*, 2005). Phenolic compounds exists as a number of different subclasses that include flavonoids, phenolics, stilbenes, lignins, tannins and polyphenols, which display a large diversity of structures (Ferreira *et al.*, 2009). The main phenolic compounds found in mushrooms tend to be phenolic acids, with hydroxybenzoic acids and hydroxylcinnamic acids being the two most important groups found in fungi (Ferreira *et al.*, 2009).

A fraction of phenolic compounds may also have been removed from the solution through the action of enzymes produced by white rot fungi. The phenolic compounds may have been oxidised by H_2O_2 using peroxidases to produce phenol radicals that can subsequently react to form polymers which are insoluble in water (Klibanov *et al.*, 1983). Furthermore, phenol oxidases can also catalyse oxidative coupling reactions of phenol compounds without the requirement for H_2O_2 (Atlow *et al.*, 1984). Dinis *et al.* (2009) investigated the modification of wheat straw with a range of white rot fungi and found that ρ-coumaric and ferulic acid composition was reduced during fermentation, through the action of ligninolytic enzymes. These phenolic compounds and have been shown to exhibit considerable antioxidants potential during testing (Dinkova-Kostova *et al.*, 1998; Stražišar *et al.*, 2008). Consequently, it may be hypothesised that during fermentation, extracellular enzymes produced from the white rot fungi may have degraded a portion of the soluble phenolic compounds, which may otherwise have resulted in a higher phenolic content in the samples during fermentation than that measured.

The β-carotene bleaching assay is a colorimetric assay based on the autoxidation of carotenoids through the formation of conjugated diene hydroperoxides by linoleic acid oxidation which is induced by light, heat or peroxyl radicals. This bleaching is inhibited by the presence of antioxidants that donate

hydrogen atoms to quench the radicals (Prior *et al.*, 2005; Tepe *et al.*, 2005). As can appreciated from Table 6.1, β-carotene bleaching inhibition was substantially increased, with maximal activity obtained following 120 and 150 days fermentation with Oyrm 1 and TV 3086, respectively. Pretreatment with TV 3086 produced the highest antioxidant activity (78 %) relative to BHT, which was used as the reference standard. BHT is a chemically synthesised phenolic antioxidant that is commonly used in food stuffs to prevent spoiling. A solution of α-tocopherol, which is a form of vitamin E and is known to be a potent antioxidant even at low concentrations, was also used as a standard (Lambert *et al.*, 1996). Whilst the antioxidant activity (as represented by the bleaching inhibition) was not as powerful as the 1 mg mL^{-1} solutions of both positive controls, the maximal relative bleaching inhibition achieved through fermentation with TV 3086 for 150 days, was still quite high. By contrast, maximal activity of 60 % was observed following 120 days of fermentation with Oyrm 1, which constituted a 18 % reduction in antioxidant activity in comparison to TV 3086 pretreatment. It is also worth noting that the bleaching inhibition of the Oyrm 1 fermentation extracts reduced considerably after maximal bleaching inhibition was achieved, which was not the case with the TV 3086 fermentation. This reduction of the antioxidant activity of the sample is likely due to the degradation of the soluble phenolic compounds that can occur by ligninolytic enzymes such as lignin peroxidase, as mentioned earlier in this Section.

The DPPH assay measures the ability of the antioxidants to scavenge free radicals prior to them attacking biologically essential molecules through donation of a hydrogen atom (Niki, 2010). It was determined during analysis (Table 6.1) that both fermentations achieved relatively low radical scavenging activity in comparison to the control standards of 1 mg mL^{-1} BHT and α-tocopherol. Despite this, a modest 11-12 % increase in activity was observed during fermentation with both Oyrm 1 and TV 3086. Maximal relative DPPH scavenging activity was achieved after 120 days fermentation with TV 3086, whilst it took 210 days for the Oyrm 1 fermentation to achieve the same level of scavenging activity. It is conceivable however, that the DPPH scavenging activity observed after 120 days fermentation with TV 3086 was an outlier as it was significantly larger than the activities observed in the rest of the samples.

Antioxidants can often act as potent reductants, therefore the reducing power was measured based on the capacity of the solution to reduce the ferric ion Fe (III) to

a lower valency state (Niki, 2010). The assay monitors the presence of reductants which exert antioxidant action on the ions, breaking the free radical chains through donation of a hydrogen atom (Jayakumar *et al.*, 2011). However, the use of ferric ions to monitor antioxidant activity can lead to an underestimation of the antioxidant potential of the substrate as some antioxidants such as thiols are not detected using this method and as such should be used in conjunction with other methods (Prior *et al.*, 2005). It was evident that the antioxidants produced from TV 3086 (Table 6.1) are more potent reducing agents in comparison to those produced from the Oyrm 1 treatment. For instance, the maximal relative reducing potential noted in extracts from TV 3086 fermentations was 2.8 times that of the equivalent Oyrm 1 sample, whilst they were also found to be a more potent reductant than a 1 mg mL^{-1} solution of α-tocopherol. Optimal relative reducing power was achieved after 180 days of pretreatment by TV 3086 (83 %), whilst maximal relative reducing activity from Oyrm 1 (32 %) was obtained after 210 days of fermentation. The reducing power in the TV 3086 fermentation was increased during the first 180 days of fermentation, with a marginal decrease detected following this. By contrast, the relative reducing power of the Oyrm 1 material was decreased by 24 % over the initial 60 days of fermentation, with the activity being marginally increased thereafter. This decrease in the reducing power during the first two months of fermentation may be as a result of indiscriminate degradation of the phenolic compounds present in the lignin substrate by peroxidase enzymes (such as lignin peroxidase, manganese peroxidase and laccase) that are produced by the white rot fungi. Laccase is a dimeric or tetrameric glycoprotein which is responsible for catalysing both phenolic and non-phenolic lignin related compounds (Rodríguez Couto *et al.*, 2006), whilst lignin and manganese peroxidases function by catalysing the reduction of phenolic substrates and as such can hydrolyse the phenolic antioxidants, thus inactivating them (Lee, 1997), as mentioned previously in this section.

The $ABTS^+$ radical scavenging assay is another method for measuring the scavenging activity of the antioxidant and is based on the generation of blue/green $ABTS^+$ molecules which can be reduced by antioxidants. This assay was also used to determine the scavenging activity of the samples as an alternative to the DPPH assay. During the initial stages of fermentation the $ABTS^+$ scavenging activity was substantially reduced, with 34.89 and 37.7 μg mL^{-1} trolox equivalents (TE) being lost during the first 60 days of treatment with Oyrm 1 and TV 3086, respectively. This

was equivalent to between 84 – 91 % scavenging activity being lost during the initial 60 days. However, as the fermentation proceeded the trolox equivalent antioxidant capacity (TEAC) of both fermentations was increased, with an activity of ~ 12 μg mL^{-1} TE obtained by both fungi. Despite this the scavenging activity of the substrate was still well below that of the untreated material.

Kim *et al.* (2012), investigated antioxidant production from the fermentation of corncob with *Pleurotus ferulae* and concluded that the main antioxidants produced from this fungal strain were phenolic compounds, with minimal quantities of other antioxidants detected, whilst no β-carotene was observed at all. In each of the samples assayed, reducing power and DPPH radical scavenging activity was detected, whilst the total phenol content was also measured, which was in agreement with the results obtained in this study.

Despite research available on the antioxidant capacity of different lignocellulosic residues and mushrooms, it can be difficult to compare the antioxidant capacity between different laboratories. Various research groups have used widely different protocols as well as a wide variety of methods to determine the antioxidant capacity. For example, Sharma and Bhat (2010) analysed a number of different procedures for the DPPH assay and determined that the concentration of DPPH varied widely among the different laboratories (20-250 mg mL^{-1}), whilst the reaction time was also vastly different. Furthermore, it can be difficult to compare the relative antioxidant activity of different materials due to the different methods of extraction that are employed resulting in different yields. For instance, Kim *et al.* (2012), investigated the scavenging activity of mycelia and fruiting body extracts using a number of different extraction techniques. They determined that the hot water extraction technique yielded almost twice as much antioxidant as the other techniques investigated, with ethanol proving to be the next best extraction method, whilst chloroform was the least effective. In addition, Cheung *et al.* (2003) compared a number of different solvents for the extraction of antioxidants from two common mushrooms and found methanol to be the most efficient extraction solvent. They also determined that the yield of mushroom extract from water extracts was approximately half of that recovered using methanol extraction. Similarly, Yu *et al.* (2005a) determined that water extracted approximately 63 % of the phenolics from peanut hulls when compared to ethanol and methanol extraction techniques. Despite the increased activities that can be achieved through changing the extraction

procedure, the present study was primarily carried out to examine the increase in activities through biological pretreatment and as such the investigation into different extraction procedures was beyond its scope.

6.1.2 Antioxidant production through fermentation of corn stover fractions with white rot fungi

Lignocellulosic biomass has the potential to provide a promising alternative to traditional sources of antioxidants. Corn stover for instance, contains significant levels of recalcitrant lignin which is recognised as possessing antioxidant and antimicrobial properties (Lu *et al.*, 1998). The antioxidant capacity of lignin depends on a number of factors such as concentration and type of material used, method of extraction, pretreatment method utilised, and oxidation conditions employed (Egüés *et al.*, 2012). For example, García *et al.* (2010) studied the characteristics of lignin obtained from different miscanthus grass fractions and determined that lignin extracted using an organosolv process exhibited the highest antioxidant capacity.

In this study the effect of pretreatment of corn stover fractions on the production of antioxidant activity with relation to fermentation time was determined (Tables 6.2-6.4). Each fermentation was monitored throughout the pretreatment with samples collected every 30 days and extracted in deionised water. The antioxidant capacity of each solution was tested using the 5 antioxidant procedures outlined in Section 2.2.7.

6.1.2.1 Corn stover upper stem

The antioxidant capacity of the upper stem of corn stover, which consisted of all herbage above the fifth node, excluding the leaf blades, husks, silks rachis and grains, was determined. This material was subsequently fermented with strains of white rot fungi and the antioxidant activity of the pretreated biomass was compared to that of the untreated material (Table 6.2).

Table 6.2 Antioxidant activity produced from corn stover upper stem fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	52.78	ND	172.31	43.31	17.04
Oyrm 1	60	61.36	ND	219.81*	39.11*	15.05*
	120	49.10	ND	234.61*	38.27*	14.97*
	150	32.27*	ND	102.04*	26.46*	15.08*
	180	79.29*	ND	161.55	36.35*	18.05*
	210	23.62*	ND	110.93*	27.34*	15.03*
	240	7.60*	ND	93.11*	27.86*	17.16
Control	0	52.78	ND	172.31	43.31	17.04
TV 3086	60	1.77*	ND	234.00*	36.35*	15.61*
	120	8.04*	ND	190.10	37.23*	17.90*
	150	3.35*	ND	176.10	41.82*	18.06*
	180	2.64*	ND	112.60*	33.44*	15.53*
	210	0.00*	ND	68.78*	33.59*	15.00*
	240	0.00*	ND	81.84*	34.48*	17.64*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: ND; not detected, RA; relative activity

Maximal reducing power, which was almost 2.5 times greater than that of the BHT standard, was observed after 60 and 120 days of fermentation with TV 3086 and Oyrm 1, respectively, with the reducing power being depreciated thereafter (Table 6.2). The upper stem of the corn stover contained a reasonably high initial relative reducing power (172 %), with the relative activity being significantly more than observed in the untreated corncob fraction. Antioxidant capacity can vary significantly among different parts of the plant, with Kähkönen *et al.* (1999) observing different antioxidant capacities between the leaf, phloem, cork, bark and needle of various trees analysed. In each case, the extracts from both fermentations appear to be powerful reductants, with the activity of both these fermentations being greater than a 1 mg mL^{-1} solution of both BHT and α-tocopherol, which are potent antioxidants.

No radical scavenging (DPPH) activity was observed during fermentation with Oyrm 1 and TV 3086, despite scavenging activity being measured in the two positive control samples of BHT and α-tocopherol. Scavenging activity of ~69 % (99-100 % relative to the BHT control) observed for a 1 mg mL^{-1} solution of each of these antioxidants indicated that antioxidant detection in the samples was not related to the assay conditions used. The inability of this assay to detect radical scavenging activity may be as a result colour interferences from the samples, which can lead to an underestimation of the sample (Teow *et al.*, 2007). Plant extracts can contain several interfering coloured compounds which can interfere in the absorbance of DPPH resulting in the failure to detect small changes in the absorbance of the DPPH (Bhandari *et al.*, 2010).

Maximal phenol content of 18 mg g^{-1} dry matter was observed following 150 and 180 days of fermentation with TV 3086 and Oyrm 1, respectively. This constituted an increase of ~1 mg g^{-1} in the phenolic content detected in the untreated material, which was found to be a statistically significant ($p<0.05$). During the first 60 days of fermentation however, the phenol content of the substrate was reduced by between 1.43 (9 %) and 2 mg g^{-1}(12 %) by TV 3086 and Oyrm 1, respectively. This variation in phenolic content during the fermentation is likely as a result of hydrolysis of the phenolic compounds by the ligninolytic enzymes. These enzymes are produced by the fungi to hydrolyse the lignin in the substrate; however, they may also impact on the total phenol content measured as they have been shown to efficiently degrade phenolic compounds, such as ρ-coumaric acid and ferulic acid (Dinis *et al.*, 2009). Jurado *et al.* (2009) monitored the removal of phenolic compounds from steam exploded straw using laccase from *Trametes villosa* and *Coriolopsis rigida*. The authors found that the total phenolic content of the straw was reduced by over 60 %, when concentrations of laccase were added to the material.

β-carotene bleaching inhibition was increased during the initial 60 days of pretreatment with Oyrm 1; however, as the fermentation proceeded the activity was decreased, with activities as low as 7.6 % of the maximal observed. Maximal inhibition was noted after 180 days pretreatment which was 27 % higher than that of the untreated material. With respect to the TV 3086 fermentation, the antioxidant activity was significantly ($p<0.05$) reduced, with minimal activity (1.77 %) observed after 60 days of fermentation, whilst no activity was detected following 210 days.

This indicated that any antioxidants that were present in the substrate following pretreatment were not effective antioxidants in an emulsion. The β-carotene bleaching inhibition of the Oyrm 1 fermentation tended to fluctuate during pretreatment of the substrate. This was also observed by Abbasi *et al.* (2007), who monitored the antioxidant capacity of pistachio hulls pretreated with *Phanerochaete chrysosporium*. The authors determined that the phenolic content and the DPPH scavenging activity fluctuated throughout the 20 days fermentation of the pistachio hulls, which they attributed to the release of antioxidants from the substrate as well as the degradation of the phenolic compounds by the ligninolytic enzymes.

As a result of the limited scavenging activity detected using the DPPH assay, an $ABTS^{+}$ radical scavenging assay was also used to give an indication of the scavenging activity of the water extracts from the fermented material. From Table 6.2 it can be determined that in both fermentations, the scavenging activity of the material was significantly reduced ($p<0.05$) during pretreatment, although the rate of reduction in activity was not as pronounced as that detected with corncobs (Table 6.1). Pretreatment with Oyrm 1 resulted in a decrease of 16 $\mu g\ mL^{-1}$ in the scavenging activity of the samples, whilst fermentation with TV 3086 led to a 9.87 $\mu g\ mL^{-1}$ reduction in the TEAC of the biomass. It was unexpected to note that the TEAC of the upper stem was decreased to such an extent during pretreatment considering white rot fungi are known producers of scavenging antioxidants in the form of phenolic compounds (Cai *et al.*, 2006). The reduction in the scavenging activity may be as a result of the degradation of the phenolic compounds in the lignin by ligninolytic enzymes (Guiraud *et al.*, 1999; Rodríguez *et al.*, 2004), which may result in the inactivation of the antioxidant compounds.

Xiang *et al.* (2012) monitored the production of extracellular phenolic compounds through submerged fermentation of corn stover by *Inonotus obliquus*. They observed an increase of 100 $mg\ l^{-1}$ during a 192 hour fermentation, which is a marked increase in phenolic content when compared to the accumulation in phenolic compounds presented in this study. It is worth noting; however, that the basal medium used by Xiang *et al.* was optimised for fungal production. Various essential nutrients were added to the fermentation, which took place under submerged conditions. This was in contrast to the fermentation set up used in this present study, in which solid state fermentation of lignocellulosic waste materials was carried out, whilst no additional nutrients were added to the substrate.

6.1.2.2 Corn stover lower stem

The untreated lower stem of the corn stover was as also evaluated for antioxidant activity. These samples were subsequently compared to the lower stem of the stover pretreated with TV 3086 and Oyrm 1 to determine whether solid state fermentation could influence the antioxidant capacity of the material (Table 6.3).

Table 6.3 Antioxidant activity produced from corn stover lower stem fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	92.29	ND	156.24	43.10	16.67
Oyrm 1	60	1.06*	ND	108.31*	29.15*	17.16*
	120	0.00*	ND	174.19*	37.20*	21.00*
	150	7.44*	ND	88.79*	28.34*	17.88*
	180	4.69*	ND	85.71*	35.88*	17.89*
	210	0.89*	ND	68.65*	33.02*	15.88*
	240	3.56*	ND	63.82*	28.81*	17.58*
Control	0	92.29	ND	156.24	43.10	16.67
TV 3086	60	7.79*	ND	125.73*	30.35*	19.22*
	120	1.33*	ND	147.20*	39.75*	20.00*
	150	4.53*	ND	142.74*	41.22*	17.69*
	180	4.85*	ND	147.48*	36.47*	16.79
	210	17.96*	ND	127.02*	30.54*	17.21*
	240	12.48*	ND	75.23*	36.82*	16.89*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: ND; not detected, RA; relative activity

As evidenced in Table 6.3, the initial total phenol content of the lower portion of the corn stalk was marginally lower in comparison to the upper portion of the stem. In addition, the reducing power of the lower corn stalk was also approximately 10 % lower than the reducing power detected in the upper stem. By contrast however, the inhibition of β-carotene bleaching by the lower stalk was 40 % higher in comparison to the upper stalk.

No DPPH scavenging activity was detected when both fungi were grown on the lower portion of the corn stover, which was unexpected considering phenolic compounds from lignin, have been shown to be effective radical scavengers (Piccinelli *et al.*, 2007). However, this lack of scavenging activity may be due to the inadequacies of the assay in which the scavenging activity could not be detected due to interfering compounds in the sample extract, as explained previously in Section 6.1.2.1. Furthermore, the limited DPPH scavenging activity detected may be due to the limits of detection of the assay, where the activity of the sample was too low to be detected. Katsube *et al.* (2004), analysed the antioxidant capacity of a number of different plant products, and found that the DPPH scavenging activity of numerous samples were below the limits of detection of the assay despite, antioxidant activities being detected using two other methods.

The total phenol content of each biomass was increased during the first 120 days, with an increase of 4.33 (25 %) and 3.33 mg g^{-1} (19 %) observed through fermentation with Oyrm 1 and TV 3086, respectively. The total phenol content subsequently decreased thereafter, although the lowest total phenol content detected was not below that of the concentration observed in the untreated material. It is likely that the initial increase in the total phenol content was due to the growth of the white rot fungi on the substrate, where phenolic compounds were produced during this cultivation. Furthermore, the mobilisation of phenolic compounds through the degradation of the lignin by ligninolytic enzymes may also play a role (Section 6.1.1.). The subsequent decrease in the phenol content (Table 6.3) during pretreatment is likely due to degradation of the phenolic compounds by the ligninolytic enzymes produced. This decrease in the total phenol content coincided with the reduction in lignin content observed during fermentation of the lower stem with both fungal strains (Section 4.1.2.1), where the ligninolytic enzymes degraded the phenolic polymers in the biomass. Ferreira *et al.* (2002) evaluated the phenolic composition of Portuguese pear and determined that after sun drying the concentration of the phenolic compounds caffeoylquinic acid and catechin was reduced. The authors attributed this to the degradation of the phenolic compounds by the oxidative enzyme polyphenol oxidase.

The reducing power was decreased during the initial 60 days of fermentation with both Oyrm 1 and TV 3086. This however, was in direct contrast to the results obtained from the upper stem treatments, where the reducing power was significantly

($p<0.05$) increased over the same period. Nonetheless, following the initial 60 days of fermentation, the reducing power of the material was subsequently increased, with the relative antioxidant capacity after 120 days of pretreatment with Oyrm 1 being 18 % higher than the relative reducing power of the untreated control material. In contrast, despite an increase in the reducing power of the TV 3086 fermentation after 60 days, the antioxidant potential after 240 days was still lower than that of the untreated material. The maximal activity of 147 % detected from the extracts of the TV 3086 fermentation was still 9 % less than the relative activity of the untreated control sample. During both treatments the final reducing power was considerably lower than the control, with the extracts from 210 days of fermentation containing between 48-59 % less activity than the control samples. When this activity is correlated to the lignin content of the sample (which contains the phenolic compounds), the reduction in the reducing power coincided with a significant reduction in the lignin content. Furthermore, a correlation was also observed between the maximal reducing power and optimal total phenol content from extracts of the Oyrm 1 fermentation, where optimal activity was achieved after 120 days in both cases.

The inhibition of β-carotene bleaching from extracts of the lower stem was relatively good, with a relative activity of 92 % detected in the untreated sample. This was appreciably higher than the upper portion of the corn stalks, where the relative activity was 53 % of the BHT standard solution. However, during pretreatment with both strains the activity was substantially reduced with less than 7.5 % inhibition being observed in the Oyrm 1 fermentation extracts. During pretreatment with TV 3086 the bleaching inhibition was also significantly ($p<0.05$) reduced during the first 180 days. Despite this, the inhibition was marginally increased thereafter, although this bleaching inhibition was still considerably lower than the antioxidant activity of the untreated material. This decrease in the bleaching inhibition indicated that the majority of antioxidants found in the extracts following pretreatment performed poorly in emulsions.

Akin to the decrease in $ABTS^{+}$ radical scavenging activity observed in the upper stem, the scavenging activity of the extracts from lower stem biomass was also decreased through pretreatment with both TV 3086 and Oyrm 1. Pretreatment with Oyrm 1 resulted in a decrease of 14 $\mu g\ mL^{-1}$ trolox equivalents after 60 days pretreatment; although, as the fermentation proceeded the activity was increased

again. This was most likely as a result of phenolic compounds being produced by the white rot fungi, which as mentioned previously are potent radical scavengers. The scavenging activity was decreased again during the final stages of pretreatment, with the final TEAC of 28.81 μg mL^{-1} detected following 240 days of fermentation being significantly ($p<0.05$) lower than the activity observed in the untreated corn stem. The reduction in TEAC noted during treatment with TV 3086 was not as substantial as that determined through fermentation with Oyrm 1, which was in contrast to the results obtained through pretreatment of the upper stem. During the first 60 days of fermentation with TV 3086 the TEAC was decreased by 13 μg mL^{-1}; however, as the fermentation continued the activity was increased. Maximal TEAC of 41 μg mL^{-1} was measured after 150 days pretreatment, which was relatively close to the value obtained with the untreated material, although it was still marginally below this value. These fluctuations in the scavenging activity were also observed by McCue *et al.* (2005) who examined the mobilisation of phenolic antioxidants through the solid state fermentation of soybean with *Lentinus edodus*. The authors determined that the phenolic content and DPPH scavenging activity fluctuated substantially during a 25 cultivation period due to the degradation of the phenolic compounds by the ligninolytic enzymes and the mobilisation of the phenolics during substrate hydrolysis.

It is apparent that this method of pretreatment may be suitable for use as a forage feed in which the material has been upgraded due to an increase in the digestibility of the forage, whilst also retaining the same (if not higher) level of antioxidant activity as the untreated material. Alternately, the antioxidants may be extracted from the material after pretreatment with this material subsequently being used as a substrate for ethanol production.

6.1.2.3 Corn leaves

The antioxidant capacity of the corn leaves were evaluated in relation to their scavenging activity, total phenolic content, reducing power and performance in an emulsion. These residues were subsequently compared to biologically pretreated corn leaves to determine the change in antioxidant capacity during fermentation (Table 6.4).

Table 6.4 Antioxidant activities produced from corn leaves fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	41.69	ND	77.46	44.65	12.36
Oyrm 1	60	19.21*	ND	97.85*	42.29*	13.90*
	120	50.76*	ND	77.68	35.32*	14.02*
	150	29.90*	ND	84.00*	29.95*	14.23*
	180	27.47*	ND	81.54	25.82*	15.21*
	210	34.49*	ND	80.69	18.53*	14.24*
	240	19.44*	ND	177.07*	18.73*	16.13*
Control	0	41.69	ND	77.46	44.65	12.36
TV 3086	60	20.87*	ND	97.55*	34.65*	13.80*
	120	15.00*	ND	73.17	29.66*	13.54*
	150	64.32*	ND	115.47*	30.73*	15.80*
	180	62.73*	ND	192.85*	25.42*	16.29*
	210	70.35*	ND	77.98	29.00*	15.98*
	240	14.76*	ND	126.19*	22.93*	16.13*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: ND; not detected, RA; relative activity

The total phenol content of the untreated leaf material was lower than that of the upper and lower portion of the stalk, which coincided with a reduced lignin content (Section 4.1.2.3) in the leaf fraction in comparison to the corn stalks. Furthermore, the reducing power of the leaf fraction was almost 3 times lower than that of the corn stems, whilst no DPPH activity was also observed in the untreated leaf fractions. This is possibly due to, as mentioned previously in Section 6.1.2.1, the colour interference from the samples masking the decrease in the absorbance of the DPPH.

The reducing power of the substrate was generally increased during pretreatment with both fungal strains, although the activities at each time point did not tend to follow any trend and were quiet variable during both treatments. The maximal relative reducing capacities of 177 and 192 % were achieved after 240 and 180 days with Oyrm 1 and TV 3086, respectively. The maximal reducing power

from both fermentations equated to over 3 times that of a 1 mg mL^{-1} α-tocopherol solution, whilst it was also significantly ($p<0.05$) higher than that of the 1 mg mL^{-1} BHT solution.

The total phenol content was also increased during fermentation with a maximal concentration of 16.13 and 16.29 mg mL^{-1} observed after 240 and 180 days pretreatment with Oyrm 1 and TV 3086, respectively. The maximal activity from the Oyrm 1 treatment coincided with the optimal reducing power which was also obtained after 240 days. The increase in the phenol content noted, which is likely as a result of fungal colonisation and subsequent production of phenolic compounds, equated to a 3.77 and 3.93 mg g^{-1} increase in phenolic content by Oyrm 1 and TV 3086, respectively.

Optimal bleaching inhibition was noted after 120 days of incubation with Oyrm 1, where a 9 % increase in the activity was measured. However, the bleaching inhibition was reduced thereafter, with a decreased of 30 % determined following 240 days of pretreatment. The bleaching inhibition of the extracts from TV 3086 fermentation was also decreased by 21 % during the first 120 days, although as the fermentation progressed the antioxidant activity increased, with maximal bleaching inhibition of 70 % achieved following 210 days of pretreatment. This equated to a 29 % increase in the activity in comparison to the untreated control sample, indicating that the sample from the corn leaf fermentations functions as potent antioxidants in emulsion.

As was observed in the other corn stover fractions (Sections 6.1.2.1 & 6.1.2.2), the $ABTS^{+}$ radical scavenging activity was reduced through pretreatment. The TEAC of the extracts from corn leaves pretreated with Oyrm 1 was significantly ($p<0.05$) decreased during fermentation, where the final activity of 18.73 μg mL^{-1} was 25.94 μg mL^{-1} lower than that of the untreated control material. Similarly, the activity of the corn leaves pretreated with TV 3086 was also reduced, although the rate of reduction in the activity was not as pronounced as that measured with Oyrm 1. In this case the activity of the fermentation was reduced by 21.72 μg mL^{-1} after 240 days fermentation to give a final TEAC of 22.93 μg mL-1 in the samples. This may be linked with the lignin hydrolysis, where the higher degree of lignin degradation found in the Oyrm 1 fermentation (Section 4.1.2.3) may have resulted in a larger reduction in the radical scavenging activity. Alternatively, the difference in the rate of reduction in antioxidant activity may be as a result of the difference in the

rate of degradation of the antioxidants, which is dependent on the phenolic compounds present in the fermentation. For example, Ferreira *et al.* (2002) determined that polyphenol oxidase has a higher affinity for caffeoylquinic acid than catechins thus causing a greater degree of caffeoylquinic degradation during hydrolysis.

It would appear that pretreatment with strains of white rot fungi is an efficient method of increasing the antioxidant potential of corn stover, depending on the method used to determine antioxidant activity. In this case an increase in the reducing power, total phenol content and β-carotene bleaching activity was observed during fermentation. However, pretreatment significantly affected the scavenging activity, with substantial losses in the TEAC of the samples observed. It can thus be concluded that this method of pretreatment would not be a viable method for the production of radical scavenging antioxidants.

6.1.3 Antioxidant production through fermentation of cottonseed hulls with white rot fungi.

Cottonseed hulls have been fed to cattle as forage feeds for generations and is an excellent source of fibre, protein, and fat in animal diets (Piccinelli *et al.*, 2007). However, secondary constituents of the cottonseed hulls have attracted interest, due to compounds such as terpenoid and phytoalexins being implicated in the anti-nutritive and toxic effects discovered when tested as animal feeds (Stipanovic *et al.*, 1975; Gambill *et al.*, 1993). As a consequence of this research, the antioxidant properties of cottonseed hulls have also been determined. Zhang *et al.* (2001) found 5 flavonoids in cottonseeds, which included an apiosyl derivative that is highly desirable in foods due to their cancer preventing and antimicrobial activity (Piccinelli *et al.*, 2007). Other antioxidants found in cottonseed hulls such as condensed tannins and selected phenolic compounds act as effective deterrents to many insects (Chan *et al.*, 1978), as well as inferring effective disease resistance (Lege *et al.*, 1995). As these phenolic compounds are regarded as being a contributory factor in the animal health and productivity, they are of importance when considering the nutritional value of the feed (Piccinelli *et al.*, 2007). Therefore, pretreatment of the cottonseed hull substrate can serve to improve the antioxidant capacity of the substrate thus increasing the nutritive value of the feed. Alternatively, the antioxidants produced from this material may be extracted for use

in the pharmaceutical and cosmetic industries. Consequently, solid state fermentation of cottonseed hulls with strains of white rot fungi was investigated to determined whether the antioxidant capacity of the material can be improved during treatment (Table 6.5).

Table 6.5 Antioxidant activities produced from cottonseed hulls fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	90.45	ND	137.19	44.71	3.25
Oyrm 1	60	29.75*	ND	56.30*	44.69	3.85*
	90	46.66*	ND	51.78*	36.35*	4.05*
	120	65.24	ND	188.98*	34.95*	6.38*
	150	105.17	ND	224.74*	42.51	8.71*
	180	53.16*	ND	218.58*	26.62*	9.35*
	210	81.02	ND	169.56	33.29*	7.12*
Control	0	90.45	ND	137.19	44.71	3.25
TV 3086	60	28.81*	ND	177.94*	36.32*	7.52*
	90	38.67*	ND	254.16*	19.42*	8.30*
	120	48.24*	ND	287.83*	26.12*	8.40*
	150	14.91*	ND	236.41*	38.08	7.20*
	180	91.39	ND	286.32*	30.20*	9.42*
	210	65.35*	ND	292.19*	33.18*	9.16*
BHT	--	100.00	100.00	100.00		--
α-tocopherol	--	99.13	98.96	57.17		--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations; ND: not detected, RA; relative activity

The total phenolic content was increased during both fermentations with an increase of over three-fold noted in both circumstances. The maximal phenol content of 9.35 and 9.42 mg mL^{-1} was observed after 180 days with Oyrm 1 and TV 3086, respectively. The phenol content was subsequently reduced, with the reduction in total phenol being markedly more pronounced in the Oyrm 1 treatments. It is apparent that the total phenol content from the cottonseed hulls was considerably lower than that of the corn stover. This indicated that there may not be a relationship between the lignin concentration of the material and the antioxidant

properties of the substrate, as the cottonseed hulls contained substantially more lignin than the corn stover substrate, although the concentration of free phenolic compounds in the substrate would also be a factor in the antioxidant activity detected (Sections 4.1.2 & 4.1.3). Furthermore, the structure of the lignin molecules may have influenced the total phenol content, as the lignin in cottonseed hulls is known to contain lignin of a high molecular mass (Abduazimov *et al.*, 1997). Pouteau *et al.* (2003) analysed the antioxidant properties of lignin in polypropylene and determined that the molecular weight of the lignin affected the antioxidant potential, with lower molecular weight lignins achieving higher antioxidant activity.

No DPPH radical scavenging activity was measured during fermentation, which again is likely due to the colour pigments in the samples. However, it is conceivable that the low radical scavenging activity may be due to limited activity in the sample, although polyphenols present in lignins are known to be excellent radical scavengers (Piccinelli *et al.*, 2007).

The reducing power of the fermentation extracts was increased through pretreatment of the lignocellulosic substrate. During the first 90 days fermentation with Oyrm 1 the reducing power was markedly decreased, with a reduction of 80 % noted during this time. Despite this, as the fermentation proceeded the activity was increased, with maximal reducing capacity of 224 % noted after 150 days pretreatment with Oyrm 1. Additionally, in the case of the TV 3086 treatment the maximal reducing power of 292 % was observed after 210 days. However, the difference between the reducing power after 120 days (287 %) in comparison to that of the 210 day fermentation was minimal, whilst the incubation time was significantly reduced. Harvesting the material after 120 days instead of 210 days would result in significant cost savings, which are likely to result in greater savings in comparison to the additional antioxidant activity produced. Both fermentations produced a significant ($p<0.05$) increase in the antioxidant activity (as measure by the reducing power) during pretreatment, although the maximal activity was measured with TV 3086, where an activity of 287 % was observed.

The β-carotene bleaching inhibition of the untreated cottonseed hulls was appreciably high, which is likely as a result of a number of the phenolic compounds which are found in cottonseed hulls. Lege *et al.* (1995) investigated the phenolic composition of cotton genotypes and determined that cinnamic, sinapic, ferulic, coumaric and benzoic acids were present in appreciable quantities whilst vanillic,

caffeic, salicylic and syringic acids were also present albeit at lower concentrations. During fermentation with Oyrm 1, the bleaching inhibition was reduced during the first 120 days of pretreatment; however, as the fermentation continued the activity of the material was subsequently increased. The maximal relative activity of 105 % was observed after 150 days fermentation, which was higher than a 1 mg mL^{-1} solution of BHT and α-tocopherol, which are known to be potent antioxidants. Optimal bleaching inhibition from the TV 3086 fermentation was detected after 180 days, with this activity correlating to a 1 % increase in activity in comparison to the untreated material.

During fermentation of the cottonseed hulls with Oyrm 1 the $ABTS^{+}$ scavenging activity was reduced, with a decrease of 10 μg mL^{-1} observed during the first 120 days. However, as the fermentation advanced the activity was increased again, with an 8 μg mL^{-1} increase in the activity being detected. This was also the case with TV 3086 pretreatment, where a significant ($p<0.05$) decrease in the scavenging activity was initially detected following 90 days of fermentation. The activity of the material was subsequently increased during the next 60 days to give an activity of 38 μg mL^{-1}. Nonetheless, this activity was still 15 % lower than that of the untreated control material. Optimal $ABTS^{+}$ activity from the Oyrm 1 treated material was detected after 60 days of fermentation with Oyrm 1, where a TEAC of 44 μg mL^{-1} was observed. The optimal harvest time to achieve maximal scavenging antioxidant production from TV 3086 fermentations (38 μg mL^{-1}) was after 150 days fermentation, although this was still lower than that of the untreated material. It would appear that in both fermentations the TEAC was reduced due to degradation of the lignin polymer which contains the phenolic polymers responsible for the antioxidant activity. Additionally, the subsequent increase in the $ABTS^{+}$ activity is likely as a result of the production of antioxidants such as carotenes and phenolic compounds from the white rot fungi and mobilisation of the antioxidants from the lignin substrate (Abbasi *et al.*, 2007; Ferreira *et al.*, 2009).

Despite it being beyond the scope of this project, it would be interesting to determine the specific antioxidants detected during these fermentations. This would help to determine whether there is a change in the antioxidant composition of the fermentation with regards to the antioxidant compounds present as the lignocellulosic material is degraded and the white rot fungi colonise the substrate. It would also be advantageous to determine whether the antioxidants present in the

substrate at the start of the fermentation are different to those found during the course of fermentation. This may shed some light on the role of ligninolytic enzymes in antioxidant degradation and mobilisation as well as the specific antioxidants produced by the white rot fungi during fermentation.

6.1.4 Antioxidant production through fermentation of cottonseed hulls/coconut fibre with white rot fungi.

Coconut is an important food around the world, whilst it also holds a valuable place in Indian folk medicine, due to its believed antiblenorrhagic, antibronchitic and antigingivitic effects (DebMandal *et al.*, 2011). Many of the medicinal effects of the coconut originate from coconut oil and the tender coconut water, which has been used due to their cardioprotective, antithrombotic, anticholecystitic, antibacterial and antiatherosclerotic properties. However, the husk and fibrous part of the coconut has also been proven to contain medicinal properties that are mainly associated with phenolic content from the lignin portion of the material. For instance, the husk fibre components of the coconut contains antioxidant compounds, such as the catechin, epicatechin and condensed tannins. Theses antioxidants have been shown to display inhibitory activities against acyclovir-resistant Herpes Simplex Virus (Esquenazi *et al.*, 2002). Similarly, aqueous extracts of the coconut husks may also have potential use as a drug with antineoplastic and anti-multidrug resistance, while antiviral and antitumor properties were also observed (Koschek *et al.*, 2007).

Despite the number of antioxidant effects, which can be increased through pretreatment with white rot fungi, coconut fibre can limit fungal growth due to the poor thermal conductivity of the substrate (Abdullah *et al.*, 2011). As a consequence, the coconut fibre was mixed with a quantity of cottonseed hulls to improve the thermal conductivity of the substrate, which allowed for superior colonisation. This material was subsequently fermented with two strains of white rot fungi, as outlined in Section 2.2.5.2 to evaluate whether solid state fermentation would impact on the antioxidant capacity of the biomass (Table 6.6).

Table 6.6 Antioxidant activities produced from a cottonseed hulls and coconut fibre mixture fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	82.41	ND	114.85	43.66	8.19
Oyrm 1	60	44.31*	13.02*	124.24*	32.71*	9.24*
	90	53.59*	20.76*	191.76*	33.55*	13.88*
	120	70.72*	11.24*	198.23*	34.39*	14.67*
	150	78.80	7.65*	100.54*	34.46*	14.91*
	180	51.82*	ND	82.49*	29.82*	13.63*
	210	60.42*	ND	77.27*	29.50*	14.70*
Control	0	82.41*	ND	114.85	43.66	8.19
TV 3086	60	57.27*	13.02*	212.10*	39.15*	13.53*
	90	63.95*	23.96*	225.36*	37.61*	14.87*
	120	68.97	9.60*	221.97*	35.65*	15.42*
	150	64.82*	10.75*	203.47*	37.05*	15.47*
	180	60.25*	12.83*	165.24*	32.71*	15.55*
	210	69.90	9.05*	105.44	33.05*	13.89*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: ND; not detected, RA; relative activity

The total phenolic content of the fermentation extracts was increased through pretreatment with both fungi (Table 6.6), in which the maximal phenolic content of 15.55 mg g^{-1} was achieved after 180 days fermentation with TV 3086. Optimal phenol content from Oyrm 1 was detected after 150 days pretreatment, with an increase of 6.7 mg g^{-1} (79 %) achieved in comparison to the control sample. Analysing both fermentations it would appear that the rate of production of phenolic compounds was optimal during the first 90 days of pretreatment with minimal product formation occurring thereafter. For instance, during the first 60 days of fermentation with TV 3086, 5.3 mg g^{-1} of phenol was produced, which would coincide with the growth and colonisation phase of the fermentation. During the next 150 days; however, only 2 mg g^{-1} of phenol was produced, which constituted a decrease of over 2.5-fold in the rate of production of the phenolic compounds. This also held true for the Oyrm 1 fermentation, where the majority of phenolic

compounds produced (5.7 mg g^{-1}) occurred during the first 90 days, with less than 1.03 mg g^{-1} being produced thereafter.

In contrast to the fermentations with corn stems (Tables 6.2 & 6.3), DPPH scavenging activity was observed during both fermentations of the cottonseed hull/coconut fibre substrate, with the maximal activity achieved after 90 days incubation with both strains. Pretreatment with Oyrm 1 and TV 3086 resulted in an increase of 20.76 % and 23.96 % in the relative scavenging activity, respectively in comparison to the untreated material, where no activity was detected. Despite the initial increase in scavenging activity observed with Oyrm 1, a reduction in activity was noted thereafter, with no activity detected during the final 60 days of fermentation. In contrast, DPPH scavenging activity was detected in all extracts from TV 3086, although similar to the Oyrm 1 fermentation the activity was considerably reduced following the initial 90 days pretreatment.

As was noted in other fermentations such as with cottonseed hulls (Table 6.5), the reducing power was increased with increasing pretreatment time. Maximal relative reducing power of 198 and 225 % was measured following 120 and 90 days of fermentation with Oyrm 1 and TV 3086, respectively. With regard to the Oyrm 1 fermentation, the reducing power of the biomass was increased during the first 120 days and subsequently decreased thereafter, where the reducing power which was observed after 210 days of fermentation (77.27 %) was notably lower than that of the untreated control sample. Similarly, the reducing power of the TV 3086 treatment was significantly ($p<0.05$) increased during the first 90 days of fermentation. However, the activity was decreased thereafter, with the lowest observed activity (105 %) recorded after 210 days of pretreatment. The untreated cottonseed hulls were a more effective reductant than the 1 mg mL^{-1} solutions of both α-tocopherol and BHT, whilst the treated sample were significantly ($p<0.05$) better reductants, with samples from the TV 3086 treatment being almost twice as powerful as the two positive controls. It is thus evident that the cottonseed hulls and coconut fibre biomass is an effective reducing agent; however, through pretreatment this can be improved further.

Analysis of the inhibition of β-carotene bleaching from extracts of the cottonseed hulls/coconut fibre fermentation (Table 6.6) revealed that a decrease in the bleaching inhibition occurred during fermentation. The maximal inhibition was noted after 150 days of pretreatment with Oyrm 1, where an activity of 79 % was

detected. This activity however, was still 4 % less than the activity of the untreated material. Additionally, the inhibition of β-carotene bleaching was also significantly ($p<0.05$) reduced through pretreatment with TV 3086, where a decrease of up to 25 % was observed. Maximal inhibition was achieved after 210 days of fermentation with TV 3086, although this was still 13 % lower than that of the untreated control material.

The $ABTS^+$ scavenging activity was also decreased during both treatments, with the TEAC of the untreated material (43.66 μg mL^{-1}) being reduced by 14.16 μg mL^{-1} (33 %) and 10.96 μg mL^{-1} (25 %) with Oyrm 1 and TV 3086, respectively. This decrease in scavenging activity was in accordance with the other fermentations studies undertaken (Tables 6.2 & 6.3), in which a reduction in the $ABTS^+$ scavenging activity was also detected.

The increase in reducing power and total phenol content during fermentation is likely as a result of the production of phenolic compounds during growth of the white rot fungi. In addition, phenolic compounds may be released from the substrate through hydrolysis of the lignin, as mentioned earlier in Section 6.1.1. Previously, dos Santos Barbosa *et al.* (2008) found that aromatic aldehydes were released from green coconut husk when the material was pretreated with *Phanerochaete chrysosporium* via solid state fermentation of the material. In particular, they monitored the release of ferulic acid and the conversion to vanillin, which is a widely used flavouring agent in a large range of foods. It was evident in this present study that through pretreatment of the cottonseed hulls and coconut fibre the scavenging activity of the material was significantly reduced, with appreciable decreases in the TEAC noted during fermentation with both fungal strains. It is possible that this decrease in the TEAC is due to the degradation of the soluble phenolic compounds by the ligninolytic enzymes, which was discussed previously in Section 6.1.1.

6.1.5 Antioxidant production through fermentation of switchgrass with white rot fungi

Switchgrass has received a significant amount of attention as a source of fuel ethanol due to its relatively high cellulose content; however, it may also be a source of potentially valuable co-products such as antioxidants (Uppugundla *et al.*, 2009). Uppugundla *et al.* (2009), analysed the antioxidant properties of switchgrass samples and found that a number of flavonoids, such as quercitrin and rutin were found to be

present in the extraction solvent. This residual antioxidant activity indicated that switchgrass may serve as an ideal source of antioxidants, whilst biological treatment of this material, with white rot fungi may result in the formation of additional antioxidants. Consequently, switchgrass may be an excellent substrate for antioxidant production, where the antioxidant could be extracted and the remaining pretreated lignocellulosic material saccharified to glucose and pentose sugars for ethanol production. As a result switchgrass was fermented with strains of white rot fungi to assess the potential of increasing the antioxidant activity through SSF (Table 6.7).

Table 6.7 Antioxidant activities produced from switchgrass fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	98.77	ND	138.09	44.45	17.98
Oyrm 1	60	69.19*	3.81*	156.77*	34.45*	20.10*
	90	73.02*	2.38*	248.48*	22.53*	19.45*
	120	77.09*	2.09*	174.64*	23.32*	19.45*
	150	87.36*	3.06*	167.71*	20.47*	19.88*
	180	81.90*	4.28*	116.82*	17.68*	19.45*
	210	88.18*	ND	133.34	22.80*	20.92*
Control	0	98.77	ND	138.09	44.45	17.98
TV 3086	60	69.35*	ND	178.50*	33.97*	22.21*
	90	73.84*	ND	139.95	36.93*	22.20*
	120	42.86*	5.89*	152.61*	38.96	20.90*
	150	57.45*	3.14	411.26*	38.81*	24.57*
	180	87.87	6.89*	68.24*	34.77*	24.30*
	210	85.42	11.89*	70.76*	33.88*	23.94*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: ND; not detected, RA; relative activity

It was evident from Table 6.7 that the total phenolic content of the samples was significantly ($p<0.05$) increased through pretreatment with both fungi. Additionally, it is clear that fermentation with TV 3086 produced the highest total phenol content during pretreatment, where an increase of 6.6 mg g^{-1} was observed.

During fermentation with Oyrm 1, an increase of less than 3 mg g^{-1} was noted, which was less than half that produced with TV 3086 treatment. The majority of phenolic compounds were produced during the initial 60 days which coincided with the colonisation of the substrate and the increase in the mycelial density of the white rot fungi, which are known to produce a significant amount of antioxidants in the form of phenolic compounds. Furthermore, the liberation of phenolic compounds from the lignocellulosic substrate during pretreatment may also have influenced the antioxidant capacity.

The reducing power of the substrate was once again significantly ($p<0.05$) increased through pretreatment with both fungi. Fermentation with TV 3086 resulted in a 3-fold increase in the reducing power in comparison to the untreated switchgrass. However, as the fermentation proceeded the reducing power was substantially reduced with the antioxidant capacity decreased to almost half that of the untreated material. The increase in the reducing power from the Oyrm 1 fermentation was less pronounced, with the activity being increased by 110 % in comparison to that of the untreated control sample. Optimal harvest time to achieve maximal reducing power would appear to be after 90 days for Oyrm 1 and 150 days for TV 3086, as the activity of the fermentations were significantly decreased thereafter, resulting in a reduced antioxidant capacity in the fermentation.

The untreated switchgrass possessed considerably higher bleaching inhibition than other substrates such as corncobs (Table 6.1) and the upper stem (Table 6.2), with over 98 % of the colour retained after 3 hours incubation, which was similar to that of the control samples of 1 mg mL^{-1} BHT and α-tocopherol. As the fermentation progressed the antioxidant activity was subsequently reduced, with maximal relative bleaching inhibition of ~88 % noted after 210 and 180 days pretreatment with Oyrm 1 and TV 3086, respectively. This however, was still 10 % lower than the antioxidant activity of the untreated control material.

The DPPH radical scavenging activity of these fermentations was relatively low, with a maximal activity of 11.89 % achieved through treatment with TV 3086 following 210 days of fermentation. During pretreatment with Oyrm 1 the activity was notably less throughout, with an optimal relative scavenging activity of only 4.28 % achieved. The $ABTS^{+}$ radical scavenging activity was decreased during fermentation with both fungal strains, with the activity from the TV 3086 fermentation being reduced by over 10 μg mL^{-1} (24 %) during pretreatment.

Additionally, through fermentation of the switchgrass substrate with Oyrm 1, the antioxidant activity of the biomass was reduced by over 50 %, which was significantly higher than the decrease observed through pretreatment with TV 3086.

It is unclear whether pretreatment of switchgrass with strains of white rot fungi is a viable method of increasing the antioxidant activity of the lignocellulosic substrate. On the one hand, the activity of the β-carotene bleaching and $ABTS^+$ radical scavenging activity were decreased thus reducing the effectiveness of the extracts from this fermentation in an emulsion and as a scavenging antioxidant. On the other hand; however, the total phenolic content and reducing power of the fermentations were increased.

6.1.6 Antioxidant production through fermentation of oat straw with white rot fungi

Straw has been used as a principle substrate for mushroom cultivation throughout Europe and America for a number of years (Chalaux *et al.*, 1995). In China, growers have also adopted straw logs as a substrate for mushroom cultivation, in comparison to the traditional methods of cultivation on wooden logs (Yildiz *et al.*, 2002; Zhang *et al.*, 2002). The waste material from mushroom cultivation may serve as an effective source of antioxidants which may be extracted and sold to add an additional revenue to mushroom cultivation. Alternatively, this material may be fed to ruminants as a forage with increased digestibility (Jalč *et al.*, 1998) thus offering a better feed (Yu *et al.*, 2005b). The increased antioxidant potential of these residues due to fungal colonisation offers important antioxidants to the animal, which are essential to the health and immune function of the ruminant (Chew, 1995). Currently, the majority of mushroom cultivation occurs on wheat straw as it is more readily available than oat straw in many countries. Nonetheless, oat straw was used to investigate the growth of common fungi in relation to antioxidant production (Table 6.8) as it is widely available in Ireland. The antioxidant capacity of each strain was monitored throughout the fermentation to determine optimal harvest time for maximal antioxidant production. The antioxidant capacity was determined using 5 different assays, which were described previously in Section 2.2.7.

Table 6.8 Antioxidant activities produced from oat straw fermented with the white rot fungi *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086.

Sample	Time (Days)	β-carotene bleaching % RA	DPPH scavenging RA %	Reducing Power % RA	TEAC (μg mL^{-1})	Total phenol (mg g^{-1})
Control	0	87.04	ND	66.59	42.57	16.16
Oyrm 1	60	95.13	ND	275.00*	32.36*	20.78*
	90	97.48*	ND	121.55*	30.98*	19.85*
	120	94.96	ND	111.50*	23.98*	19.65*
	150	95.13*	ND	178.19*	29.27*	18.50*
	180	81.76	ND	176.65*	34.91*	18.70*
	210	91.44	ND	79.21	14.92*	18.70*
Control	0	87.04	ND	66.59	42.57	16.16
TV 3086	60	16.07*	ND	74.82	36.74*	19.36*
	90	37.65*	ND	99.58*	35.48*	19.33*
	120	62.13*	ND	147.37*	32.11*	18.92*
	150	89.26	ND	243.86*	37.27*	20.57*
	180	70.17*	ND	206.55*	27.13*	19.83*
	210	32.82*	ND	119.42*	25.40*	19.37*
BHT	--	100.00	100.00	100.00	--	--
α-tocopherol	--	99.13	98.96	57.17	--	--

- Results displayed are a representation of the means of triplicate fermentations. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, where the confidence level was set at 95 %. An asterisk (*) signifies a significant change in the composition of a particular fraction in comparison to the control sample.
- The β-carotene bleaching activity, DPPH radical scavenging activity and reducing power of each sample were expressed as a relative percentage of a 1 mg mL^{-1} solution of BHT.
- Abbreviations: ND; not detected, RA; relative activity

The total phenol content of the fermentations was increased during pretreatment with both fungal strains. The primary increase in the phenol content was determined during the initial 60 days of fermentation with both TV 3086 and Oyrm 1, where an increase of 3.2 and 4.62 mg g^{-1}, respectively was reported. This was also noted in a number of other fermentations i.e. the lower stem of the corn stover (Table 6.3), where a larger portion of the increased phenol content occurred during the initial stages of fermentation, coinciding with the colonisation of the substrate. The phenolic content from the Oyrm 1 extracts was decreased following an initial increase of 4.62 mg g^{-1}, with the reduction in antioxidant activity being less than half (2.28 mg g^{-1}) of the total increase observed during the initial 60 days fermentation. By contrast, the maximal phenolic content of 20.57 mg g^{-1} was determined after 150 days treatment with TV 3086, with the concentration of phenolic compounds being reduced thereafter.

The TEAC of the material was decreased during pretreatment with both fungi. Fermentation with TV 3086 decreased the scavenging activity by up to 17.17 μg mL^{-1}, which equated to a 40 % reduction in activity. Similarly, the activity of the substrate pretreated with Oyrm 1 was also decreased, with a reduction of up to 27.65 μg mL^{-1} observed. These were comparable to those of the other fermentations analysed in this section, where a reduction in the TEAC of the substrate was also determined. In contrast to the TEAC assay which also measures the scavenging activity of the fermentation, no DPPH radical scavenging activity was observed. This was, as explained previously in Section 6.1.2.1 l, due to the colour interference in the assay from the samples.

The reducing power of each fermentation (Table 6.8) was increased significantly ($p<0.0.5$), with a maximal activity of 275 % achieved after 60 days of pretreatment with Oyrm 1. Optimal reducing activity from the extracts from TV 3086 was noted after 150 days of fermentation, with an increase of almost 4-fold in comparison to the activity observed from the untreated material. Similarly, the reducing power of the Oyrm 1 fermentation was increased by over 208 % during pretreatment, which is likely as a result of phenolic compounds released from the substrate resulting in an increased antioxidant potential.

β-carotene bleaching activity was increased during fermentation with both fungi, although the initial activity of the untreated sample was also appreciably high, with an activity of 87 % detected. Maximal bleaching inhibition of 97 % was noted after 90 days pretreatment with Oyrm 1, with the activity being decreased marginally thereafter. With respect to TV 3086, the activity was significantly ($p<0.05$) decreased during the initial stages of fermentation. As the fermentation proceeded; however, the activity was increased, with an optimal antioxidant capacity of 89 % achieved following 150 days of pretreatment. This represented an increase of 2 % on the activity of the untreated material, which was a marginal increase in the activity from the substrate through fermentation.

Limited research is available on the production of antioxidants from oat straw as oats tend not to be extensively produced around the world in comparison to other cereal crops such as wheat. Consequently, the availability of oat straw is limited in many countries and as such a lot of focus has been put on the utilisation of wheat straw as a substrate for antioxidant production (Arora *et al.*, 2011; Koncsag *et al.*, 2012). Sharma *et al.* (2010), investigated the antioxidant properties of wheat straw

pretreated with the two strains of white rot fungi, *Phanerochaete chrysosporium* and *Daedalea flavida* in solid state fermentation. They found significant increases in the antioxidant properties of the wheat straw, with the radical scavenging activity, reducing power and total phenolic content being increased during fermentation. Their results are broadly in line with the results achieved in this study, although the radical scavenging activity from this study tended to be decreased during pretreatment.

6.2 General conclusions

It was apparent that through biological treatment of these substrates the total phenol content and reducing power activity can be increased in all of the lignocellulosic substrates tested, with the fermentation extracts also acting as effective reducing agents. This increase in antioxidant activity was as a result of the production of antioxidants by white rot fungi during fermentation as well as the mobilisation of phenolic compounds during lignin hydrolysis.

Analysis of the scavenging activity using the DPPH assay indicated that minimal scavenging activity was present in the fermentation extracts. However, phenolic compounds such as those present in lignin are widely regarded as being excellent scavenging antioxidants. This was attributed to the colour interference from the samples, masking the change in the absorbance during the assay. Consequently, an alternative scavenging assay was used, which indicated the presence of scavenging antioxidants. These results illustrated that the scavenging activity was reduced during pretreatment, demonstrating that the extracts were not as effective scavenging antioxidants following fermentation with white rot fungi.

The inhibition of β-carotene bleaching was increased in a number of fermentations, including the corncob, upper stem, corn leaves, cotton seed hulls and oat straw, with the activity of a number of extracts being close to that of a 1 mg mL sample of BHT, demonstrating that these antioxidants worked well in an emulsion. On the other hand, fermentation of other substrates such as the lower corn stem and switchgrass reduced the inhibition of β-carotene bleaching, with the lower corn stem fermentations causing almost complete loss of bleaching activity.

It appears that no one substrate is optimal for antioxidant production as maximal activity from each assay was achieved with different substrates. For instance, maximal inhibition of β-carotene bleaching was observed during solid state

fermentation of cottonseed hulls, whilst the optimal phenolic concentration was noted following pretreatment of the switchgrass. In any case, it is evident that pretreatment of switchgrass and oat straw would be the most viable substrates for antioxidant production, as appreciably higher total phenol content, reducing power and bleaching activity were observed in these fermentations. In contrast, pretreatment of corncobs produced extremely low antioxidant activities with the extracts from the switchgrass fermentation containing over 10 times more total phenol than that of corncobs.

Solid state fermentation of lignocellulosic waste materials looks to be a viable method for the production of natural antioxidants, which could potentially be used to replace chemical antioxidants which are currently used, such as BHT.

7. Conclusions

The assessment of alternative uses for lignocellulosic materials including bioethanol fermentation, antioxidant and hydrolytic enzyme production as well as upgrading the nutritional value of the residues with regard to digestibility and protein content was investigated.

The production of hollocellulosic enzymes from the SSF of corncobs was examined in relation to its potential as a substrate for enzyme production. A number of fungal strains were assessed for protease, cellulase and xylanase activity when grown on the lignocellulosic substrate corncob. *Aspergillus* spp. typically produced some of the highest cellulase and xylanase activities, which were the two enzymes of most interest in this study. The two strains selected for further study were *A. niger* 102.12 and *A. oryzae* 553.65, due to the production of appreciable quantities of both cellulase and xylanase during SSF. Strains with low protease activity were preferred so as to limit hydrolysis of the hollocellulosic enzymes. Typically, *Rhizopus* spp. produced the lowest xylanase activities achieved, whilst they also had some of the highest protease activities during fermentation and were thus not selected for enzyme production. Of the range of fungal strains tested, *Trichoderma* spp. generally produced the lowest enzyme titres, despite them being known as good producers of cellulase. This indicated that *Aspergilli* spp. would be the most suitable fungal genus for producing hollocellulosic enzymes from corncobs, whilst *Trichoderma* spp. would generally be regarded as unsuitable, despite them being widely used in the production of cellulase and xylanase in SmF.

Cellulase and xylanase production from *A. niger* 102.12 and *A. oryzae* 553.65 was subsequently optimised so as to achieve maximal enzyme production using corncob as a substrate. A selection of different carbon and nitrogen sources at different concentrations were investigated to determine which supplement would have the greatest impact on the enzyme yields. Whilst a number of carbohydrates resulted in an increase in the yield of both enzymes, the addition of supplemental nitrogen had a greater influence on enzyme production. In a number of fermentations containing supplemental carbon, catabolite repression was observed in which the availability of free sugars such as glucose reduced the production of hollocellulolytic enzymes. Yeast extract in particular, resulted in a substantial

increase in the enzyme production, with the addition of 5 % yeast extract yielding a 31 and 45-fold increase in xylanase and cellulase respectively, from *A. oryzae* 553.65. Optimal enzyme production from *A. niger* 102.12 also occurred through the addition of 5 % yeast extract, although the effect on cellulase and xylanase production from *A. niger* 102.12 was not as considerable, with yields increased by 14 and 17-fold, respectively. This substantial increase in enzyme yield with yeast extract was attributed to the varying length chain lengths of the nitrogen source, whilst other growth factors such as vitamins and trace elements, which may be available from the yeast extract may also have played a role.

A number of other fermentation parameters were optimised in this study, namely temperature, time and initial moisture content. Optimal fermentation temperature for both fungal strains was observed at 30 °C, with cellulase and xylanase production being considerably reduced as the temperature of the fermentation was adjusted above or below this temperature. The initial moisture content of the fermentation also had a discernible effect on enzyme production. Optimal cellulase and xylanase production from *A. niger* 102.12 occurred with an inoculum volume of 14 mL, with enzyme production decreasing thereafter, with increasing moisture levels. The optimal inoculum volume for *A. oryzae* 553.65 (18 mL/20 g substrate) was higher than that of *A. niger* 102.12, with the productivity from this strain not being as considerably affected by higher moisture levels. At lower moisture levels (10g mL/20 g substrate) the production of cellulase and xylanase was considerably lower, with the production being reduced by between 25-40 %. *A. oryzae* 553.65 took a relatively short period of time (72 hours) to achieve optimal cellulase and xylanase production during fermentation, with the volumetric productivity being considerably reduced thereafter. By contrast, *A. niger* 102.12 took a further 48 hours to achieve optimal enzyme production. This illustrated that *A. oryzae* 553.65 would be the most suitable strain for enzyme production from corncob as appreciably shorter fermentation periods are required, thus potentially reducing the cost of fermentation.

The optimal assay temperature and pH for cellulase and xylanase activity from both fungal strains was also assessed. It was apparent that the pH of the assay had a considerable affect on the detection of cellulase and xylanase during fermentation. Optimal pH for cellulase detection from both strains occurred at pH 4.15, with the activity of SSF extracts being decreased as the pH was increased and

reduced beyond this point. Maximal xylanase activity tended to be achieved using a higher pH, with a pH of 5.7 and 5 resulting in optimal xylanase activity from *A. oryzae* 553.65 and *A. niger* 102.12, respectively. The assay temperature also affected the detection of cellulase and xylanase. In general moderately high temperatures were required to achieve maximal enzyme activity from both strains. The optimal temperature for xylanase activity was at 60 °C for both fungal strains, with a substantial (>90 %) decrease in activity noted with increasing temperature. The optimal temperature for cellulase activity was slightly higher than that of xylanase, with a temperature of 70 °C required to achieve maximal relative activity from both fungal extracts.

The stability of the enzymes from both strains was examined at a range of different temperatures and pHs over a defined period of time. Typically, both cellulase and xylanase were most stable between 24 and 30 °C and least stable at elevated temperatures (60 °C). Furthermore, cellulase from both strains tended to be more thermostable than xylanase at 60 °C, with xylanase from both strains being almost completely denatured after 30 minutes of incubation. By contrast, cellulase from *A. oryzae* 553.65 at pH 5.1 retained 33 % relative activity following 48 hours of incubation at 60 °C.

The pH of the storage solution also affected the stability of the enzymes at different temperatures, with xylanase from both strains typically being more stable at pH values of pH 5.1 to pH 8. Cellulase was most unstable at pH 7 and 8, whilst at moderate temperature of 40 and 50 °C, it was also unstable at lower pHs such as pH 3.1. At 60 °C, the cellulase from both strains were most stable at pH 5.1, whilst the relative activity of the sample decreased as the pH increased or decreased beyond this. These enzymes could suit a variety of processes, in particular processes in which the temperature is moderately high as cellulase tended to be relatively thermotolerant. For example, they would be suitable for enzymatic hydrolysis of pretreated lignocellulosic residues prior to fermentation to ethanol. Depending on the method of bioethanol production, enzymes can be required to work at a number of different temperatures in lignocellulosic ethanol production. For instance, in a SHF technique the enzymes would be required to operate at their optimal temperature to ensure efficient hydrolysis, whereas in a SimSF process the enzyme would be required to work at between 35-45 °C. The cellulase enzymes would also be suitable for the detergent industry, as they as relatively stable over a range of pHs

at temperatures of between 24-30 °C. Enzymes are required to function at low temperatures in detergents as this can lead to energy savings during washing. In addition, the cellulase and xylanase enzymes would also be suitable for processes such as pulp and paper production and in animal feeds to improve digestibility.

Biological pretreatment of lignocellulosic biomass was investigated as a method of delignification of such material for ethanol production. Two strains of white rot fungi were selected due to their ability to grow rapidly on a range of substrates and produce low sporulation. The two strains selected were *Pleurotus ostreatus* Oyrm 1 and *Trametes versicolor* 3086. In total 10 different lignocellulosic substrates were utilised during this present study. A good degree of delignification was noted during pretreatment with both TV 3086 and Oyrm 1 in most substrates, where as much as 75 % of the lignin was removed during fermentation. Both fungal strains did not colonise coconut fibre or pine wood shavings during fermentation, with relatively little growth observed on these substrates following 180 days of cultivation. Consequently, the degradation profile of the substrates were not analysed. The least amount of lignocellulosic degradation occurred during pretreatment of corncobs and cottonseed hulls with both fungal strains, despite complete colonisation of the substrate being observed. During fermentation of corncobs with Oyrm 1 only 14 % of the insoluble lignin fraction was removed, which was in stark contrast to a number of other fermentations in which a significantly higher degree of lignin degradation occurred. For instance, during pretreatment of the upper stem of corn, 63 % of the insoluble lignin fraction was removed, which was 4.5 times as much as corncob. The highest degree of delignification occurred when oat straw was pretreated with Oyrm 1, where 75 % of the lignin was removed during fermentation. In addition the glucose concentration was also increased by 20 %, which is likely a concentration affect due to the significant loss of xylose and lignin from the material. This increase in the glucose content tended to occur in every fermentation with Oyrm 1, indicating that the glucose remained relatively unutilised. This would influence the potential yield of ethanol from the substrate as less cellulose was utilised during pretreatment, thus increasing the final ethanol yield.

It was apparent during fermentation that the Oyrm 1 strain selectively degraded most of the lignocellulosic substrates, with reductions in xylan and lignin detected. By contrast, TV 3086 non-selectively degraded the substrate with

substantial lignin, hemicellulose and cellulose losses noted. For example, TV 3086 degraded 30 % of the lignin fraction during fermentation of the upper stem, whilst 30 % of the cellulose fraction was also removed. This was in contrast to the Oyrm 1 strain, which degraded 63 % of the lignin, whilst the cellulose fraction was increased. Oyrm 1 also tended to remove a higher proportion of the lignin fraction in all substrates tested, with in some cases (oat straw), it removing over twice as much lignin from the material than TV 3086. This in turn would impact on the potential to produce ethanol from the substrate as the greater the degree of delignification the more cellulose available for conversion to glucose.

It was clear that biological pretreatment is an effective method for pretreatment of a number of lignocellulosic substrates, with Oyrm 1 appearing to be the most suitable strain for lignin removal. Furthermore, less degradation of the cellulose fraction was noted during pretreatment with Oyrm 1 which would dramatically increase the economic viability of future ethanol fermentations in comparison to pretreatment with TV 3086.

These lignocellulosic residues were subsequently tested as a means of ethanol production. The yeast strain *Pichia thermotolerans* 7012 was selected during screening, due to its rate of ethanol production, as well as the higher final ethanol yield observed. This strain was subsequently utilised to ferment a corn mash medium, in which a portion of the mash was removed and replaced with pretreated lignocellulosic biomass. The lignocellulosic residues which were pretreated with Oyrm 1 were used due to the higher degree of delignification noted in these fermentations.

The addition of these residues did not have a discernible effect on the overall rate of ethanol production during fermentation. Furthermore, the final ethanol concentration of the substrate was not increased through the addition of these residues to the mash. However, when it was considered that a lower amount of corn mash was utilised to achieve the same concentration of ethanol in the fermentation it was determined that a reduction in the substrate cost could be achieved through the use of these residues. Exogenous cellulase was also added to a number of fermentation mashes to hydrolyse the lignocellulosic substrate and thus increase the final ethanol yield. This did not appear to be the case however in a number of fermentations, with no increase in the yield of ethanol observed through the addition of exogenous cellulase. This was attributed to the large amount of lignin remaining

in a number of the substrates (corncobs and cottonseed hulls), inhibiting the cellulose degradation. However, despite pretreatment of other lignocellulosic such as switchgrass resulting in substantial lignin degradation of (52 %), no increase in the ethanol yield was observed when exogenous cellulase was added. This could be due to other factors such as the cellulose crystallinity or the formation of compounds such as furfural and phenolics that can affect cellulose hydrolysis. In the oat straw fermentation, a minor increase in the ethanol yield was noted through the addition of the cellulase to the mash. The oat straw fermentation had the highest degree of delignification during pretreatment, which most likely aided the hydrolysis of the cellulose fraction during fermentation.

Instead of using exogenous cellulase, pretreated residues were fermented with *Aspergillus* spp. in the anticipation that endogenously produced cellulolytic and hemicellulolytic enzymes may increase the ethanol potential. The optimal conditions for enzyme synthesis, as outlined previously, were employed to try and obtain the maximal cellulase hydrolysis during this secondary fermentation. This material was then used to replace 6 % (w/v) of the corn mash substrate. It was evident through the analysis of the results obtained in this study that the rate of ethanol fermentation was significantly increased through the addition of pretreated corncob to the mash. This increase in fermentation rate was thought to be as a consequence of an increased yeast cell biomass, due to the larger concentration of available sugars at the start of fermentation. For instance, the addition of corncobs pretreated with *P. ostreatus* 32783 and *A. oryzae* 553.65 resulted in a 9 hour reduction in the fermentation time, with minimal (0.09 % v/v) reduction in the final ethanol concentration. Fermentation time could be reduced even further in a number of these fermentations, although the difference in the final ethanol yield would be slightly larger in comparison to the control sample. In spite of this increased reduction in fermentation yield it may be possible that the culture with a reduced fermentation time would be more economically viable than the control fermentation with a higher ethanol yield, due to the reduced running costs. However, the potential loss in revenue would need to be weighed against the potential savings that could be achieved through a reduced fermentation time. This increase in the rate of fermentation was noted in each sample fermentation, although in a number of fermentations, the reduction in the final concentration was larger than that noted in the *P. ostreatus* 32783 / *A. oryzae* 553.65 fermentation. For example, the rate of

fermentation time was considerably reduced when corncobs were pretreated with *P. salmoneostramineus* 5338 and *A. niger* 102.12. However, the final ethanol concentration also decreased, with a reduction of 0.61 % (v/v) noted between the sample fermentation after 34 hours and the control after 41 hours. Although a decrease in the final ethanol yield was observed during, the efficiency in the sample fermentations were typically lower than the controls. It was thought that the reduction in fermentation efficiency was partly responsible for the reduction in ethanol yield observed. This reduction was attributed to one of a number of reasons, such as a stuck fermentation or retrogradation of the starch molecules in the mash.

Pilot scale fermentations were investigated to determine whether the results obtained from laboratory scale could be scaled up to a larger process. However, the results from these pilot scale fermentations indicated that this may not be the case. The addition of corncobs pretreated with Oyrm 1 and TV 3086 to the mash reduced the rate of ethanol fermentation substantially in comparison to the control. In addition, the final yields of these fermentations were notably lower. However, the overall efficiency of the fermentations were also lower, thus indicating that the lower yield was as a consequence of the slower fermentation rate. The addition of corncobs which were pretreated with Oyrm 1 and *A. niger* 102.12 did not appear to negatively affect the rate of ethanol production, such as those containing corncobs pretreated with TV 3086 and Oyrm 1. However, in the laboratory scale fermentations a notable increase in the fermentation rate was observed, which was not the case here. This was attributed to the differences in the vessel design, in which a higher degree of mixing was carried out in pilot scale, potentially affecting a number of fermentation parameters. The corn mash fermentation containing oat straw performed relatively similar to laboratory scale, in which the rate of ethanol production was comparable with the control fermentation. In addition, the fermentation efficiency was also similar between the two fermentations, although the final yield of ethanol was moderately lower. These results indicated that the addition of pretreated lignocellulosic residues decreased the rate of ethanol production from the fermentations containing corncobs pretreated with TV 3086 and Oyrm 1. Furthermore, the rate of ethanol production from the dual treated fermentations was also decreased in comparison to the laboratory study. Further tests would need to be carried out to confirm these results, as singular fermentations were investigated due to time constraints in the pilot facility.

The simultaneous saccharification and co-fermentation of pretreated lignocellulosic residues was carried out. A sterilised enzyme cocktail from the fungal strains *A. niger* 102.12 and *A. oryzae* 553.65 was used to saccharify the substrate. The optimal fermentation conditions, which were determined during enzyme production, were utilised to achieve maximal enzyme production from the fungus. In these fermentations, ethanol production from these residues was typically slow. Maximal ethanol yield was obtained following hydrolysis of cottonseed hulls, in which a concentration of 0.41 % (v/v) ethanol was obtained after 48 hours. This was unexpected considering that the degree of delignification from cottonseed hulls was considerably lower than that of the other residues. The rate of ethanol production appeared to be higher when the residues were pretreated with Oyrm 1, in comparison to when TV 3086 was utilised. This was not unexpected considering that a higher degree of delignification occurred during pretreatment with Oyrm 1. The efficiencies of the fermentations also tended to be quite low, with maximal fermentation efficiency of 85 % noted after 72 hours of pretreatment of the upper stem with Oyrm 1. It was evident that SimSF is a potential method of ethanol production from lignocellulosic residues, although a number of key issues such as the low fermentation efficiencies and the slow rate of cellulose hydrolysis will have to be investigated further to make this method a viable alternative to corn mash ethanol production.

The potential of upgrading the commercial value of a number of forages through the pretreatment with white rot fungi was also examined. During fermentation, the crude protein content of the residues tended to be increased through pretreatment with both white rot fungal strains. A difference between the amount of crude protein produced by each fungus during fermentation was typically observed. This difference in the crude protein content between the fermentations can most likely be attributed to the differences in the protein content of the fungal biomass generated during fermentation. Furthermore, the loss of structural carbohydrates and lignin during fermentation may also have had a concentration effect on the protein content. Pretreatment of the corncob residues resulted in a relatively small increase in the crude protein content in comparison to fermentation of the other residues. For instance, fermented upper stem of corn had a maximal crude protein content of 13.9 % (w/v), which constituted a 200 % increase in the crude protein content of the forage. Comparatively, the maximal crude protein

content from corncobs was 2.3 %. The maximal crude protein content observed during this study was 32 % (w/v). This was noted through the pretreatment of corn leaves with Oyrm 1, although it should be noted that the initial crude protein of the corn leaves tended to be higher than the other lignocellulosic residues investigated.

The *in vitro* true digestibility (IVTD) of a number of the residues was also increased through fermentation with white rot fungi. Pretreatment of oat straw with Oyrm 1 resulted in the greatest increase in the *in vitro* true digestibility of the substrate, with a 2.2-fold increase observed. Enhanced IVTD was also noted during pretreatment of a range of other residues such as corncobs, cottonseed hulls and switchgrass. Conversely, pretreatment of the lower stem resulted in a decrease in IVTD, which was surprising considering the NDF and the ADF of the substrate were reduced during fermentation. Generally, pretreatment with Oyrm 1 tended to result in a more digestible substrate than with TV 3086, which would have been expected considering the higher degree of delignification occurred through pretreatment with this strain.

The total digestible nutrient content was also marginally increased during pretreatment in the vast majority of fermentations, which was anticipated as the digestibility of the forages was increased. The largest increase in TDN content came through the pretreatment of cottonseed hulls on their own and cottonseed hulls and coconut fibre. It was also worth noting the TDN content of these residues tended to be almost twice as low as the other lignocellulosic residues prior to treatment. However with pretreatment, the TDN of the cottonseed hulls was greater than any of the other lignocellulosic residues examined, whilst the TDN of the cottonseed hulls and coconut fibre biomass was similar to a number of the other materials, such as switchgrass and oat straw prior to pretreatment.

The acid detergent fibre (ADF) content was also increased during fermentation in a number of forages, suggesting that the digestibility of the forage was being increased, which was in agreement with the IVTD data. In contrast to the IVTD results however, in which the Oyrm 1 fermentation resulted in the greatest increase in digestibility, it was the TV 3086 fermentations which typically had the most significant impact on the ADF content. However, as mentioned previously in Section 5.1.1.1, the two methods were poorly correlated. It is likely that the IVTD results are more representative of the true digestibility as it closely mimics that of rumen degradation. Neutral detergent fibre (NDF) was also improved which would

lead to an increased intake from the ruminant. Cottonseed hulls and corncobs had the highest initial NDF of all the residues investigated; indicating that intake by ruminants would be considerably reduced if they were fed these forages. Through pretreatment however, the NDF content was significantly reduced in these two forages. The greatest reduction in the NDF content came through the pretreatment of the corn leaves, in which almost 50 % of the NDF was removed during fermentation with TV 3086. This correlated with the reduction in the lignin content observed, where almost complete removal of the lignin fraction was detected.

It was clear that pretreatment of lignocellulosic residues enhanced the nutritional value of these potential forages. However, the degree to which the forage was improved depended on the type and composition of the lignocellulosic substrate. Pretreatment with TV 3086 appeared to be the best strain to improve the ADF and crude protein content of forage. As a consequence, the use of this strain for pretreatment of forages for dairy cattle forages would be ideal, as the higher the crude protein content the greater the milk yield that can be achieved. Conversely, Oyrm 1 appeared to be more suitable to improve the *in vitro* true digestibility of the forage and thus increase the overall nutritional value of the residues as a feed crop.

Assessment of the lignocellulosic residues indicated that they contained appreciable quantities of antioxidants prior to pretreatment with white rot fungi. Following pretreatment, the antioxidant potential of the residues generally tended to be enhanced, all be it in an assay dependent manner. An assessment of scavenging activity using the DPPH assay indicated that there was minimal activity in the fermentation extracts. However, analysis using the TEAC scavenging assay indicated that the same extracts contained good scavenging potential. It was postulated that colour interference in the DPPH assay may have affected the detection of the antioxidant capacity. In any case, the TEAC of the fermentation extracts was generally decreased during fermentation of the residues, which was unexpected considering white rot fungi are generally known to produce phenolic acids such as flavonoids, which have previously been shown to be good antioxidant scavengers. This decrease observed during treatment was thought to be as a result of the degradation of phenolic compounds by the oxidative enzymes produced by white rot fungi during fermentation.

A direct assessment of the phenolic compounds indicated that the total phenol content of the substrates was generally increased through the pretreatment

with both TV 3086 and Oyrm 1. Maximal phenolic activity occurred during pretreatment of switchgrass with TV 3086, although this substrate also contained appreciably higher initial total phenol content than the other residues. The greatest increase in phenolic content was detected when cottonseed hulls and coconut fibre were pretreated with TV 3086, where an increase of 7.36 mg g^{-1} was determined. Meanwhile, the lowest phenolic content was observed during fermentation of the corncob substrate, although cottonseed hulls also contained a relatively low total phenol content. This increase in phenolic content would indicate that although the phenolic compounds are increased during fermentation they are typically poor radical scavengers, as the scavenging activity was reduced. The increase in the phenolic content of the samples was likely due to the production of antioxidants by white rot fungi during fermentation, whilst the mobilisation of phenolic compounds during lignin hydrolysis may also have influenced the antioxidant activity.

The reducing power of the fermentation extracts appeared to be the most notably affected by the pretreatment of the biomass. In all fermentations, the reducing power was increased through pretreatment, most notably when switchgrass was pretreated with TV 3086, where a reducing power of over 400 % was observed. The inhibition of β-carotene bleaching was also enhanced through pretreatment, although in a number of fermentations, the activity of the initial substrate was also quiet high, with bleaching inhibition of 92 % observed in some of the control samples. A number of the fermentation extracts such as the cottonseed hulls pretreated for 180 days with TV 3086 were potent antioxidants, with the antioxidant capacity being almost as potent as a 1 mg mL^{-1} of BHT.

Pretreatment of the lignocellulosic biomass with white rot fungi typically improved the antioxidant capacity of lignocellulosic residues. As a consequence, these residues may be ideal as a forage feed in which the digestibility and crude protein content would be increased as well as the antioxidant potential, thus further increasing the potential value of the material. Furthermore, the extraction of antioxidants from mushroom waste in which straws and grasses are typically used may offer an additional revenue stream for mushroom producers which would increase the profitability of the business with relatively little processing required to extract the antioxidants from these materials.

In general biological pretreatment of lignocellulosic materials appears to be a viable method of treating a number of the lignocellulosic waste products analysed, to remove the lignin fraction. Additionally, it would also appear to have potential as a method of upgrading the digestibility and crude protein of the majority of forage feeds analysed, where the antioxidant content of the feed could also be enhanced thus further improving the value of the forage as an animal feed.

While this technique does suffer from being a relatively slow process in which long fermentation times are required, it appears that it can be relatively selective in its degradation of lignin and hemicellulose. Consequently, this would leave the cellulose fraction in the material more exposed for hydrolysis to glucose for ethanol production. In addition, this method could be relatively low cost as the fermentation temperature is comparatively low and the labour costs would be minimal, as the material can be inoculated and left to ferment for a number of months in a type of ensiling process before it is harvested. Furthermore, the fermentations conditions were not strictly controlled which would also lend itself to a less costly process in comparison to a more controlled environment, such as fermentations where the air flow was more strictly controlled. By comparison, although other methods of pretreatment such as chemical and physico-chemical methods generally require shorter treatment times, they are typically very energy intensive and in some cases use harsh chemicals, which may be costly to purchase and difficult to dispose of.

Although the fermentation time was long and thus could influence the viability of this process in industry, optimisation of the fermentation conditions could offer an opportunity to reduce the time required for fermentation. For instance, increasing the inoculum volume of the fermentation would reduce the time that was required for initial colonisation of the substrate thus reducing the overall fermentation time. In addition, optimisation of other conditions such as moisture, temperature, substrate size, oxygen supply and pH could also help to reduce the time required for fermentation. Consequently, further research aiming at reducing the fermentation time would be beneficial as at present, the time which is required to achieve the significant amount of lignin degradation observed may hinder this technique being viable as a treatment technique for both ethanol production and the upgrading of the nutritional value of forage feed.

Furthermore, an investigation into the specific antioxidants present in the lignocellulosic material prior to treatment and during pretreatment may shed some light on the antioxidants that were degraded during fermentation as well as those that were produced by the fungi as a consequence of growth on the fermentation medium. In addition, an investigation as to why a greater amount of ethanol production did not occur from some of the treated materials would also be beneficial. This may indicate the factors which were hindering ethanol production from the lignocellulosic material, as in some cases as much as 75 % of the lignin was degraded, which should have left a considerable amount of cellulose available for hydrolysis. If this could be achieved the potential of producing ethanol from this material could be enhanced thus aiding in making this method of pretreatment economically viable.

In conclusion, biological pretreatment of lignocellulosic biomass can help realise the potential of these materials, which would otherwise be left unutilised. Whilst traditional methods of antioxidant and enzyme production are still viable, this approach could offer an alternative to these methods. A reduced process cost due to mild conditions used during fermentation and the low cost associated with obtaining lignocellulosic material are of particular note. Furthermore, with the anticipated growth in the world population which will lead to a greater need for corn and other grains as a food source, alternative substrates are required for producing fuel ethanol to meet the needs of this growing population. In addition, the cultivation of lignocellulosic residues could help countries around the world sustainably produce fuel ethanol and not rely on petroleum from oil producing countries such as Russia and Saudi Arabia. Although the present study has investigated potential uses of these residues in the production of a number of value added products, further research into the production and optimisation of these processes is required to fully release the potential that these substrates have to offer.

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