

Optimisation of phytase production by *Aspergillus niger* using solid state fermentation



NUI MAYNOOTH

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Declaration

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: _____

Tânia Santos

Date: _____

Dedication

Esta tese é dedicada a ti Mãe, porque me ensinaste que até a batalha mais dura pode ser enfrentada. É também dedicada a ti Pai, porque sempre me disseste para acreditar em mim mesma e no meu potencial, e a ti Fábio, porque nunca duvidaste e sempre acreditaste em mim.

“This thesis is dedicated to my Mum, who taught me that even the hardest battle can be fought. It is also dedicated to my Dad that always said to me to believe in myself, and to my Brother, Fábio, for always believing in his big sister.”

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Abstract

The use of phytases as an alternative to the use of inorganic phosphorous has resulted in improved utilisation of phytate-phosphorous by monogastric animals. Solid state fermentation (SSF) has been proven as a valuable production strategy for phytase.

*The objective of this study was the stabilisation and optimisation of phytase production by a non-genetically modified strain of *Aspergillus niger* using SSF. The variability of phytase production in replicate SSF flasks produced by individual clonal isolates of the *A. niger* Phy-A strain was successfully decreased via optimisation of the agar slant media; however the variability in enzyme productivity noted between slants remained. Twelve days old slants of wheat germ agar were optimal for maximal phytase production. In the liquid seed media optimisation, tapioca flour at 2% (w/v) inclusion was found to be the most suitable starch and carbon source, producing increased phytase activity (7%) relative to the control ($p \leq 0.05$). Peptone at 1.2% (w/v) inclusion was the optimal nitrogen source, yielding a 12% increase in enzyme production ($p \leq 0.05$). Optimisation of the SSF system contributed the most to the enhancement of phytase production by the *A. niger* Phy-A strain. Wheat middlings (WM) were shown to be a better substrate than wheat bran for phytase production in SSF; phytase production was increased by 34% when grown on WM ($p \leq 0.05$). However, the best substrate was found to be a combination of WM and wheat germ (WG) (8:2, w/w) resulting in an additional 19% increase in phytase production ($p \leq 0.05$). The optimum inoculum size was 8 mL per 10 g of mixed substrate. The optimised incubation parameters for phytase production in SSF were temperature at 30°C for 5 days corresponding to a 15% increase in phytase production by the *A. niger* Phy-A strain. Analysis of the finished SSF product verified the presence of ancillary enzymes such as cellulase, xylanase, β -glucanase and protease. In vitro thermostability and proteolytic analysis indicated that the *A. niger* Phy-A phytase exhibited minimal activity after incubation at 80°C during 5 minutes. However, this phytase retained its activity post incubation with pepsin but was nearly inactivated after incubation with pancreatin. A novel study demonstrated that the *A. niger* Phy-A phytase was stable in vitro in the presence of ruminal fluid (pH 6.0) over a 24 hour period.*

Abbreviations

ANF	Antinutritional actor
IP6	Phytic acid
P	Phosphorous
Phytate-P	Phytate phosphorous
Total-P	Total phosphorous
SSF	Solid state fermentation
SmF	Submerged fermentation
GRAS	Generally Regarded As Safe
A _w	Water activity
C/P	Carbon/phosphorous
C/N	Carbon/nitrogen
CMC	Carboxymethyl cellulose
DNS	Dinitrosalicylic acid
HCL	Hydrochloric acid
PDA	Potato dextrose agar
TCA	Trichloroacetic acid
WGA	Wheat germ agar
WM	Wheat middlings
WG	Wheat germ
WB	Wheat bran
SD	Standard deviation
SPU/g	Standard phytase units per gram
XU/g	Xylanase units per gram
HUT/g	Protease units per gram
CMCU/g	Carboxymethyl cellulase units per gram
rpm	revolutions per minute
RH	Relative humidity
μmolIP/mL	Micromolar per militer of inorganic phosphorous
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

Chapter 1

1. Introduction

In an era where we are part of an increasingly health conscious society and where there is a paradigm change from cure to prevention; where climate change, pollution and waste are high on the agenda, consumers are looking more and more for reassurance that what they buy is safe and environmentally friendly. Health promotion is becoming of greater significance and the concern with food safety is of supreme importance. The role of animal feed in the production of safe food is recognised worldwide (Collins and Wall, 2004) and recent events have underlined its impacts on public health, feed and food trade, and food security.

Economic and technological advances are driving the development of new feed products, especially products of biotechnology. Enzymes as additives to animal feed have an important role to play in current farming systems. Not only have they improved the digestibility of nutrients, leading to greater efficiency in the production of animal products such as meat and eggs, but they have also improved the quality of the environment by allowing better use of natural resources and reducing pollution by nutrients (Acamovic, 2001).

The global industrial enzyme market is currently valued at \$2 billion with an annual growth rate of 3-5% (Kangasmäki, 2009). However, recently, the report “World Enzymes Market” by The Freedonia Group (2009) announced that the world market for enzymes will recover from a difficult year in 2009 to reach \$7 billion in 2013. According to this report, the animal feed enzyme industry is expected to achieve its fastest growth in developing markets where rising per capita incomes will continue to increase demand for meat in the local diets.

Phytase is an enzyme in which interest has increased remarkably in the past 20 years, not only because of its wide range of applications in animal and human nutrition, but also, in response to heightened concerns over phosphorous pollution in the environment (Lei and Porres, 2003). Suzuki *et al.* first detected phytase activity in rice bran in 1907, but it was not until 1991 that the first phytase feed enzyme became commercially available (Haefner *et al.*, 2005; Cao *et al.*, 2007). The past five decades have seen a rapid increase in the use of filamentous fungi for the production of industrial enzymes and phytases are very much part of this trend. Submerged fermentation (SmF) has primarily been promoted as the best production technology. However, in recent years, interest in solid state fermentation (SSF) has increased as it provides various advantages from both practical and economic perspectives.

These include, higher product concentration, better product recovery, low-technology cultivation equipment, reduced waste-water output, lower capital investment and lower plant operation cost (Muniswaran *et al.*, 1994; Zhu *et al.*, 1994; Becerra and Siso, 1996; El-Batal and Karem, 2001).

Phytase can be found in plants, bacteria, fungi, yeast and animals. However, phytase activity in microorganisms has been found most frequently in fungi, in particular, *Aspergillus* species (Kim *et al.*, 1998a). To date, only a handful of commercial phytase products are available (Haefner *et al.*, 2005). Ongoing research projects continue to focus on the discovery or improvement of phytases that could become even more suitable to animal feed applications than current commercial preparations (Vats *et al.*, 2009). Despite its value as a feed additive, problems are still arising with some of the characteristics of phytase. Thermostability at the elevated temperatures that occur during hydrothermal treatment of animal feed processing (e.g. pelleting) and stability at different gastrointestinal pH are issues that continue to raise the bar in this field of study (Zhao *et al.*, 2010). Hence, the ideal phytase is yet to be found and its suitable biochemical characteristics to be agreed on (Fu *et al.*, 2008). Even though phytase supplementation effectively improves nutritional quality while decreasing phosphorous waste, the high cost of the phytase enzyme preparations currently limits its commercial use, thus economical alternatives are in need.

1.1 *Phytic Acid Overview*

1.1.1 Phytic acid origin and structure

Phytic acid or *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate), IP6 (IUPAC-IUB Commission on Biochemical Nomenclature, 1977) is a naturally occurring compound that can significantly influence the functional and nutritional properties of foods. Phytic acid is a simple ringed carbohydrate with one phosphate group per carbon (Figure 1.1), its molecular formula is $C_6H_{18}O_{24}P_6$ and its molecular weight is 660.035 g/mol.

Three terminologies, namely phytate, phytin and phytic acid, are used in the literature to describe the substrate for phytase enzymes. The most commonly used term, phytate, refers to the mixed salt of phytic acid. The term, phytin, specifically refers to the deposited complex of IP6 with potassium (K), magnesium (Mg) and calcium (Ca) as it occurs in plants, whereas phytic acid is the free form of IP6 (Selle and Ravindran, 2007).

Phytic acid is a strong chelator and is the principal storage form of phosphorous and other minerals and trace minerals in many plant tissues. It is the major phosphorous storage compound in the plant seed and can account for up 80% of the total phosphorous in seed (Lopez *et al.*, 2002). Phytic acid is found within legumes, cereals, oil seeds, pollens, as well as in the hulls of nuts, constituting about 1 to 5% of their weight (Vats and Banerjee, 2004). Oilseed meals and cereal by-products contain large amounts of phytate phosphorous, whereas cereals and grain legumes contain moderate amounts (Ravindran *et al.*, 1995). Total phosphorous concentration in cereals (%) ranges from 0.35 to 0.45 and between 0.65 and 1.12 in oil seeds and by-products (Eeckhout and De Paepe, 1994). Table 1.1 outlines various feedstuffs and the levels of phytate phosphorous present.

The existence of phytic acid has been known for over a century, although its role is not completely understood and consequently it still constitutes an area of active research.

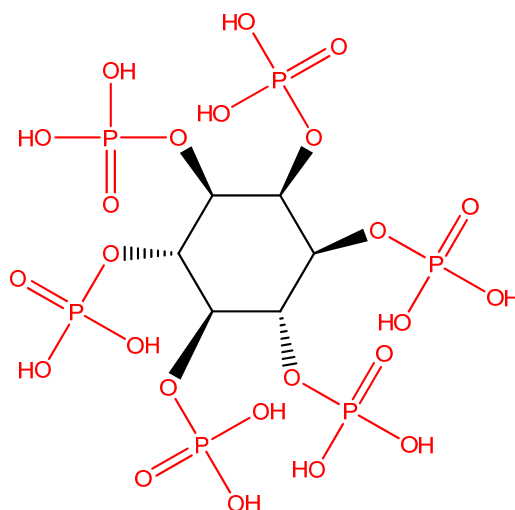


Figure 1.1 Phytic acid molecular structure

Table 1.1 Phytate phosphorous content of various feed ingredients

Adapted from Ravindran *et al.* (1995).

	Phytate-P (g/100 g DM)	Phytate-P (as % of total)
Cereals		
Corn	0.24	72
Wheat	0.27	69
Barley	0.27	64
Sorghum	0.24	66
Rice (unpolished)	0.27	77
Grain legumes		
Field peas	0.24	50
Oilseed meals		
Soybean meal	0.39	60
Rapeseed meal	0.70	59
Sunflower meal	0.89	77

Note: DM, dry matter; Phytate-P, phytate-phosphorous

1.1.2 Phytic acid as an antinutritional factor

The ability to interact with other food ingredients makes phytic acid an antinutritional factor (ANF) in several ways as described below.

The unique phytate ion structure, with 12 replaceable protons and high density of negatively charged phosphate groups (responsible for its characteristic properties), allows it to form very stable complexes with multivalent cations (Dost and Tokul, 2006). A considerable number of researchers have reported the chelating ability of the phytate ion with several mineral elements including, Cu^{2+} , Zn^{2+} , Co^{2+} , Cd^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Fe^{3+} , Ni^{2+} and Ca^{2+} to form phytate-mineral and/or other protein-mineral-phytate

complexes (Wise, 1995; Persson *et al.*, 1998; Liu *et al.*, 1998; Maenz *et al.*, 1999). Intestinal absorption is a key and complex stage for maintaining normal mineral homeostasis and requires that minerals remain in the ionic state for absorption (Lopez *et al.*, 2002). Therefore, mineral-phytate complexes are not readily absorbed under gastrointestinal pH conditions found in monogastric animals and humans (Maga, 1982) due in part to the lack of hydrolytic enzymes capable of breaking down these complexes. A number of studies have found that phytic acid reduces the digestibility of proteins, starch and lipids (Yoon *et al.*, 1983; Nyman and Björk, 1989; Dvoráková, 1998).

Proteins are able to bind directly with phytic acid through electrostatic charges at low pH or through salt bridges formed at high pH (Thompson, 1986). This binding or interaction with dietary proteins reduces their digestibility through changes in protein solubility or by altering the protein structure and thus reducing the activity of endogenous proteases because of steric hindrance (Cowieson *et al.*, 2006). If these protein-phytate complexes are insoluble in the aqueous environment of the gastrointestinal tract, it is more difficult for proteolytic enzymes to hydrolyze the proteins. Consequently, protein digestion may be reduced (Kies *et al.*, 2006).

The mechanism whereby phytic acid affects starch digestion and blood glucose is unclear because of the various types of interactions that can occur. Phytic acid may affect starch digestibility either through its binding with proteins which are closely associated with starch, through association with digestive enzymes which are themselves proteins, through chelation with Ca^{2+} required for the activity of amylase, or due to direct binding with the starch, since phytic acid is structurally capable of binding to starch through phosphate linkages (Thompson, 1986).

To date there is little known about the effect of dietary phytate on lipid profiles in chickens or other farm animals. However, in a recent study, Liu *et al.* (2010) suggested that phytate can interfere with lipid metabolism, and consequently energy regulation, including energy ingestion and lipid deposition in chickens.

Phytic acid inhibits the action of enzymes such as α -amylase, trypsin, lipase, acid phosphatase and pepsin (Harland and Morris, 1995; El-Batal *et al.*, 2001). These enzymes play an important role in the digestive system and their inhibition may cause a deficient digestion as well as absorption of nutrients.

1.1.3 Phytic acid significance in agriculture

1.1.3.1 Phosphorous: a vital source of animal nutrition

Phosphorous (P) is an essential nutrient to biological systems and is one of the most important minerals in animal nutrition. It is the second most abundant element in an animal's body after calcium, with 80% of phosphorous found in the bones and teeth and the remainder located in the body fluids and soft tissue (Hegsted, 1968).

Phosphorous plays a key metabolic role and has more physiological functions than any other mineral. These functions involve major metabolic processes such as development and maintenance of skeletal tissue, maintenance of osmotic pressure and acid base balance, energy utilisation and transfer, protein synthesis, transport of fatty acids, amino acid exchange, growth and cell differentiation, appetite control, efficiency of feed utilisation, and fertility (Dobrota, 2004). Animals require an adequate supply of phosphorous in their diet; otherwise they can suffer from a phosphorous deficiency. This can affect the animal's physical well-being in several ways: compromise of the immune system, bone breakage, loss of appetite, reduction in fertility and loss in live weight gain due to low feed efficiency (Aehle, 2007).

1.1.3.2 Phytate phosphorous, animal nutrition and environmental pollution

Extensive studies have examined the availability of phytate phosphorous to monogastric animals. Feeds for pigs and poultry are traditionally supplemented with inorganic phosphate to meet the nutritional requirements for optimal growth of these animals (Reddy, 1989a).

In providing required levels of phosphorous, the primary concerns of the livestock producer are animal welfare and productivity. However, undigested phytate-P is excreted through the faeces and spread as manure into the soil (Bohn *et al.*, 2008). Manure-borne phosphorous is a serious environmental hazard that has been reviewed by several researchers (Hooda *et al.*, 2000; Sharpley *et al.*, 2001, 2004; Centner, 2004; Shigaki *et al.*, 2006; Powers and Angel, 2008). The potential eutrophication of fresh water streams, lakes and near coastal areas can then cause cyanobacterial blooms, hypoxia and death of aquatic animals followed by production of nitrous oxide, a potential green-house gas (Naqvi *et al.*, 2000; Mallin and Cahoon, 2003).

Improved utilisation of phytate-phosphorous by farming animals, as an alternative to the use of inorganic phosphorous resulted in the increased use of phytase enzymes as a feed supplement. Numerous animal trials have shown that adding phytase

to feed at 500 to 1,000 phytase unit' s kg⁻¹ may replace inorganic-phosphorous supplements for pigs and poultry and reduce their phosphorous excretion by approximately 50% (Lei *et al.*, 1993; Augspurger *et al.*, 2003).

An alternative approach to decrease the phytic acid content of the agricultural products could be the use of chemical methods (e.g. extraction and precipitation), although, such methods affect the nutritional quality of the product and are generally expensive (Pandey *et al.*, 2001a).

1.2 Phytase Overview

Phytase is an acid phosphohydrolase that catalyses the hydrolysis of phosphate from phytic acid to inorganic phosphate and *myo*-inositol phosphate derivatives (Roopesh *et al.*, 2006). Phytases can be classified into three classes depending on the position of the first dephosphorylation of phytate, namely, 3-phytases, 4/6-phytases and 5-phytases (Figure 1.2). Within each class, not only can structural differences be found but also different mechanisms for the hydrolysis of phytic acid. The 3-phytase (*myo*-inositol hexakisphosphate-3-phosphohydrolase, E.C.3.1.38) removes the phosphate from the 3-position of phytate (Cosgrove, 1969, 1970; Johnson and Tate, 1969) and is found typically in microorganisms. The 4/6-phytase (*myo*-inositol-hexakisphosphate 4/6-phosphohydrolase E.C.3.1.3.26) hydrolyzes the phosphate ester at the L-6 (or D-4) position of phytic acid (Johnson *et al.*, 1969; Barrientos *et al.*, 1994) and is generally present in seeds of higher plants (Irving, 1980). The 5-phytase (*myo*-inositol-hexakisphosphate 5-phosphohydrolase, E.C.3.1.3.72) was recently identified by Barrientos *et al.* (1994) in pollen from the lily flower and the initial hydrolysis of the phosphate ester occurs at the D-5 position of phytic acid.

Phytase was first discovered by Suzuki *et al.* (1907) in the course of rice bran hydrolysing studies. They found an enzyme present in the rice bran which catalyzed the hydrolysis of phytic acid to inositol and orthophosphoric acid (Nagai and Funahashi, 1962). Over the past fifty years the scientific community has come to the consensus that phytases are widespread in nature. These can be found in plants, certain animal tissues and microorganisms like fungi, bacteria and yeast.

The phytase activity of microorganisms has been comprehensively studied. Shieh and Ware (1968) screened more than 2,000 cultures of microorganisms isolated from 68 soil samples and identified *Aspergillus niger* as the most active group producing phytases. In 1982, Powar and Jagannathan showed that an enzyme which hydrolysed

only phytate was present in culture filtrates of *Bacillus subtilis* (Powar and Jagannathan, 1982). The first report on phytase from yeast was in 1984, where Nayini and Markakis extracted phytase from baker's yeast, *Saccharomyces cerevisiae* and carried out purification and some characterisation studies (Nayini and Markakis, 1984).

The first commercial phytase was prepared by fermentation of a genetically modified *A. niger* strain in 1991 by Gist-Brocades and marketed by BASF in Europe under the brand-name NatuphosTM (Haefner *et al.*, 2005). Ever since, the commercial application and the research on phytase developed a symbiotic relationship and became an increasingly important area of interest.

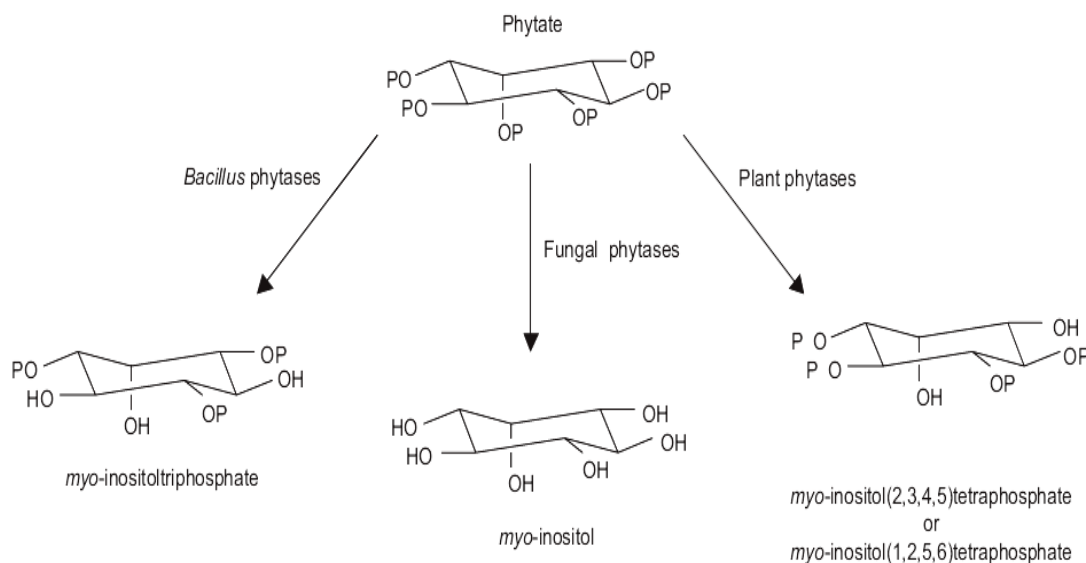


Figure 1.2 Schematic diagram showing end products from phytate hydrolysis mediated by different phytases

Adapted from Rao *et al.* (2009).

1.2.1 Sources of phytase

Phytases occur widely among plants, animals and microorganisms. Microbial sources of phytase are widespread and can be found in soils, aquatic systems and in animals. In the last fifteen years, research has indicated that several strains of bacteria, yeast and fungi can produce high yields of phytase with application at the industrial scale. With this objective in mind, scientists started to purify and express phytase in a wide range of hosts using various biochemical methods. Depending on the source and/or expression host, phytases can present different biophysical and biochemical properties (Rao *et al.*, 2009).

1.2.1.1 Fungal phytases

One of the first systematic studies on fungal phytase was reported by Shieh and Ware (1968), where various microorganisms were tested for extracellular phytase production and a strain of *Aspergillus niger* known as *Aspergillus ficuum* NRRL 3135 was identified as the most efficient. They were particularly impressed with this strain not only because it produced the highest phytase activity but it also had favourable production characteristics i.e. no sporulation. Since then, this strain of *A. ficuum* has been investigated in numerous studies by many researchers (Ullah and Gibson, 1987; Ullah, 1988; Ullah and Phillippy, 1994; Chelius and Wodzinski, 1994; Ronglin and Qirong, 1996; Mullaney *et al.*, 2002). According to a review by Wodzinski and Ullah (1996), *A. ficuum* NRRL 3135 produces more phytase activity in liquid culture than any other naturally occurring organism. Numerous studies have documented other phytase producing fungi (see Table 1.2.), however these yielded lower activity.

The genus *Aspergillus* (*A. niger* in particular) continues to be favoured for production of phytase, other enzymes and organic acids. This is not only due to its GRAS (Generally Recognised As Safe) status, but also due to its great secretory potential and the in-depth knowledge regarding its growth cultivation (Shivanna and Govindarajulu, 2009). Two pH optima, at 2.5 and 5.0–5.5, can be observed for the *A. niger* NRRL 3135 phytase, phy-A (Wodzinski and Ullah, 1996; Dvoráková, 1998). Only one pH optimum has been noted for the pH 2.5 optimum acid phosphatase, which has been referred to as phy-B phytase (Ehrlich *et al.*, 1993). Phytases from *Aspergillus* species usually exhibit optimum temperature between 50 and 65°C (Vats and Banerjee, 2004). *A. niger* phytase (EC 3.1.3.8) has been well characterised by Ullah and Gibson (1987) and it is an extracellular glycoprotein with the mass of 85 kDa. *A. niger* phy-B phytase has also received attention from enzymologists and protein chemists because of its high catalytic activity and enhanced thermal stability (Ullah *et al.*, 2008). However, due to its restrictive and narrow pH optima, this biocatalyst has not drawn attention from animal feed industries or enzyme producers (Ullah *et al.*, 2008). The production of phytase from this fungus has been achieved by three different cultivation methods, i.e. solid state (Ebune *et al.*, 1995a), semi-solid (Han *et al.*, 1987) and submerged fermentation (Howson and Davis, 1983; Vats and Banerjee, 2004). Due to their acid tolerance and higher yield (Kim *et al.*, 1998a) fungal phytases are widely used as an animal feed additive in comparison with bacterial phytases (Soni and Khire, 2007).

Table 1.2 Examples of phytase producing fungi

Fungi	Reference
<i>Aspergillus terreus</i>	Yamada <i>et al.</i> (1968); Yamamoto <i>et al.</i> (1972); Mitchell <i>et al.</i> (1997)
<i>Aspergillus carneus</i>	Ghareib (1990)
<i>Aspergillus oryzae</i>	Shimizu (1993); Fujita <i>et al.</i> (2000)
<i>Aspergillus carbonarius</i>	Al-Asheh and Duvnjak (1994)
<i>Aspergillus niger</i>	Volfova <i>et al.</i> (1994)
<i>Aspergillus fumigatus</i>	Mullaney <i>et al.</i> (2000)
<i>Rhizopus oligosporus</i>	Wang <i>et al.</i> (1980)
<i>Myceliophthora thermophila</i>	Mitchell <i>et al.</i> (1997)
<i>Penicillium simplicissimum</i>	Tseng <i>et al.</i> (2000)
<i>Mucor racemosus</i>	Bogar <i>et al.</i> (2003b)

1.2.1.2 Yeast phytases

Yeast are ideal candidates for phytase and phosphatase research due to their mostly non-pathogenic and GRAS status; however, they have not been utilized to their full potential (Satyanarayana and Kunze, 2009). To date, only a few studies have been published on yeast phytase, such as *Saccharomyces cerevisiae* (Howson and Davis, 1983; Greiner *et al.*, 2001; Anandan *et al.*, 2007), *Saccharomyces castellii* (Segueilha *et al.*, 1993) and *Arxula adenivorans* (Sano *et al.*, 1999). Nakamura *et al.* (2000) identified among numerous yeast species that *Pichia spartinae* and *Pichia rhodanensis* exhibited the highest levels of extracellular phytase with optimal temperatures at 75-80°C and 70-75°C and optimum pH at 3.6-5.5 and 4.5-5.0, respectively. They have also verified that an intracellular phytase occurs in *S. cerevisiae*. In a recent work, Olstorpe *et al.* (2009) developed a reliable, fast and easy-to-use screening method to clarify the ability of different yeast strains to utilise phytic acid as sole phosphorous source. After measuring the specific phytase they established that *A. adenivorans* displayed the highest intra- and extracellular specific activities and that the extracellular phytase activity detected in *Pichia anomala* was strain specific. The authors also concluded that there were large differences in both extra- and intracellular phytase activities amongst the screened species.

In recent times, yeast present in the gut of aquatic species have been also studied for phytase activity. Hirimuthugoda *et al.* (2007) isolated and identified two phytase producing strains, *Yarrowia lipolitica* and *Candida tropicalis* in the intestine of sea cucumber. These strains produced high amounts of extracellular and cell bound phytase.

Li *et al.* (2008) isolated a marine yeast strain *Kodamea ohmeri* BG3 in the gut of a marine fish that produced phytase and showed its highest activity at pH 5.0 and 65°C.

Yeast have been reported to be a rich genetic resource for heat-resistant phytase, however, the possibility of applying these phytase in the industry, has not been extensively investigated (Kaur and Satyanarayana, 2009).

1.2.1.3 Bacterial phytases

Phytases have been detected in several types of bacteria, such as bacilli, enterobacteria, anaerobic ruminal bacteria and pseudomonads (Jorquera *et al.*, 2008). Cosgrove (1970) reported the dephosphorylation of the hexaphosphates of myo-inositol by *Pseudomonas sp.* phytase. Although, it was only in the last 20 years that several bacterial strains (wild or genetically modified) such as *Lactobacillus amylovorus*, *Escherichia coli*, *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Klebsiella sp.* have been applied for phytase synthesis (Pandey *et al.*, 2001a). Gram-negative bacteria are known to produce phytase intracellularly while gram-positive bacteria and fungi produce it extracellularly (Greiner *et al.*, 1993). An enzyme which liberated phosphate from phytic acid was shown to be present in culture filtrates of *B. subtilis*. This enzyme differed from other previously known phytases in its metal requirement and in its specificity for phytate. It required Ca^{2+} specifically for its activity (Powar and Jagannathan, 1982). Greiner *et al.* (1993) purified two periplasmatic phytases, named P1 and P2 from *E. coli*. The P2 enzyme was characterised as a 6-phytase based on its hydrolysis of phytate. Sreeramulu *et al.* (1996) identified that *L. amylovorus* could have the potential to improve the nutritional qualities of cereal and pulse-based food fermentations. After the screen of a range of strains of lactic acid-producing bacteria, for the synthesis of extracellular phytase, they verified that *L. amylovorus* B4552 under submerged cultivation conditions was the highest producer. The strain *Bacillus sp.* DS11A was isolated by Kim *et al.* (1998a) as producer of a thermostable phytase (DS11 phytase) which could improve the value of some grains, rice flour in particular. In their work, Sajidan *et al.* (2004) showed that a *Klebsiella sp.* strain ASR1 hydrolysed phytate. A recombinant version of this enzyme was identified as a 3-phytase and was different from other general phosphatases and phytases. These researchers proposed the *phyK* gene product as an interesting candidate for industrial and agricultural applications. In general, the phytases from bacteria have a pH optimum between neutral and alkaline (Vats and Banerjee, 2004) and have temperature optima from 40°C up to 70°C (Kim *et al.*, 1998a; Cho *et al.*, 2003). According to Igbasan *et al.* (2000) within bacterial

phytases, an enzyme with high thermal stability (*Bacillus* phytase) or high proteolytic stability (*E. coli* phytase) does exist. The future of bacterial phytases will depend on them being developed for their favourable properties as feed additives.

1.2.1.4 Plant phytases

Many plant seeds contain significant amounts of phytic acid which is degraded during germination by one or more phytases (Scott and Loewus, 1986). Seeds contain both constitutive phytase activity and phytases that are synthesized again during germination, however this last mechanism is not well understood (Nayini and Markakis, 1986).

The activity of phytase has been well described in many plants, including, wheat, rice, corn, peanut, mung beans, dwarf beans, soybeans, lettuces, rye, and other legumes and oil seeds (Chang, 1967; Mandal and Biswas, 1970; Yoshida *et al.*, 1975; Sutardi, 1986; Eskin and Johnson, 1987; Eeckhout and De Paepe, 1994).

The optimum temperature and pH measured for most plant phytases ranges from 45 up to 60°C and from 4.0 to 7.2 respectively (Reddy, 1989b). Despite numerous attempts to purify plant phytases, only a few have been purified to homogeneity or near homogeneity (Greiner *et al.*, 1998). Alkaline phytases with unique catalytic properties have been identified in plants. Garchow *et al.* (2006) purified alkaline phytase from pollen grains of *Lilium longiflorum*. These investigators suggested that the unique properties of this alkaline phytase attributed it the potential to be useful as a feed and food supplement.

1.2.1.5 Animal phytases

The existence of the first animal phytase was demonstrated in the blood and liver of calves in 1908 by McCollum and Hart. Since then, controversy has persisted regarding the existence of phytases in animals in the digestive tract of animals (especially monogastric animals). According to Rapoport *et al.* (1941), other investigators failed to find phytase in the extracts of intestine, pancreas, kidney, bone, liver and blood of several species of animals.

Preliminary work on the activity of phytase produced by rumen microorganisms was initiated by Raun *et al.* (1956) and undertaken again by Yanke *et al.* (1998). They have examined the presence of phytase activity in species of obligatory anaerobic ruminal bacteria and concluded that the most highly active strain was *Selenomonas ruminantium*.

With the objective of outlining the complete system of phytate degradation in the gut of humans and the enzymes involved, Schlemmer *et al.* (2001) carried out a study using pigs as model for humans. They concluded that negligible amounts of endogenous phytase activity were found in stomach chyme and small intestine, though, in the colon the phytate hydrolysis was of an endogenous origin.

Intestinal bacteria with endogenous phytase activity were discovered in several species of fish. Huang *et al.* (2009) screened the intestinal contents of grass carp and found the phytate-degrading isolates, *Pseudomonas*, *Bacillus* and *Shewanella* species.

1.3 Fermentation Strategies

1.3.1 Solid State Fermentation versus Submerged Fermentation systems

Enzymes are typically produced using submerged fermentation (SmF) (Krishna, 2005). This method requires the submersion of the microorganism in an aqueous solution containing all the nutrients required for growth. However, in recent years the use of filamentous fungi in solid state fermentation (SSF) for the production of commercially important products has increased (Pandey *et al.*, 1999), becoming an attractive alternative method to submerged microbial fermentation for enzyme production.

Solid state fermentation is defined as the fermentation that occurs in the absence (or near absence) of free water; thus, the substrate must possess enough moisture to support the growth and metabolism of the microorganism (Pandey, 2003). Solid state fermentation is far from being a recent technology. Its origin can be traced back thousands of years, to the use of soy sauce, Koji, in Japan (Lambert *et al.*, 1983) and bread making in ancient Egypt (Sato and Sudo, 1999). Koji is an enzyme preparation usually obtained by growing *Aspergillus oryzae* on steamed rice, this mixture is then used as inoculum and added to the main substrate, e.g. soya beans, used for soy sauce production (Lambert *et al.*, 1983). In Asia, enzymes and metabolites have continued to be produced by SSF, initially in the form of labour-intensive, hand-made processes. However, these traditional processes have been automated into higher industrial SSF systems (Hölker and Lenz, 2005). In Western countries SSF was neglected for the most part in the 1940s, due to the importance of penicillin during World War II.

Penicillin was produced in large quantities in submerged fermentation thus transforming this technology into a role model for production of any compound by fermentation (Pandey, 2003). Over the past 30 years considerable scientific interest emerged promoting SSF as a science for researchers and as an industry for manufacturers. Some of these areas comprised the pollution of soils and the potential use of bioremediation, and the necessity to find alternatives for animal feeding (Durand, 2003). Consequently, the application of upgraded SSF technologies has supplied a wide range of new and useful products not only environmentally friendly but also commercially viable. Some of these goods included industrial enzymes (e.g. phytase, cellulase and xylanase), antibiotics (e.g. penicillin and cyclosporine) and other secondary metabolites, enriched food stuffs, biofuels (bioethanol and biodiesel), organic acids (citric and lactic acid), and aromatic compounds (Krishna, 2005).

The main advantage of SSF is that the enzyme yields achieved are generally higher than through SmF (Viniestra-González, 1998). Table 1.3 compares and contrasts solid state and submerged fermentation systems.

Table 1.3 Differences between solid state fermentation and submerged fermentation

Adapted from Mitchell and Lonsane (1992).

Solid State Fermentation	Submerged Fermentation
Culture medium is not free flowing	Culture medium is always free-flowing
Depth of the medium is usually shallow	Medium depth varies with bioreactor type
Single water insoluble substrate provides carbon, nitrogen, minerals and energy	Different water soluble sources of nutrients are used
Gradients in nutrient concentration are common	Nutrients are uniformly distributed throughout the fermentation
Water availability is just sufficient to support optimum growth of the culture	Water availability is abundant
Culture system involves three phases, solid, liquid and gaseous	Involves two phases, liquid and gaseous
Culture system is not aseptic beyond the medium sterilisation	Whole system is always under aseptic conditions
Rigorous control of parameters is not required, except for heat removal, oxygen supply and moisture	Rigorous control of all parameters during fermentation is essential
Inoculum ratio is always large	Inoculum ratio is usually low
System may or may not involve agitation	Agitation is often essential
Fungal growth involves penetration of the hyphae deep into solid substrate particles	Fungal mycelial cells grow in the form of individual mycelium or mycelial pellets
Bacterial and yeast cells grow by adhering to solid particles	Bacterial and yeast cells are uniformly distributed throughout the liquid

From an engineering and economic point of view, SSF offers a number of advantages over SmF systems; these have been reviewed by several authors (Lambert *et al.*, 1983; Viniegra-González *et al.*, 2003; Hölker and Lenz, 2005; Krishna, 2005) and can be summarised as follows:

- Raw materials can be used as substrates and the media preparation is simple and inexpensive.
- Aeration is easily achieved due to the spaces existing in the medium and agitation is not mandatory.
- Lower energy is required while better productivity (higher biomass production) and higher reproducibility are achieved.
- Solvents use and liquid effluent treatments are expected to be less.
- The final products are more concentrated so less downstream processing is required.
- The treatment of the fermented solids is very simple, since these can be extracted immediately, dried and used directly in animal feed preparations or as fertilisers.

Nevertheless, solid state fermentation systems also offer some limitations described by Lambert *et al.* (1993), Sato and Sudo (1999) and Krishna (2005), such as:

- This type of fermentation is limited to moulds that are able to tolerate the low moisture conditions.
- Agitation of the substrate bed can be difficult, resulting in uneven distribution of the cell mass, nutrients and moisture content.
- The substrate bed presents low thermal conductivity therefore, control of the temperature and heat resultant of microbial respiration or metabolism can be problematic.
- Rapid determination of growth or other fermentative processes is a problem, as they can not be rapidly measured by direct methods.
- The factors that contribute to high productions in SSF are not fully understood; therefore the cultivation and optimisation process is still an empirical strategy.

Some of the above limitations are being overcome. The past few years have seen major developments in biochemical engineering for SSF technology. These include areas like modelling and bioreactor design (Singhania *et al.*, 2009); hence

solid state fermentation is becoming a more modernised and economical industrial production system.

1.3.2 Factors affecting enzyme production in SSF

In nature fungi can grow on solid materials, like wood, plant stems and even rocks. Filamentous fungi are the group of microorganisms that is best adapted to SSF. Their hyphal mode of growth enables them to penetrate solid substrates. In low water activity (A_w), the efficient secretion of the hydrolytic enzymes occurs at the hyphal tip. Consequently, the penetration in most solid substrates is possible and it increases the access to all available nutrients within particles (Raimbault, 1998). The solid substrate acts as source of carbon, nitrogen, minerals and growth factors, and has the ability to absorb water which is required to promote growth (Goes and Sheppard, 1999). The main factors to be monitored and controlled in SSF processes include moisture content, growth matrix and temperature.

1.3.2.1 Moisture content and water activity (A_w)

The water present in SSF systems exists in a complex form within the solid matrix or as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary regions of the solid (Raimbault, 1998). Water activity values of 0.95-0.98 are usually typical for solid substrates. These values are particularly ideal for the growth of filamentous fungi, which grow relatively well at A_w values as low as 0.9 (Mitchell *et al.*, 2000). During fungal growth in SSF, higher A_w favours sporulation, while low A_w promotes spore germination or mycelial growth (Krishna, 2005). Higher moisture may result in bacterial contamination; however on the other hand, a dry substrate hinders the access to nutrients decreasing growth (Pandey, 2003). Therefore, balanced substrate moisture content is essential. Different SSF systems have distinct water retention capacities and have various optimal values of initial moisture content (Barrios-González and Mejía, 1996). Initial moisture content is a critical factor for growth and enzyme production. The control of the water activity in the substrate is essential for mass transfer of the water and solutes across the cells and it can be used to modify the metabolic production and excretion of the microorganisms (Pandey, 2003). If the water content and constant substrate volume is increased, the air content of the substrate is reduced (Gautam *et al.*, 2002). El-Batal and Karem (2001) found that the optimum moisture content was 60% for production of phytase and reduction of phytic acid in rapeseed meal by *Aspergillus niger* A-98 during solid state fermentation.

Roopesh *et al.* (2006) also verified that 60% moisture promoted the highest phytase yield produced by *Mucor racemosus* in a mixed substrate of wheat bran and sesame oil cake.

1.3.2.2 Growth matrix

Enzyme productivity is affected by the nature of the solid substrate, particle size of the substrate, moisture content, incubation temperature, presence or absence of carbon, nitrogen and mineral supplements (Chandran and Pandey, 2005). Therefore, the selection of the substrate is of critical importance in SSF.

The physical support and the energy required for a fungus to grow and produce the desired metabolite is primarily provided by a substrate (Pandey *et al.*, 2001b). Typically, substrates for SSF are complex and heterogeneous products from agriculture or by-products of agro-industry (Raimbault, 1998). The selection of a substrate for SSF possibly involves the screening of a large number of raw materials for microbial growth and enzyme formation. This selection also depends on cost and availability of the substrate (Krishna, 2005). The substrate that provides all the necessary nutrients to the microorganisms growing in it should be considered as the ideal substrate (Pandey *et al.*, 1999). However, in some cases the nutrients may be available in sub-optimal concentrations, or even absent in the substrates thus external supplementation becomes necessary (Pandey *et al.*, 1999). Examples of the SSF substrates used for phytase production are summarised in Table 1.4.

Table 1.4 Substrates used for phytase production by SSF

Substrates	Microorganism	Reference
Wheat bran, soybean meal, cottonseed meal, corn meal, canola meal, citric pulp, corn vinegar residue	<i>Aspergillus ficuum</i>	Han and Wilfred (1988); Ebune <i>et al.</i> (1995a,b); Spier <i>et al.</i> (2008); Wang <i>et al.</i> (2010)
Coconut oil cake	<i>Rhizopus oligosporus</i> <i>Mucor racemosus</i>	Sabu <i>et al.</i> (2002) Bogar <i>et al.</i> (2003b)
Rapeseed meal, cassava dregs, cowpea meal, wheat bran	<i>Aspergillus niger</i>	El-Batal <i>et al.</i> (2001); Hong <i>et al.</i> (2001); Mandviwala and Khire (2000); Gunashree and Venkateswaran (2008)
Soybean meal	<i>Aspergillus oryzae</i>	Chantasartramee <i>et al.</i> (2005)
Canola meal	<i>Aspergillus carbonarius</i>	Al-Asheh and Duvnjak (1994)
Cassava waste meal	<i>Pseudomonas</i>	Esakkiraj <i>et al.</i> (2010)
Sesame oil cake	<i>Sporotrichum thermophile</i>	Singh and Satyanarayana (2008a)

The size and the shape of the prepared substrate particles influence the accessibility of nutrients to the organism (Mitchell *et al.*, 2006). Substrates with a smaller particle size present a larger specific surface area, which is advantageous for the surface growth of filamentous fungi. It also promotes better heat transfer and exchange for oxygen and carbon dioxide between the air and the solid surface (Sato and Sudo, 1999). However, if substrate particles are too small they might agglomerate, which could interfere with microbial respiration/aeration resulting in poor growth. In contrast, larger particles uphold improved respiration/aeration (due to increased inter-particle space), but provide limited surface for microbial attack (Pandey *et al.*, 1999). da Silva *et al.* (2005), found that the particle size of lupin flour affected both phytase activity and phytic acid hydrolysis by *Aspergillus niger* in SSF and better results were obtained with medium and coarse particle sizes. The ability of wheat bran particles to remain free under high moisture conditions providing large surface area is the main reason why it is one of the most commonly used substrates for phytase production in SSF. Several reports have detailed wheat bran as the ideal substrate for phytase production (Kim *et al.*, 1998a; Pandey *et al.*, 1999; Pandey *et al.*, 2000).

1.3.2.3 Temperature

Temperature control in the substrate bed is very important for SSF since growth and production of enzymes or metabolites are usually sensitive to temperature (Sato and Sudo, 1999). However, the optimal temperatures for growth may not be the same as for product formation, so temperature changes in later stages of the fermentation might be needed (Mudgett, 1986). Temperature control during SSF can be quite difficult not only because of the usually static nature of this process, but also because most of the solid substrates present poor thermal conductivity (Krishna, 2005). Mandviwala and Khire (2000) studied the effect of temperature on phytase production by *Aspergillus niger* in SSF. They verified that the enzyme was secreted at both 30°C and 37°C; however the phytase activity was significantly higher when the fungus was grown at 30°C. Gautam *et al.* (2002) also found that the incubation temperature influenced the rate of phytase production by *A. ficuum* and *R. oligosporus* during SSF. A temperature of 30°C promoted the highest enzyme production for both fungi.

1.4. Optimisation of Microbial Phytase Production

Phytases can be produced by several sources. However, a commercial relationship exists between the animal feed industry and phytase production from microbial sources. This relationship is essentially based on finding the most cost-efficient production technique. In the last 30 years, the enzyme industry has become highly competitive, so reliable estimations of the degree to which different fermentation techniques are used are not easy to establish (Lambert *et al.*, 1983). As discussed in Section 1.3.1, there are two principal methods of cultivation, i.e. solid-state (SSF) and submerged fermentation (SmF) (Lambert *et al.*, 1983) and both methods have been widely used in phytase production. The type of strain, culture condition and the availability of nutrients, are fundamental factors that affect the yield and makes them a critical consideration before selecting a particular technique (Pandey *et al.*, 2001a). Traditionally, filamentous fungi have been employed by the animal feed industry for the production of phytases. A number of studies have found that phytase production by filamentous fungi is growth-associated (Al-Asheh and Duvnjak, 1995; Krishna and Nokes, 2001a; Vats and Banerjee, 2002; Greiner, 2005). Whilst many microorganisms including fungi, yeast and some bacteria can be used in SSF and SmF systems, this review focuses on phytase production by *Aspergillus* species and *A. niger* strains in particular.

1.4.1 Strain improvement and mutation studies for phytase production

The production level of the protein of interest in naturally occurring strains is usually too low for commercial exploitation (Punt *et al.*, 2002). Therefore, the success of any fermentation is dependent on finding an appropriate microorganism and improving its performance if necessary. Mutagenesis and genetic recombination are the conventional methods that were applied for strain improvement (Parekh *et al.*, 2000); however, in recent times, the general approach consists on the generation of overproducing strains through genetic engineering (Verdoes *et al.*, 1995). Technological developments have allowed scientists to create a new generation of strains according to a design and build concept, in which genes are introduced to a predetermined region in the genome of the strain (van Dijck *et al.*, 2003). As a result, most of the commercial phytase producing strains are genetically modified organisms

(Selinger *et al.*, 1996). The first example was a commercial phytase named NatuphosTM, a preparation of 3-phytase produced by a genetically modified strain of *Aspergillus niger* (Han *et al.*, 1999).

Classical mutagenesis with physical and/or chemical agents has been used successfully to improve the productivity of several fungal metabolites and enzymes (Baltz, 1999). Because the genetic and metabolic profiles of mutant strains are poorly characterised, mutagenesis remains a random process (Bai *et al.*, 2004). Diverse mutagens can be used to induce mutations, the most widely used of which are methyl-methane sulfonate (MMS), hydroxylamine (HA), ethyl-methane sulfonate (EMS), N-methyl-N-nitro-N-Nitrosoguanidine (MNNG), and ultraviolet (UV) radiation (Baltz, 1999). The improvement in product yield that can be achieved by mutating a single gene (once or repeatedly) is limited, therefore in a strain improvement programme a series of mutagenic agents is used (Carlile *et al.*, 2001). In their investigation Chelius and Wodzinski (1994), developed a strain improvement program where UV radiation was the only mutagen used to increase extracellular phytase production by *A. niger* (synonym *A. ficuum*) NRRL 3135. A combination of two UV treatments resulted in a 3.3-fold increase in phytase activity compared to the wild-type strain. In their recent work, Shah *et al.* (2008) applied a combination of physical and chemical mutagens to isolate hyper secretory strains of *A. niger* NCIM 563 for phytase production. Their treatment of the spore suspension consisted of an incubation period of 24 hours with 0.2% (v/v) EMS, followed by UV-radiation for 4 min. Two mutants, N-1 and N-79 were selected for further optimisation, with phytase activities 17 and 47% higher than the parent strain, respectively. Shivanna and Govindarajulu (2009) obtained an asporulating strain of *A. niger* by UV radiation and chemical mutation using different concentrations of EMS and NTG. In the mutant strain M-1, not only was the sporulation significantly reduced, but more importantly, the phytase yield was unaffected.

1.4.2 Composition of fermentation medium for phytase production

Pirt (1975) identified five essential conditions for the growth of microorganisms in a culture. These include, an energy source, nutrients to provide the essential materials from which biomass is synthesised, absence of inhibitors which prevent growth, a viable inoculum and suitable physico-chemical conditions.

The correct nutrient conditions must be optimised for maximal product formation applying the most cost-efficient approach. Constant medium development

remains a prerequisite so that an established product retains its competitive edge in the marketplace (Dahod, 1999). To improve results, it is critical to make the fermentation system as effective as possible.

Phytase production is highly influenced by the media components, in particular, the chemical composition of the different ingredients, the quality of carbon and nitrogen sources, in addition to minerals and metal ions, particularly phosphorous.

1.4.2.1 Carbon sources

The organic compounds that support growth are usually simple sugars which are rapidly metabolised. Glucose is by and large the most utilised carbohydrate source in the fermentation industry (Papagianni, 2004). For that reason, several studies contemplating the effect of glucose on phytase production by filamentous fungi on submerged (Shieh and Ware, 1968; Gibson, 1987; Martin *et al.*, 2003) and solid state fermentation conditions (Al-Asheh and Duvnjak, 1994; Ebune *et al.*, 1995a; El-Batal *et al.*, 2001; Bogar *et al.*, 2003a) have been carried out. Shieh and Ware (1968) demonstrated that the use of simple sugars such as glucose and sucrose as a single carbon source promoted the formation of mycelial pellets and a strong repression of phytase synthesis in *Aspergillus ficuum* NRRL 3135. These researchers also verified that the production of phytase in pure corn starch synthetic medium increased with an increase of the carbon: phosphorous (C/P) ratio with low levels of phosphate in the media. In addition to glucose, starch is also used as a carbon source in the liquid fermentation media. As a polysaccharide, starch is gradually assimilated by the microorganism during the synthesis of its metabolites. When heated and dissolved with water, its gelatinisation increases its viscosity. Corn starch or soluble starch have also been reported for phytase production from numerous strains of *Aspergillus sp.* (Vats and Banerjee, 2002; Gargova and Sariyska, 2003) and other filamentous fungi (Bogar *et al.*, 2003b; Singh and Satyanarayana, 2008b). Dextrin, which is partially hydrolysed starch, was utilised by Soni and Khire (2007) for phytase synthesis (224 IU/mg) from a strain of *A.niger* NCIM 563.

Other sugars used less frequently in the fermentation industry include mannitol, sorbitol and xylose. Oils can also be an option as they supply both the energy and carbon needs of the organism (Dahod, 1999).

1.4.2.2 Nitrogen sources

An adequate supply of nitrogen is a requirement to obtain rapid growth of the organism in the both initial and final phases. In filamentous fungi 15% of the mycelial dry weight may be composed of nitrogen and the media should include an appropriate source (Corbett, 1980). *Aspergillus* species are capable of utilising a wide range of nitrogen-containing compounds as an exclusive nitrogen source; this includes ammonia, nitrate, nitrite, purines, amides and most amino acids (Marzluf, 1997). Beet or cane molasses, corn-steep liquor, whey powder, soy flour, and yeast extract are also used as industrial raw materials rich in nitrogen (Papagianni, 2004). In their overview, Vats and Banerjee (2004) mentioned the importance of nitrogen for growth and phytase production by *A. niger* under submerged fermentation conditions. Ramachandran *et al.* (2005) demonstrated that some inorganic nitrogen sources such as ammonium sulphate and sodium nitrate inhibited phytase production by *Rhizopus* spp. in solid state fermentation. However, ammonium nitrate and yeast extract both had a positive influence on the phytase yield. In a recent study, Vassilev *et al.* (2007) optimised the solid state fermentation medium for *A. niger* and verified that corn steep liquor as the nitrogen source significantly enhanced phytase production.

1.4.2.3 Effect of phosphorous concentration

Shieh and Ware (1968) studied the factors affecting phytase production by fungi and concluded that production of extracellular fungal phytase was negatively correlated with the concentration of inorganic phosphate in the growth medium. Low concentrations of phosphorous in the media limited the mycelial growth, but phytase production was maximal. Conversely, an increase in phosphate in the medium promoted growth of the fungi, although phytase synthesis decreased significantly. Kim *et al.* (1999a) found that both phytase and phosphatase synthesis by *Aspergillus* sp. 5990 were maximal at low phosphate concentrations (<50 mg/L) while the opposite response occurred for higher concentrations of phosphate (>100 mg/L). In similar way, Vats and Banerjee (2002) observed that even at 0.05% (w/v) final concentration of phosphate, an abrupt decline in phytase production by *A. niger* occurred with no production at 0.1% (w/v) or higher. Both the investigations carried by Kim *et al.* (1999a) and Vats and Banerjee (2002) verified that biomass and protein concentration were not affected by the phosphate concentration in the medium.

The phenomenon by which phosphorous concentration in the growth medium acts as a regulator of phytase production has been reported by many investigators (Howson and Davis, 1983; Gibson, 1987; Ullah *et al.*, 1987; Chelius and Wodzinski, 1994; Gargova *et al.*, 1997; Nakamura *et al.*, 2000; Vuolanto *et al.*, 2001) and was observed not only in fungi, but also in yeast. However, in contrast to most reports, some researchers have found that the expression of phytase activity by bacteria in the rumen was neither inhibited nor stimulated by phosphate supplementation (Yanke *et al.*, 1998; Lan *et al.*, 2002). Also, Fredrikson *et al.* (2002) showed that in a more complex medium, access to inorganic phosphorous was not sufficient to repress phytase synthesis. They interpreted that this was the result of either an inability of inorganic phosphorous to repress enzyme production or that phytase synthesis was promoted by a component in that medium. The reasons behind the phosphorous effect in phytase production have become an area of growing interest. Recently, Makarewicz *et al.* (2006) studied the regulation of *phyC* gene expression in strains of the genera *Bacillus* and concluded that expression of the *phyC* gene was strictly dependent on phosphate starvation.

The most commonly used phosphorous sources for phytase production are KH_2PO_4 and K_2HPO_4 (Vohra and Satyanarayana, 2003), however most substrates used for both submerged and solid state fermentation have phosphorous in their composition. Bhavsar *et al.* (2010) in their study also concluded that phytase synthesis in *A. niger* NCIM 563 was affected by the inorganic phosphate content of agricultural residues in the medium under submerged fermentation conditions, which ranged from 2.8 to 8 mg/g. In contrast, agricultural residues that contained less than 4 mg/g inorganic phosphate sustained phytase production. Wheat bran as a cereal by-product contains high levels of phosphorous; however as considered in Section 1.3.2.2, is one of the most widely used substrates for solid state fermentation (Kim *et al.*, 1998a; Pandey *et al.*, 1999). Wheat bran can also be used in submerged culture to form a complex liquid medium as tested by Krishna and Nokes (1999b) and Papagianni *et al.* (1999).

1.4.2.4 Inoculum age, size and quality

The amount, type (spore or vegetative) and age of the inoculum are some of the major factors that determine the general course of fungal fermentations and its morphology (Papagianni, 2004). Wodzinski and Ullah (1996) reviewed how the properties of the inoculum affected phytase production. The inoculum size or its viscosity influenced the morphology of the fermentation (pellets or open mycelia).

Several optimisation studies for phytase production focused on the inoculum characteristics for both submerged (SmF) and solid state fermentation (SSF) conditions (Krishna and Nokes, 2001a; Papagianni *et al.*, 2001; Ramachandran *et al.*, 2005; Roopesh *et al.*, 2006; Vassilev *et al.*, 2007). The final inoculum for production fermentation must be of adequate size, which usually means having a biomass of about 10% of that to be grown in the production fermenter (Carlile *et al.*, 2001). Krishna and Nokes (2001a) demonstrated that the age of the liquid inoculum treatment and fermentation time has a strong influence on the fermentation process for phytase synthesis. It has been observed that the presence of wheat bran as a slow-releasing phosphate source can influence the form of growth of *Aspergillus* (Papagianni, 2004). The low solubility of phytate in the wheat bran is thought to result in a controlled release of substrate which directly regulates phosphate in the medium and therefore, enzyme production (Hill and Richardson, 2007). This occurrence was also verified by Papagianni *et al.*, (1999) when inclusion of wheat bran in the medium induced a filamentous morphology and improved both growth and phytase production by a strain of *A. niger*.

1.5 The Use of Phytase in Animal Nutrition

Over the past twenty years there has been a dramatic rise in the pressures placed upon animal producers demanding a more-efficient, economical and environmentally friendly approach to the industry. Regardless of location, standards for compliance are increasing and the extent of problems becomes wider each year in spite of little change in turnover and producers alike (Powers and Angel, 2008). Nowadays, feed manufacturers use enzymes as processing aids in order to enhance the nutritive value of feed raw materials, especially cereals (Sabatier and Fish, 1996).

Phytase supplementation is an environmentally friendly solution that can be adopted not only to reduce the nutrients excreted by the animals but also to provide nutritional benefits (Ramachandran *et al.*, 2005). Wodzinski and Ullah (1996) recognised that the addition of phytase to the diet of every monogastric animal reared in the US would not only diminish the phosphorous released into the environment by 8.23×10^7 Kg but also would save the animal producers 1.68×10^8 US dollars per year in its supplementation. Since then, the use of phytase as a feed additive has become widely accepted and several commercial phytase preparations (e.g. NatuphosTM, Ronozyme P, Phyzyme XP) are used in Europe and the US (Selle and Ravindran, 2007).

From their commercialisation in the early 1990s the sales value for phytase was estimated at USD\$50 million within the decade (Sheppy, 2001), where today it represents more than half of all feed enzyme sales (<\$250 million) (Wyatt *et al.*, 2008).

Several studies focusing on phytase application in animal feed (Table 1.5) have demonstrated the efficacy of using this enzyme as a tool to enhance phosphorous and nutrient utilisation in various production animals (for a review, see Cao *et al.*, 2007; Selle and Ravindran, 2007, 2008). Depending on the diet, the species and the level of phytase supplemented, phosphorous excretion can be reduced between 25 and 50% (Kornegay, 1999).

Table 1.5 Use of phytase in animal nutritionAdapted from Rao *et al.* (2009).

Phytase Source	Animal	Diet/Feed	Response criteria
<i>Escherichia coli</i>	Poultry	Maize-soybean	Increased intake of apparent metabolisable energy (AME) and metabolisable N, P and acids
<i>E. coli</i>	Pig	Maize-wheat-soybean	Improved growth performance and energy utilisation
Natuphos TM <i>Aspergillus niger</i> phytase	Pig	Barley-based diet	Improved P digestibility and utilisation and decreased P excretion
Phyzyme XP phytase	Pig	Corn-wheat-soy-canola-based diet	Improved P digestibility and P retention
Natuphos TM	Poultry		Increased plasma levels of Ca and P and reduced activity of alkaline phosphatase
Microbial phytase	Fish	Plant-based basal diet	Significant increase of bone Na, Ca, K, P and Fe contents
<i>E. coli</i>	Poultry	Corn-soybean meal	Improved growth performance, bone characteristics and retention of P, Ca, N and amino acids
<i>E. coli</i>	Poultry	Corn-soybean meal	Improved growth performance, bone mineralization, P utilisation
NovoNordisk	Poultry	Cereal-soybean meal	Increased body weight gain and feed consumption
<i>E. coli</i>	Poultry	Wheat-canola-soybean meal	Positive effect on growth, nutrient digestibility and toe ash
Ronozyme	Fish	Soybean meal based diet	Enhanced protein and P utilisation; decreased phytic acid in excreta
Natuphos TM 500	Poultry	Corn-soybean meal	Improved Ca, P, Mg, Zn retentions, tibia weight, tibia ash, Mg and Zn content in tibia
Finnfeed	Poultry	Maize-soybean	Improved ileal digestibility of nitrogen, amino acids, starch and lipids
Natuphos TM and phytaseed	Poultry	Corn-soybean meal	Increased body weight gain, feed intake, gain: feed, retention of dry matter, P and Ca; decreased P excretion
Microbial phytase	Fish	Fish meal-soybean	Positive effect on P digestibility and retention
Recombinant <i>A. niger</i> phytase	Pig	Corn-soybean meal	Improved bioavailability of phytate P

1.5.1 Phytase in poultry diets

In their groundbreaking work, Nelson *et al.* (1968) demonstrated that addition of exogenous phytase enzyme to broiler diets was an effective means of improving the availability of phytate-bound phosphorous. In 1990, Simons *et al.* showed that phytate dephosphorylation by microbial phytase added to poultry feeds increased P availability to over 60%, decreasing its amount in the droppings by 50%. Recently, Maguire *et al.* (2004) demonstrated that diets containing non-phytate phosphorous (NPP) closer to the requirements along with the use of supplemental phytase decreased the total P concentrations in litters without affecting P solubility in litters and amended soils. Ravindran *et al.* (2001) also verified that phytase supplemented to a wheat-soybean meal-sorghum-based diet improved performance and digestibility of other nutrients, such as nitrogen and amino acids, as well as energy. In a similar way, phytase added to corn-soybean diets for broilers improved phytate-P and total P digestibility (Rutherford *et al.*, 2004) in addition to improved ileal digestibility of nitrogen, amino acids, starch and lipids that were ultimately reflected in enhancements in energy (Camden *et al.*, 2001). In a comparable study, Denbow *et al.* (1995) showed that supplemental phytase increased responses in body weight (BW), ash percentage of toes and tibia, shear force and stress of tibia of the animals as well as lower mortality. Studies on the supplementation of microbial phytase to the diets of laying hens reported improved egg production, decreased broken and soft egg production rate with reduced P excretion (Um and Paik, 1999; Lim *et al.*, 2003).

1.5.2 Phytase in pig diets

Simons *et al.* (1990) found that the addition of microbial phytase to the diets of growing pigs increased the apparent absorbability of P by 24% and the P excreted in the faeces was 35% lower. This investigation was the first to describe a successful application of microbial phytase in pig nutrition (Selle and Ravindran, 2008). Lei *et al.* (1993) conducted two experiments to verify the effects of phytase addition on the utilisation of dietary zinc by weanling pigs. The supplementation of corn-soybean meal diets with microbial phytase significantly enhanced weight gain, feed intake, gain: feed ratio, plasma concentrations of inorganic phosphorous, and retention of phosphorous and calcium without affecting zinc retention. Several studies attested the capability of phytase to increase P availability to pigs at the end of the starter, grower, and finisher phases (Harper *et al.*, 1997; Gentile *et al.*, 2003; Johnston *et al.*, 2004; Shelton *et al.*, 2004; Jendza *et al.*, 2005). In these reports, the feed intake, body weight, trace mineral

availability, energy digestibility and bone mineralization were improved by supplementation of a P-deficient diet with phytase. The supplementation of phytase to a diet of gestating and lactating sows improved Ca, Mg, and total apparent total tract digestibility (ATTD) of P (Kemme *et al.*, 1997). Liesegang *et al.* (2005) suggested that addition of phytase to sow diets could help with the prevention of problems such as lameness.

1.5.3 Phytase supplementation in aquaculture species

Studies regarding the effects of supplemental phytase on the nutrient utilisation or growth of fish in common aquaculture species (e.g. rainbow trout, catfish and salmon) increased in the mid 1990s (Cao *et al.*, 2007). In an attempt to improve utilisation of phytate phosphorous, Jackson *et al.* (1996) and Robinson *et al.* (2002) considered the use of microbial phytase as a substitute for inorganic phosphorous supplementation in catfish diets an efficient and economical solution. Recently, Sajjadi and Carter (2004) confirmed higher phosphorous digestibility and retention efficiency through lower phosphorous excretion in fish supplied with a phytase-rich diet. They concluded that supplemental phytase decreased the P waste from plant-meal-based diets for Atlantic salmon and therefore the need for inorganic P supplementation. Other investigations have demonstrated further benefits of supplementary phytase on aquaculture. As an example, Storebakken *et al.* (1998) showed that a diet for Atlantic salmon supplemented with phytase resulted in improved protein digestibility, feed conversion, protein retention, and reduced metabolic N-excretion. Similarly, Cheng and Hardy (2002) demonstrated that supplemental phytase improved the digestibility of Ca, Mg, Mn, total-P, phytate-P, and gross energy (GE) in the rainbow trout.

1.5.4 Determination of phytase activity in feed

Despite considerable research on the use of phytase as an additive for animal feed, a consensus on the efficacy and amounts supplied for the different commercial phytase preparations has not yet been reached (Cao *et al.*, 2007). One major obstacle is the fact that until recently there was no International Standard Assay for expressing phytase activity. This led to confusion when analysing different phytase sources, especially when the same designation of units (phytase units) was used in different procedures (Powers and Angel, 2008). The specifics of the assays (substrate, buffer, pH, temperature) change for different experiments and this can induce errors in the measured phytase units (Selle and Ravindran, 2007).

In 2009, a standardised method that allows the analysis of all currently authorised commercial phytases in feed samples and other matrixes was finally published in the EU. This standard, known as ISO 30024:2009, is based on the principle that inorganic phosphate is released from the substrate phytate under defined assay conditions, and it has been validated for its suitability to measure enzyme activity of various phytase sources and products (FEFANA, 2009).

1.5.5 Thermostability of phytases

One of the limitations of the enzyme's efficacy as a feed additive is reduced thermostability. Pelleting is a process applied to feed to increase its uniformity and preservation, reducing the prevalence of pathogenic bacteria (Taylor and Harman, 1990). In this process smaller feed particles are agglomerated with the help of mechanical pressure, moisture and heat, to larger particles. Enzymes used as animal feed supplements should be able to survive temperatures of 60-90°C, which may be reached during pelleting (Igbasan *et al.*, 2000). Otherwise, the post-pelleting application of liquid enzymes is necessary. However, this can constitute a problem due to a requirement for expensive equipment and space restriction in the feed mills (Mascrell and Ryan, 1997). Therefore, progress in the manufacture of more thermotolerant enzymes will allow dry, powder enzymes to be used in high temperature processed feeds (Bedford, 2000).

Thermostability is an increasingly important area in the improvement or development of phytases. The temperature optima of microbial phytases are in the range of 40 to 77°C (Vats and Banerjee, 2004). According a study carried out by Wyss *et al.* (1998), *Aspergillus niger* phytase and *A. fumigatus* phytase were not thermostable. *A. niger* phytase at temperatures between 50 and 55°C underwent a permanent conformational change that resulted in 70-80% loss of enzyme activity. In contrast, the *A. fumigatus* phytase refolded completely into its fully active conformation after 20 minutes heat denaturation at 90°C. The *A. niger* acid phosphatase (pH 2.5) had higher intrinsic thermostability (up to 80°C), although the inactivation was irreversible and the enzyme activity was completely lost after treatment at 90°C. However, when Rodriguez *et al.* (2000) expressed the *A. fumigatus* phytase in *Pichia pastoris*, the recombinant phytase retained 20–39% of residual activity in sodium acetate buffer after 20 minutes of exposure to temperatures ranging between 65–90°C. It was demonstrated by Kim *et al.* (1998a, b) that the phytase from *Bacillus sp.* DS11 exhibited optimal activity at 70°C and stability at 90°C for 10 minutes incubation. The cation Ca²⁺ exerted a protecting

effect on the enzyme against thermal inactivation. Park *et al.* (2003) performed a follow up on the study of Kim *et al.* (1999b) and examined the thermostability of *A. ficcum* and *Bacillus amyloliquefaciens* DS11 phytases throughout the pelleting process at temperatures from 60 to 120°C. The activity of the fungal phytase started to decline at 70°C; however, the DS11 phytase maintained more than 85% of its activity. Most of the thermostability studies are performed *in vitro* and this can impact on the validity of the results, since the experiments performed *in vitro* may not reflect the conditions in the feed pelleting process (Park *et al.*, 2003). It is possible that some feed matrix components can promote a protective or stabilising influence on the enzymes (Walsh *et al.*, 1994). Further research on the nature of the interactions of the enzyme with the feed matrix should be considered and could provide fundamental information on the mode of action of enzymes and on the optimisation of diets under different dietary conditions (Marquardt and Bedford, 1997).

The development of thermotolerant phytases is still challenging, even though recently, pelleting stability has to some degree been improved by protected formulations or thermostable coatings that better resist pelleting.

1.5.6 Proteolysis resistance

Resistance to proteolysis and stable activity are important characteristics that should be inherent to phytases for a proper functionality within the digestive tracts of animals (Huang *et al.*, 2008). However, according to Boyce and Walsh (2006) most of the commercial phytase products available do not possess the physicochemical properties suited to their use in animal feed, such as digestive tract stability. Several research papers focusing on phytases with desirable properties were published in the last 10 years. Phillippy (1999) compared the activity of wheat and *Aspergillus niger* phytases in the presence of pepsin or pancreatin to study their capability to survive in the gastrointestinal tract. They verified that after incubation with pepsin at pH 3.5, the *A. niger* phytase retained 95% of its original activity, but only 70% of the wheat phytase activity was recovered. Similar results were obtained for the phytases in the presence of pancreatin at pH 6.0. The authors recognised the increased level of glycosylation on the *A. niger* phytase as the most probable justification for its greater resistance to proteolysis. Matsui *et al.* (2000) evaluated the stability of phytase from yeast or *A. niger* in simulated gastric conditions of pigs. They concluded that the yeast phytase was less efficient than the *A. niger* phytase during incubation in acidic solutions with pepsin.

Wyss *et al.* (1999) demonstrated that through side-directed mutagenesis, the exposed surface loops in the phytase molecules may be engineered as a strategy for improving phytase stability during feed processing and in the digestive tract. Simon and Igbasan (2002) analysed phytases from different microorganisms and showed that *Escherichia coli* and consensus phytases presented the highest residual activities in a simulated stomach digestion. Thus, the *Bacillus* phytase was more resistant to pancreatin. In more recent work, Zhang *et al.* (2010) compared *Aspergillus* phytases for proteolysis resistance and found that *A. ficuum* NTG-23 had increased stability in the presence of pepsin and trypsin than *A. niger* and *A. oryzae*. However, it is important to consider, that despite the convenience of the *in vitro* characterisation as an indicator of the commercial potential of phytases, the actual functional applicability can only be fully determined *in vivo* by direct animal trials (Boyce and Walsh, 2006).

1.5.7 Future perspectives

The development of enzyme technologies based on supplementing diets with microbial phytase has proven to be a practical and effective method of improving phytate digestibility in monogastric animal diets. A thermostable and proteolytic resistant phytase continues to be highly desirable for the animal nutrition industry. However, this ideal phytase will only be competitive if produced in high yield by a relatively inexpensive system (Vats and Banerjee, 2004).

Future developments in molecular biology may increase phytase efficacy, reduce phytate accumulation in plants or increase endogenous phytase synthesis in both plants and animals (Selle and Ravindran, 2007). Therefore ongoing interest in developing more adapted phytases with the ultimate goal to produce this enzyme through cost-effective processes and establish the required suitability for its industrial application (Vats *et al.*, 2009).

1.6 Project Objectives

The overall goals of this project were as follows:

- Stabilise the growth and phytase production of a strain of *Aspergillus niger* (*A. niger* Phy-A) through optimisation of slant media composition and growth conditions.
- Optimise the liquid seed media through the assessment of the effects of thickening agents and media supplementation on phytase production.
- Optimise the solid state fermentation conditions through assessment of different substrates, examining the effect of liquid inoculum age and volume, in addition of media supplementation, followed by determination of optimal incubation period and temperature.
- Assess the thermostability of the phytase enzyme.
- Examine the proteolytic stability of the enzyme using *in vitro* simulated gastrointestinal digestion.
- Determine the enzyme stability in ruminal fluid

Chapter 2

2. Materials and Methods

2.1 Materials

Chemical, solvents and other reagents:

All materials used were of molecular biology grade or the highest grade available.

All bacteria and fungal culture media reagents were supplied by Difco Laboratories, Inc., Michigan, U.S.A., or Sigma-Aldrich, St. Louis, U.S.A.

Acetic Acid, acetone, agar, alginic acid sodium salt, ammonium acetate, ammonium nitrate, ammonium molybdate, ammonium phosphate, ammonium sulphate, arabinose, beef extract, bovine haemoglobin, carrageenan, citric acid, carboxymethyl cellulose (CMC), corn starch, corn steep liquid, 3,5-Dinitrosalicylic acid (DNS), galactose, D-(+)-glucose, gelatine, guar, gum arabic, glycine, hydrochloric acid (HCl), magnesium sulphate, maltose, mannose, oatmeal agar, pancreatin, pectin, pepsin, potato dextrose agar (PDA), phytic acid, potassium chloride, potassium phosphate, sulphuric acid, sodium acetate, sorbitol, sucrose, trichloroacetic acid (TCA), Triton[®]X-100, whey, xylan from birchwood and D-(+)-xylose were obtained from Sigma-Aldrich, St. Louis U.S.A..

Glass Microfibre Filters GF/A 25 mm from Whatman[®], Kent, U.K..

Barley β -Glucan was acquired from Megazyme, Bray, Ireland.

Peptone, PDA, skimmed milk and yeast extract were supplied by Difco Laboratories Inc., Michigan, U.S.A..

Ethanol was purchased from BDH laboratory Supplies, Poole, England.

All maltodextrins were obtained from Cargill, Incorporated, Minneapolis U.S.A..

Lodex 5 starch was obtained from Cerestar, Neuilly-sur-Seine, France.

Tapioca flour was supplied by Lifestyle Healthcare Ltd., Mamhilad Technology Park, Pontypool, South Wales.

Organic Beetroot juice was from HeartBeet, White's Fruit Farm, Ipswich, England.

Wheat bran and wheat middlings were sourced from Glasson Grain Ltd, West Quay Glasson Dock, Lancaster, U.K..

Wheat germ oil was supplied from Atlantic Aromatics, Bray, Ireland.

Oatmeal, soy flour, soy meal and wheat germ were obtained locally.

Green and red algae were a kind gift donated by Dr. Becky Timmons, Alltech Inc. Kentucky, U.S.A..

Phyzyme XP2500, Norkem phytase and Natu-mix were a kind gift donated by Dr. Becky Timmons, Alltech Inc. Kentucky, U.S.A.

The *Aspergillus niger* Phy-A strain used in this work was a kind gift donated by Dr. Richard Murphy, Alltech Inc. Dunboyne, Ireland.

2.2 Methods

2.2.1 Growth of *A. niger* Phy-A

The *A. niger* Phy-A strain was grown at 30°C. The solid growth media for this strain consisted of 39 g/L PDA.

2.2.1.1 Storage of Fungal Spores

To increase its storage time, fungal spores were gently harvested from PDA slants using 20% (w/v) skimmed milk, previously sterilised (105°C for 30 minutes). Following this, 1.0 mL aliquots of the spore mixture were aseptically transferred into sterile glass lyophilisation vials (Wheaton, New Jersey, U.S.A) and freeze-dried (Virtis Freezemobile 25 XL) for 48 hours. The vials were then sealed under vacuum and stored in a cool dry place, until use.

2.2.1.2 Isolation of Colonies from Lyophilised Vials and Slant Media Preparation

A lyophilised vial was prepared as outlined in Section 2.2.1.1 and opened in a sterile environment; sterile water (1.0 mL) was added and carefully mixed. Aliquots (0.5 mL) of the spore stock (1:50 dilution) were mixed with PDA (pre-cooled to 50°C) using the pour plate technique. The plates were incubated for 72 hours at 30°C. Subsequently, single colonies (clonal isolates) were selected and transferred to the centre of PDA slants under aseptic conditions (unless otherwise stated). Finally, the slants were incubated at 30°C for 14 days to promote adequate spore formation.

Table 2.1 describes the different compositions of culture media used for slants. All media were prepared in 1 L deionised water, with the exceptions of wheat germ extract and beetroot juice extract.

Wheat germ extract: 15.0 g of wheat bran was grounded for 2 minutes and boiled in 700 mL of deionised water for 10 minutes. When cooled the solution was filtered through cheesecloth and the supernatant volume was brought up to 1 L. This procedure was adapted from Benny (1972);

Beetroot juice extract: 400 mL of beetroot juice was diluted up to 1 L of deionised water. This procedure was adapted from Hays (1951).

2.2.1.3 Slant Selection

After the incubation period, slants were stored at room temperature (maximum two weeks). Slants were selected by visual observation of the level of sporulation; slants of this particular strain have a positive correlation between level of sporulation and high enzyme titres.

2.2.1.4 Liquid Seed Medium and Growth Conditions

The liquid seed growth medium consisted of (g/L deionised water): corn starch, 60.0; wheat bran, 20.0; peptone, 18.0; glucose, 5.0; magnesium sulphate, 1.5; potassium phosphate, 1.0; potassium chloride, 0.5. The matured clonal isolate obtained as outlined in Section 2.2.1.3 was inoculated into 100 mL of sterile liquid seed medium (105 °C for 30 minutes). Subsequently, the cultures were incubated for 72 hours at 30°C in a Gallenkamp orbital shaker (Surrey, UK) at 200 rpm.

Table 2.1 Culture media composition for slants

Medium	Abbreviation	Components	Concentration (g/L)
Difco PDA	DifPDA	Agar	15
		Dextrose	20
		Potato starch	4
Sigma PDA	SigPDA	Agar	15
		Dextrose	20
		Potato starch	4
Glucose agar	GA	Agar	15
		Glucose	20
Potato starch agar	PSA	Agar	15
		Glucose	20
		Potato starch	4
Corn starch agar	CSA	Agar	15
		Glucose	20
		Corn starch	4
Wheat starch agar	WSA	Agar	15
		Glucose	20
		Wheat starch	4
Maltodextrin 14.4 agar	MDA14.4	Agar	15
		Glucose	20
		Maltodextrin 14.4	4
Maltodextrin 17.2 agar	MDA17.2	Agar	15
		Glucose	20
		Maltodextrin 17.2	4
High glucose lodex5 agar	HGLA	Agar	15
		Glucose	20
		Lodex5	4
Low glucose lodex5 agar	LGLA	Agar	15
		Glucose	1
		Lodex5	0.11
Lodex5 agar	LA	Agar	15
		Lodex5	10
Maltose agar	MA	Agar	15
		Glucose	20
		Maltose	4
Glucose peptone agar	GPA	Agar	15
		Glucose	20
		Peptone	20
Wheat germ agar	WGA	Agar	15
		Glucose	5
Beet root juice agar	BRJA	Agar	15
		Peptone	3
		Potassium Phosphate [KH ₂ PO ₄]	2.5
Peptone PDA from Difco	PPDA	PDA	39
		Peptone	10
Phytic acid PDA from Difco	PhyPDA	PDA	39
		Phytic acid	0.25

2.2.1.5 Solid State Fermentation Conditions

Solid state fermentation (SSF) consisted of 10 g sterilised wheat bran (105°C for 30 minutes) in 250 mL Erlenmeyer flasks inoculated with 8 mL of the diluted liquid culture (Section 2.2.1.4). The flasks were then vigorously shaken to ensure a homogenous mixture and incubated for 96 hours in a relative humidity (RH) chamber (Sheldon Manufacturing Inc., Oregon, U.S.A) at 30°C and 80% RH.

2.2.1.6 Preparation of Cell Free Extracts from Liquid Culture

Cell free extracts (1.0 mL) from liquid culture were prepared by centrifuging at 4°C at 14,000 rpm in 1.5 mL eppendorfs for 2 minutes. An aliquot was retained for High Performance Liquid Chromatography (HPLC) analysis (Section 2.2.1.8).

2.2.1.7 Preparation of Cell Free Extracts from Solid State Fermentation

Following the SSF (Section 2.2.1.5), enzyme extraction was conducted for an hour by adding 90 mL of 5 mM sodium acetate buffer (pH 5.5) to each flask containing 10 g of koji. After filtration through cheesecloth to remove the wheat bran and solids, 1.0 mL aliquots were centrifuged at 14,000 rpm for 2 minutes in 1.5 mL eppendorfs to remove any remaining cellular material. Cell free extract samples were retained to assay for enzyme activity (Section 2.2.2).

2.2.1.8 Analysis of Carbohydrates by HPLC

The quantitative determination of carbohydrates (glucose, xylose, maltose) was carried out by HPLC using an Aminex HXP 87-P (Bio-Rad Laboratories Ltd., Hertfordshire, UK) for separation (T = 80°C) and refractive index detector (Waters 2410) for detection. Millipore filtered water was used as mobile phase; the flow rate was 0.6 ml/minute (Dionex D-50).

2.2.1 Enzyme Assays

2.2.2.1 Phytase Activity Assay

Crude enzyme extracts were examined for total phytase activity using a modification of the assay described by Engelen *et al.* (1994). Aliquots (0.5 mL) of the enzyme sample were appropriately diluted in 5 mM sodium acetate buffer, pH 5.5 and added to 0.5 mL of substrate solution (2.5 mM phytic acid sodium hydrate in 0.2 M sodium acetate buffer pH 5.5) for 10 minutes at 37°C in a water bath. The reaction was stopped with

the addition of 2 mL of ice-cold colour stop solution (10 mM ammonium molybdate: 5 N sulphuric acid: acetone, in the ratio 1:1:2), followed by the addition of 100 μ L of 1 M citric acid. The assays were carried out in triplicate. After the incubation and subsequent substrate hydrolysis, the enzyme activity was quantified from the amount of orthophosphate released by the hydrolysed substrate upon determining the increase in absorbance at $\lambda_{380\text{ nm}}$ (Shimadzu 160-A). The results of the unknowns were compared to a standard curve prepared with inorganic phosphate (K_2HPO_4) and the phytase activities were determined. One solid state fermentation phytase unit (SPU) is defined as the amount of enzyme that will liberate 1 μ mol of inorganic phosphate per minute at pH 5.5 and 37°C and is calculated as outlined below:

$$\text{SPU} / \text{g} = \left(\frac{\Delta A_{380} \times F \times 2 \times D}{10} \right)$$

Where:

- ΔA_{380} is the difference in absorbance between the sample and the blank
- F is the phosphate concentration ($\mu\text{mol/mL}$) corresponding to the absorbance ($\lambda_{380\text{nm}}$) 1.0 obtained from the Standard Curve
- 2 is a multiplication to a standard of 1 mL
- 10 is the time of the reaction
- D is the required dilution to be within the range of the assay

2.2.2.2 Protease Activity Assay

Protease activity was determined by a modification of the method published in the Food Chemicals Codex (Food and Nutrition Board, 1981). Aliquots (1.0 mL) of the enzyme sample were diluted in 5 mM of glycine/HCL buffer pH 2.5 and preincubated at 40°C for 5 minutes. An aliquot (5.0 mL) of pre-incubated substrate (2% (w/v) bovine haemoglobin in 5 mM glycine/HCl buffer pH 2.5) was added to the sample and incubated for 30 minutes at 40°C in a water bath. The reaction was stopped by the addition of 5 mL of 0.3 M TCA. After settling, the samples were filtered through 25 mm Glass Microfiber Filters (Whatman[®], U.K.). The absorbance reading was measured at $\lambda_{275\text{nm}}$ using quartz cuvettes (Shimadzu 160-A) to determine the levels of enzyme activity against the blanks (1 mL aliquot of the diluted enzyme sample, 5 mL of TCA and 5 mL of haemoglobin). The assay results were expressed as protease units per gram of dry weight (HUT/g) using the formula outlined below. The assays were performed in triplicate.

$$\text{HUT} / \text{g} = \left(\frac{\Delta A_{275}}{0.0084} \right) \times \left(\frac{11}{30 \times W} \right)$$

Where:

- ΔA_{275} is the difference in absorbance between the sample and the blank
- 0.0084 is the accepted absorbance of 1.1 $\mu\text{g/mL}$ tyrosine at $\lambda_{275\text{nm}}$
- 11 is the final volume of the test solution
- 30 is the reaction time in minutes
- W is the weight of the original sample

2.2.2.3 Cellulase Activity Assay

Cellulase activity was determined by a colorimetric method using a modification of the procedure described by Miller (1959). The chemical method is based on the quantification of the absorbance at $\lambda_{540\text{nm}}$ (Shimadzu 160-A) of the colour developed after the reaction between the reduced sugar and DNS. Essentially, a 0.3 mL aliquot of enzyme sample was suitably diluted in 50 mM sodium acetate buffer pH 4.8 and added to 0.3 mL of substrate solution (1% (w/v) CMC in 50 mM sodium acetate buffer pH 4.8), followed by incubation of 10 minutes in a 50°C water bath. The reaction was then terminated by the addition of 0.9 mL of DNS and incubated at 100°C for 5 minutes. The samples were cooled for 5 minutes prior to absorbance readings. The results of the unknowns were compared to a standard curve prepared with glucose. All the assays were carried out in triplicate and the results were expressed as carboxymethylcellulase units per gram of dry weight of wheat bran (CMCU/g) as calculated using the formula below:

$$\text{CMC units} / \text{g} = \left[\left(\frac{1}{\text{slope}} \right) (\Delta A_{540}) + C_o \right] \times \frac{D}{1.8}$$

Where:

- ΔA_{540} is the difference in absorbance between the sample and the blank
- C_o is the intersection of the x -axis
- 1.8 is the reaction time multiplied by the molecular weight of glucose
(10 minutes \times 0.18016 mg/micromole)
- D is the required dilution to be within the range of the assay ($0.5 < \Delta A_{540} < 0.6$)

2.2.2.4 Xylanase Activity Assay

Xylanase concentrations were determined using a modification of the method described by Bailey *et al.* (1992). An aliquot (0.2 mL) of enzyme sample was appropriately diluted in 0.05 M sodium citrate buffer pH 5.3 and added to 1.8 mL aliquot of substrate (1% (w/v) xylan, in 0.05 M sodium citrate buffer) and the reaction mixture was incubated for 5 minutes in a 50°C water bath. The reaction was stopped by the addition of 3 mL DNS and incubated for 5 minutes at 100°C. The samples were cooled for 5 minutes before taking the absorbance reading at $\lambda_{540\text{nm}}$ (Shimadzu 160-A). The results of the unknowns were compared to a standard curve prepared with xylose. The assays results were expressed as xylanase units of dry weight of wheat bran (XU/g) as determined by the formula below. All the assays were carried out in triplicate.

$$\text{XU } \mu\text{mol/mL per minute} = \frac{\Delta C \times D}{5}$$

Where:

- ΔC is the concentration
- 5 is the reaction time
- D is the required dilution to be within the range of the assay

2.2.2 β -Glucanase Activity Assay

β -Glucanase activity was determined using the method outlined in Section 2.2.2.3, using β -Glucan as substrate.

2.2.3 Inorganic Phosphorous Determination

Phosphorous was determined by the adaptation of the procedure of Engelen *et al.* (1994). The reaction mixture contained 1 mL of sample with 2 mL of the colour stop reagent (as described in section 2.2.2.1), followed by the addition of 100 μL of 1 M citric acid. The assays were carried out in triplicate. The amount of phosphorous was determined by absorbance at $\lambda_{380\text{nm}}$ (Shimadzu 160-A). The results of the unknowns were compared to a standard curve prepared with inorganic phosphate (K_2HPO_4).

2.2.4 Determination of the Viscosity of the Liquid seed medium

Samples were placed in a 30°C water bath and allowed to equilibrate to temperature (30° +/- 0.2°C). The viscosity was measured using the DV-II⁺Pro Viscometer (Brookfield Labs Inc, Middleboro, U.S.A.), with the RV spindles, in a 250 mL beaker for 5 minutes, without the guardleg. The aim was to obtain a viscometer display (% torque) reading between 10 and 100%, therefore spindle selection is critical. In the case of the liquid culture samples (Section 2.2.1.4) no pre-existing method existed, so spindle and speed selection were determined by trial and error. If the reading was over 100 a smaller spindle or slower speed was selected. Conversely, if the reading was lower than 10 a larger spindle or higher speed was chosen. The spindle was carefully attached to the viscometer and immersed into the sample (avoiding trapping air bubbles under the spindle disk) so that the annular groove was at the surface level of the sample and in the centre of the jar opening. The viscosity readings were recorded using the Rheocalc software provided by Brookfield Labs Inc.

2.2.5 Stability of phytases

2.2.5.1 Determination of Thermostability

Enzyme preparations (0.5 g) were extracted for 30 minutes at (350 rpm) in 10 mL of 5 mM sodium acetate buffer, pH 5.5.

Thermal stability was determined by measuring the residual phytase activity after incubating the enzyme extract in 5 mM sodium acetate buffer, pH 5.5 at 80°C for 1, 2, 3, 4 and 5 minutes using a thermal cycler (Grant Instruments Ltd., U.K.). This procedure was adapted from Boyce and Walsh (2006). The enzyme samples were then immediately cooled in ice and promptly assayed for phytase activity (Section 2.2.2.1).

2.2.5.2 Effect of Simulated Monogastric Digestive Tract Conditions

Enzyme preparations (0.5 g) were extracted for 30 minutes at (350 rpm) in 10 mL of 100 mM sodium acetate buffer, pH 5.5.

Residual phytase activity was determined after exposure to:

➤ Simulated gastric digestion at pH 2.5 for 2 hours (37°C):

0.1 mL freshly prepared pepsin solution (10 mg porcine pepsin in 0.2 M Glycine-HCl buffer, pH 2.5) was added to 0.1 mL of enzyme diluted in 2.5 mL 100 mM sodium acetate buffer (pH 5.5), after the pH of the mixture was adjusted to pH 2.5 with 0.5 M HCl.

➤ Simulated upper intestinal digestion at pH 6.8 for 4 hours (37°C):

0.5 mL of simulated intestinal fluid (50 mg porcine pancreatin in 0.2 M tris-maleate-NaOH buffer, pH 6.8) was added to 0.1 mL of enzyme diluted in 2.5 mL 100 mM sodium acetate buffer (pH 5.5), after the pH of the mixture was adjusted to pH 6.8 with 0.5 M NaOH.

➤ Total gastrointestinal tract simulation (37°C).

For each experiment a control was included in which the enzyme was maintained at pH 5.5 and 37°C without addition of gastrointestinal (GI) enzymes. After incubation, samples were immediately placed on ice and the pH was adjusted to pH 5.5 using 0.5 M HCl/0.5 M NaOH. Samples were diluted to 5.0 ml with 100 mM sodium acetate buffer, pH 5.5 and centrifuged (4000 rpm; 2 min; 4°C). The resulting supernatant was diluted appropriately and assayed for phytase activity (Section 2.2.2.1). Simulation of gastric and intestinal digestion was also carried out with omission of GI enzymes to establish if any activity loss was due to pH instability, susceptibility to digestive proteases, or a combination of both. This procedure was adapted from Boyce and Walsh (2006).

2.2.5.3 Effect of Ruminal Fluid on Phytase Stability

Enzyme preparations were extracted as outlined in Section 2.2.5.2.

Ruminal fluid, 0.5 mL, (pH 6.0) was added to 0.1 mL of enzyme diluted in 2.5 mL 100 mM sodium acetate buffer (pH 5.5), after the pH of the mixture was adjusted to pH 6.0 with 0.2 M sodium acetate. Two experiments were performed at 37°C with incubation period of 6 and 24 hours.

Two controls were included, one in which the enzyme was maintained at pH 5.5 and a second control where the pH was adjusted to pH 6.0. Both were maintained at 37°C, but without addition of ruminal fluid. After incubation, samples were immediately placed on ice and the pH was adjusted to pH 5.5 using 0.2M of acetic acid. The samples were prepared as described in Section 2.2.5.2 and assayed for phytase activity (Section 2.2.2.1).

Chapter 3

3. Results and discussion

3.1 Overview

Application of microbial phytase to feed ingredients has been demonstrated as a practical and successful method for improving phytate-phosphorous utilisation by monogastric animals and thus reducing phosphorous output in the manure (Maenz, 2001). However, the rate of phytase inclusion in animal diets depends on both the degree of phytic acid reduction and economical considerations (Wang and Yang, 2007). The phytase enzymes produced by conventional submerged fermentation are expensive and commercial preparations are highly priced, potentially increasing feed costs by US \$2–3 per metric ton. Therefore, solid state fermentation has gained in significance as an economical production alternative (Bogar, *et al.*, 2003).

The SSF industrial process methodology is generally performed as follows. The initial phases of the SSF process are carried out in a lab environment. The process begins with screening (if applicable in the case of non stable mutants), selection and propagation of a microbial culture that produces large amounts of the desired enzyme. The production of the culture used as inoculum can be developed on a small scale and a traditional submerged fermentation system may be used to generate the seed culture (Filer, 2001). Subsequently, the industrialisation of the process begins, as the shake flask inocula are transferred and passaged through a number of fermenters of increasing volume before transfer to the sterile solid substrate. Substrate sterilisation involves the removal of microbes and also effects the conversion of starch to sugars, modifies fibre and hydrolyses protein into peptides. Ideally the solid substrate should be a cheap agricultural by-product rich in fibre with available starch and protein in addition to providing a large surface area for mycelial growth. The inoculum and substrate are well mixed to ensure good fibre colonisation by the fungal mycelia and then evenly applied to trays. Finally, the trays are incubated in chambers with controlled temperature and humidity as well as gas exchange for the duration of the fermentation. Following drying, the crude product can be directly mixed into feed.

Phytase from *Aspergillus niger* assumed crucial industrial importance in the early 1990's when the first commercial phytase preparation became available. Since then, phytase from *A. niger* has been intensively investigated and numerous patents have been obtained to develop cost-effective microbial expression systems for production of the enzyme (Maenz, 2001).

The aim of this study was to stabilise and optimise phytase production by a non-genetically modified strain of *A. niger* in solid state fermentation for possible application in the animal feed industry. During this thesis the author attempted to mimic the SSF process on a smaller scale. The *A. niger* Phy-A strain used in this study is a hyper secretory mutant phytase strain, selected after multiple screening procedures, although, it was phenotypically unstable. Therefore, clonal isolates of the *A. niger* Phy-A strain exhibited slant to slant variability (characterised by high standard deviations in phytase activities) when cultivated in replicate solid state cultures. Consequently, if the enzyme titres of the final product varied greatly, the product would not be viable for commercialisation.

The isolation of a more stable *A. niger* Phy-A clonal isolate, maintaining and if possible, increasing its hyper-phytase secretory ability was attempted. This investigation focused on achieving the best growth strategy to lessen the noted strain variability and also optimisation of the necessary parameters for increasing phytase production in the SSF system. For this purpose, the agar slant medium composition was optimised to minimise its influence on stability of the strain. Following on from this, optimisation of the liquid seed medium was attempted, involving changes to its composition. Additionally, efforts to enhance phytase production under solid state fermentation conditions were carried out. This involved evaluating the solid substrate and investigating the effects of inoculum properties and media supplementation, in addition to optimisation of the incubation period and temperature on the fermentation. These optimisation studies were designed with a view to their potential implementation at industrial scale, thus all possible changes to the process had to be economically justified.

Stabilisation and optimisation procedures are discussed in detail in the following sections. Furthermore, the thermal and proteolytic stability characteristics of the phytase enzyme were also assessed.

3.2 Stabilisation of the *A. niger* Phy-A Strain

A high producing mutant strain of an appropriate organism is a prime requirement for all commercial fungal fermentation processes (Corbett, 1980). Strain improvement of microorganisms has been conventionally achieved through mutation and selection, and is an important requisite for enzyme production, because in general wild type strains produce low yields of product (Mala *et al.*, 2001).

Generally, the mutation methods that comprise the steps in a strain improvement programme do not affect the morphology and promote a 10-15% increase in product yield (Carlile *et al.*, 2001). Occasionally a mutation can result in a higher increase in yield, although, this event can be accompanied by changes in the strain morphology and behaviour that requires extensive modification of the medium and fermentation process (Carlile *et al.*, 2001). The *A. niger* Phy-A strain used in this study was the result of a strain improvement programme where a combination of physical and chemical mutagens was applied. However, the strain undergoes genetic drift and may revert rapidly to a low phytase producing phenotype. This reduced enzyme production correlates with the appearance of a morphological variant, visually characterised by the production of a yellow colouration by the *A. niger* Phy-A clonal isolates when cultivated on agar media. In addition, slants of this particular strain have a positive correlation between a high level of sporulation and high enzyme titres. Therefore, visual slant selection is an important empirical procedure that considerably contributes to the level of phytase production by this strain in SSF.

Initial stabilisation studies of the *A. niger* Phy-A strain focused on the design of a new slant media and verification of optimal slant incubation time.

Standard deviation is a measure of the variability or dispersion of a population, a data set, or a probability distribution. In the following experiments, the standard deviation represents the variability that was obtained from the phytase activities produced by clonal isolates of the *A. niger* Phy-A strain.

3.2.1 Screening of phytase activities produced by clonal isolates of the *A. niger* Phy-A strain

To assess the potential for phytase production, approximately 80 clonal isolates of the *A. niger* Phy-A strain were cultivated using the growth conditions described in Sections 2.2.1.2 to 2.2.1.5. Cell free extracts were obtained as outlined in Section 2.2.1.7 and assayed for total phytase activity by the modified method of Engelen *et al.* (1994) (Section 2.2.2.1).

A break down of the phytase activities produced by a representative group of clonal isolates (80) indicative of the variability observed by this strain is illustrated in Figure 3.1. The clonal isolates were isolated from single colonies originating from the same lyophilised vial prepared as described in Section 2.2.1.2, and following the incubation period the slants were selected by visual observation of the level of sporulation (Section 2.2.1.3). Fungal cultures can be stored after they have grown on

agar media and they generally survive for a number of weeks or even months (Carlile *et al.*, 2001); however, in this study, the *A. niger* Phy-A slants have a shelf life of a maximum of two weeks. Therefore, to increase its storage time and preservation, fungal spores were routinely lyophilised and maintained in vials until use (Section 2.2.1.1).

The instability of the strain and its reversion to a lower phytase-producing ability, in conjunction with a morphological change noted visually by the yellow colouration of the mycelia in the slant media, confirmed what was previously described (Dr. Richard Murphy, personal communication).

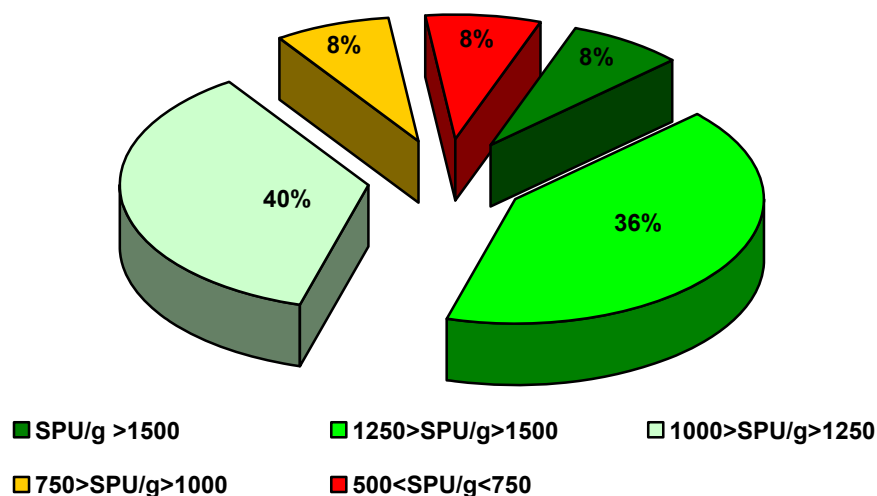


Figure 3.1 Screening of phytase activities produced by clonal isolates of the *A. niger* Phy-A strain.

A representative selection of the phytase activities from a group of 80 clonal isolates is illustrated.

The screen of clonal isolates produced an average phytase activity of 1226 SPU/g. Individual clonal isolates when cultivated in triplicate solid state culture flasks produced highly variable levels of phytase with a high standard deviation of ± 273 SPU/g between flasks. Typically, a lyophilised vial of a high producing isolate, produces daughter isolates with high variability, from which clonal isolates with phytase activities as low as 509 SPU/g and as high as 1966 SPU/g were noted. In all cases a high standard deviation was noted when clonal isolates were cultivated in replicate solid state cultures. Figure 3.1 indicates that from the 80 clonal isolates analysed for phytase activity, 8% ($n = 6$) produced between 500 and 750 SPU/g. In contrast, 8% ($n = 6$) of the clonal isolates were hyper producers of the enzyme activity (SPU/g > 1500). Most of the clonal isolates (40%) produced phytase activities within the range 1000 - 1250 SPU/g, followed by 36% ($n = 29$) of the clonal isolates that had phytase activities between 1250 and 1500 SPU/g.

The *A. niger* Phy-A hyper activity, which was the reason for its selection, was confirmed to be highly variable between clonal isolates, making it inapplicable for potential commercial production. Therefore this strain required stabilisation before any further optimisation.

3.2.2 Slant medium design

Medium design is the most important aspect to take into consideration when growing any organism (Corbett, 1980). In its composition the culture medium should include all the indispensable nutrients that an organism requires. Quantitatively, the nutrients should be present in correct and non-toxic amounts, in such a way, that any deficiency will not stop any of the other nutrients being utilised. Preferably, knowledge of an individual microorganism's biochemical characteristics and nutritional demands fosters an environment that promotes their growth in culture media *in vitro* (Dahod, 1999). However, as the *A. niger* Phy-A utilised in this thesis was a mutant strain, no prior information was available about any of these factors. Additionally, in the literature most mutants detailed are stable for the production of the desired enzyme or metabolite (Chand *et al.*, 2005; Purohit *et al.*, 2006; Himabindu *et al.*, 2007; Shah *et al.*, 2008), and optimisation studies detail fermentation media only (Adsul *et al.*, 2007; Chidananda *et al.*, 2008; Shafique *et al.*, 2010). Thus, all the solid slant media optimisation in this study was solely empirical. The main objective was to stabilise the *A. niger* Phy-A strain with the design of a new slant medium.

The strain was initially cultivated using the growth conditions outlined in Section 2.2.1., although for this experiment, clonal isolates were cultivated in each of the slant media outlined in Table 2.1 before transfer to liquid seed media (Section 2.2.1.4). Subsequent growth in SSF was carried out as described in Section 2.2.1.5 and cell free extracts (Section 2.2.1.7) were assayed for phytase activity as indicated in Section 2.2.2.1. For all the media tested, triplicate slants were individually assessed in triplicate SSF flasks.

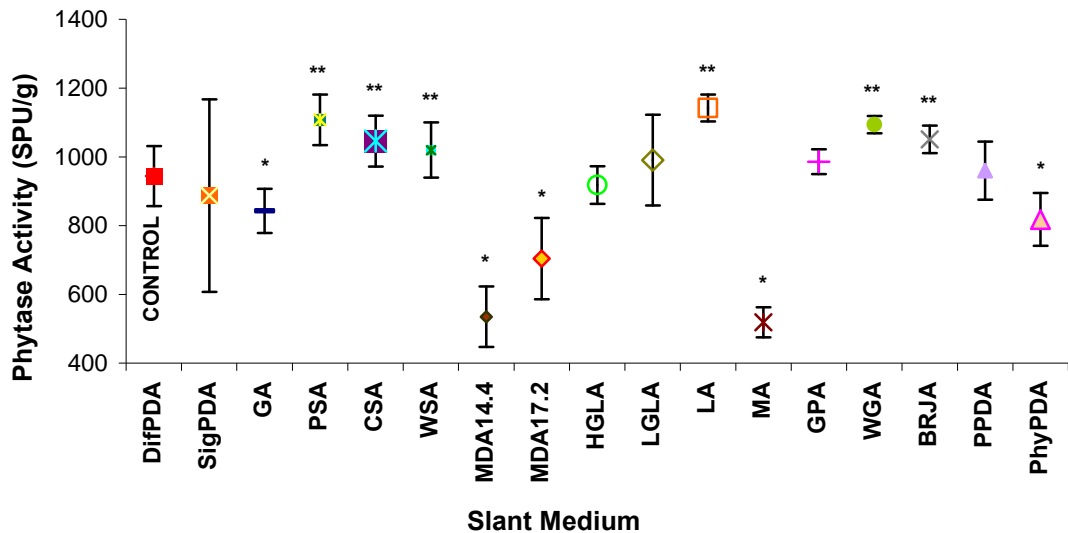


Figure 3.2 Effect of slant media composition on the variability of phytase activity.

DifPDA = Difco potato dextrose agar; SigPDA= Sigma potato dextrose agar; GA = glucose agar; PSA = potato starch agar; CSA = corn starch agar; WSA = wheat starch agar; MDA14.4 = maltodextrin 14.4; MDA17.2 = maltodextrin 17.2; HGLA = high glucose lodex5 agar; LGLA = low glucose lodex5 agar; LA = lodex5 agar; MA = maltose agar; GPA = Glucose peptone agar; WGA= wheat germ agar; BRJA = beet juice agar; PPDA = peptone potato dextrose agar from Difco; PhyPDA = phytic acid potato dextrose agar from Difco.

Data represent MEAN value \pm SD of nine measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

As can be appreciated from Figure 3.2, all clonal isolates grew on all seventeen media tested and the phytase activity results obtained for each media are individually plotted. From Figure 3.2, it is clear that the effect of the treatment (slant medium composition) resulted in a range of enzyme yields with varying standard deviations. The standard deviation was the measurement of the efficacy of the treatment to reduce the variability of the expressed phytase activity.

Statistical analysis of this data indicated that a number of treatments significantly influenced phytase production ($p \leq 0.05$) relative to the standard slant medium (Difco PDA). Six formulations had positive effects on enzyme yield, four of which included starch (PSA, CSA and WSA) or hydrolysed starch (LA) in its composition, demonstrating that starch inclusion in slant media might positively influence phytase production by this strain. Two media, lodex5 agar (LA) and wheat germ agar (WGA) resulted in increased phytase activity with reduced variation also being noted (1142 ± 39 SPU/g and 1094 ± 25 SUP/g, respectively). Phytase production was significantly reduced in five treatments ($p \leq 0.05$), including glucose (GA), phytic acid (PhyPDA), maltodextrins with higher dextrose equivalents (MDA 17.2 and 14.4) and maltose (MA). Maltose agar (MA) resulted in the lowest, yet stable, phytase activity with 519 ± 44 SPU/g. Visually, the morphology of the strain also changed with

the composition of the medium, however none of the clonal isolates exhibited the yellow pigmentation. Instead, they produced visually different levels of sporulation and mycelia sizes. In some of the slants the growth of the colony ceased, as noted with the use of all the starch agars and maltodextrin agars (except LA). This effect is known as 'staling' and is due to the production of toxic substances through metabolism (e.g. hydrogen ions and ammonia) and its occurrence implies an inadequately buffered or otherwise unsuitable medium (Carlile *et al.*, 2001). The use of SigPDA resulted in growth of clonal isolates with reduced sporulation and high variability (SD= ± 280 SPU/g).

The media that produced the highest and least variable phytase activities were LA and WGA, respectively. However, whilst the differences between enzyme yields expressed in the two media were not considerably different from each other, WGA exhibited the lowest standard deviation and for that reason was selected for further optimisation studies.

3.2.3 Optimisation of slant incubation time

The incubation period is associated with the rate of the substrate utilisation and the production of enzymes and other metabolites. When enzyme production and growth of the microorganism decreases, this can be attributed to the reduced availability of nutrients and the production of toxic metabolites (Romero *et al.*, 1998). The age of the plate culture and its influence on performance of the fermentation process is of concern for successful industrial applications, given that these processes can use primary, secondary, as well as tertiary and quaternary seed cultures as inoculum and the efficiency of the seed culture depends on the quality of the plate culture used (Krishna and Nokes, 2001a).

In order to determine the optimal incubation period for maximum phytase production by the *A. niger* Phy-A strain in the selected medium (WGA), agar cultures were allowed to incubate over a 14 day period (Figure 3.4). Triplicate slants which had been incubated from 7 - 14 days were then used to inoculate liquid seed media (Section 2.2.1.4). Subsequent to this, solid state fermentations were performed using triplicate flasks as described in Section 2.2.1.5. Cell free extracts were prepared (Section 2.2.1.7) and assayed for phytase activity as outlined in Section 2.2.2.1.

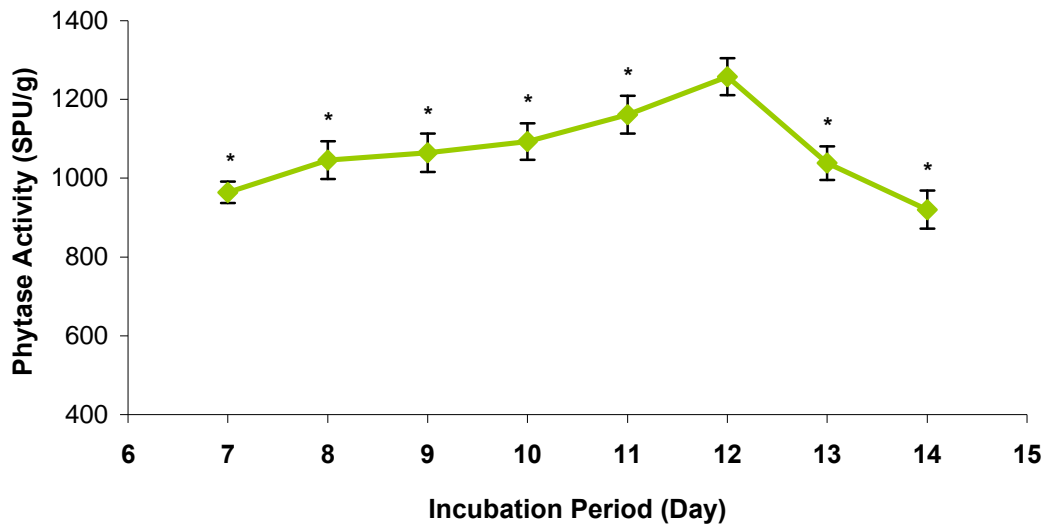


Figure 3.3 Time course of phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the optimum incubation period. * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

It is evident from Figure 3.3 that maximal phytase production was observed from slants which were incubated for 12 days (1258 ± 48 SPU/g) ($p \leq 0.05$). Longer incubation time beyond the optimum decreased phytase production by more than 200 SPU/g. The standard deviation was again low at all time points over the course of the incubation period (SPU/g < 50), this further highlights that replacing DifcoPDA with WGA reduced the variable nature of phytase production in replicate SSF flasks from single clonal isolates.

Before attempting further optimisation studies, a series of experiments were performed to confirm if the reduction in the variability of enzyme production by clonal isolates cultivated on WGA slant medium could be replicated. In these experiments slants were incubated for 12 days and the average of the phytase activities obtained was 979 ± 33 SPU/g. A low standard deviation was recorded (SD < 50 SPU/g) when clonal isolates were cultivated in replicate solid state cultures, however the phytase activity recorded was lower than the initial experiment. This confirmed that WGA reduced the standard deviation in the individual SSF flasks from single clonal isolates of the *A. niger* Phy-A strain, however the variability in enzyme productivity noted between slants still remained.

Due to the highly variable phytase productivity noted between individual slants, all subsequent optimisation studies were assessed and compared on the basis of relative activity between treatment and control.

3.3 Optimisation of the Liquid Seed Media

The quality of the inoculum contributes considerably to the success of and the high yield of fermentations (Krishna and Nokes, 2001a). The *A. niger* Phy-A mutant strain does not produce significant levels of spores in either solid or liquid media and for this reason a vegetative inoculum is required for the solid state fermentation system. The purpose of the liquid culture is to grow cells as fast as possible and usually this is achieved by supplying the essential nutrients for growth, disregarding the product formation needs (Dahood, 1999). However, in this study the liquid culture is being developed as a medium for the fermentation stage of the process. Therefore, the objective is not only to increase cell mass but also to minimise any potential impact on subsequent enzyme production. This means that the selection of the components and the optimisation of their concentrations in the medium are more complex (Dahood, 1999).

Filamentous fungi are morphologically complex microorganisms, exhibiting different structural forms throughout their life cycles. When grown in submerged culture, these fungi exhibit different morphological forms, ranging from dispersed mycelial filaments to densely interwoven mycelial masses referred to as pellets (Papagianni, 2004). Fungal growth whether as pellets or free mycelia depends on the culture conditions and the genotype of the strain. These forms can affect the process productivities by influencing the mass transfer rates (Thomas, 1992). Papagianni *et al.* (1999) found that media composition and fungal morphology affected phytase production by *A. niger* in submerged fermentation (Section 1.4.2.4). As discussed in Section 1.4.2.3, Shieh and Ware (1968) also demonstrated that depending on the phosphorous content of the cornmeal added to the media, the mould displayed a filamentous growth and higher phytase yield.

In the present work, optimisation of the liquid seed medium was performed by varying the thickening agent and nutrient sources (carbon and nitrogen) and determination of their optimal concentrations. This optimisation occurred in terms of the liquid seed medium suitability for use as inoculum for the solid state phytase fermentation system. The effects of medium composition in influencing phytase production through SSF were evaluated by changing one parameter at a time while keeping all the others constant, as recommended by other authors (Thiry and Cingolani, 2002; Nampoothiri *et al.*, 2004). The optimum conditions established for a specific factor were adopted into the basal liquid seed medium composition and used in subsequent experiments, unless otherwise stated.

3.3.1 Effect of thickening agents on phytase production

Thickening agents are widely applied in several industries, in particular in the food industry to provide body, increase stability and improve suspension of ingredients. Chen *et al.* (1997) demonstrated that through addition of thickening agents to the fermentation medium, the fungal growth form could be manipulated. Depending on the concentration of thickening agent added, the change in morphology went from compact smooth pellets to various intermediate forms and finally to filamentous mycelia.

Cornstarch medium is widely used for phytase production by *Aspergillus niger* (Shieh and Ware, 1968; Gibson, 1987; Chelius and Wodzinski, 1994; Gargova *et al.*, 1997; Papagianni *et al.*, 1999). Although starch is a carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds, the cornstarch media in previous investigations (Section 2.2.1.4) were supplemented with glucose. This suggests that starch in the liquid seed medium acts not solely as a carbon source, but also as a bulk agent conferring its viscosity.

The following experiments focused on verifying the effect of various thickening agents on the liquid seed medium, their optimal concentration, and correlation with the viscosity conferred by these agents to the medium. All the results were expressed in terms of phytase production in solid state fermentation.

3.3.1.1 Assessment of thickening agents

A study on the effect of several thickening agents on the liquid seed medium was performed by replacing the cornstarch in the basal medium (Section 2.2.1.4) with tapioca flour, oatmeal, gelatine, guar gum, pectin, agar, oat agar and carrageenan. These studies were based on previous practical trials (Dr. Richard Murphy, personal communication). The *A. niger* Phy-A strain was cultivated on wheat germ agar slants at 30°C for 12 days as optimised in Section 3.2.2. For each thickening agent tested, triplicate flasks of the liquid seed medium were inoculated as detailed in Section 2.2.1.4 apart from the modification discussed above. The liquid seed medium with cornstarch served as a control. Solid state fermentations were carried out using triplicate flasks for each of the triplicate liquid cultures (Section 2.2.1.5). Cell free extracts were prepared as described in Section 2.2.1.7 and assayed for phytase activity (Section 2.2.2.1).

Table 3.1 Effect of thickening agent on phytase production

Thickening Agent	Concentration % (w/v)			
	0.25	0.5	2.00	6.00
Cornstarch (control)				100±2
Tapioca flour				115±4**
Oatmeal				115±7**
Gelatine			86±6*	
Guar Gum			95±5*	
Pectin			105±6**	
Agar		90±3*		
Oat Agar		100±4		
Carrageenan	77±5*			

Phytase activity is expressed as a percentage the activity in the control and represents MEAN value ± SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

The results in Table 3.1 indicate that use of tapioca flour and oatmeal both resulted in a significant 15% increase in the enzyme yield ($p \leq 0.05$) relative to the standard cornstarch medium. Pectin also had a positive influence on phytase production, however in comparison with the previous treatments the increase in activity was less pronounced (5%). Additionally, whilst a low concentration of pectin was utilised, the slight increase in phytase productivity could not be justified economically. Phytase activity was not influenced by the use of oat agar, however all the other thickening agents significantly decreased the enzyme titres ($p \leq 0.05$) and was most pronounced through the use of carrageenan with a 23% decline in activity. The thickening agents influenced the morphology of the mycelia under liquid fermentation. Through the use of tapioca flour, oatmeal and pectin a more open mycelial growth was observed with a reduction in pellets and clumps relative to the control medium (Figure 3.4). Some of the other media resulted in the formation of large pellets, as can be seen from the use of carrageenan in Figure 3.4. These findings are in agreement with Papagianni *et al.* (1999) who showed that when *A. niger* grew in the form of fine pellets and clumps, it produced a more suitable inoculum for phytase production in both submerged and solid state fermentations, in contrast to inocula with large pellets which resulted in lower activities. As a consequence of the initial results, tapioca flour and oatmeal were chosen for subsequent assessment of the effects of viscosity on phytase production.

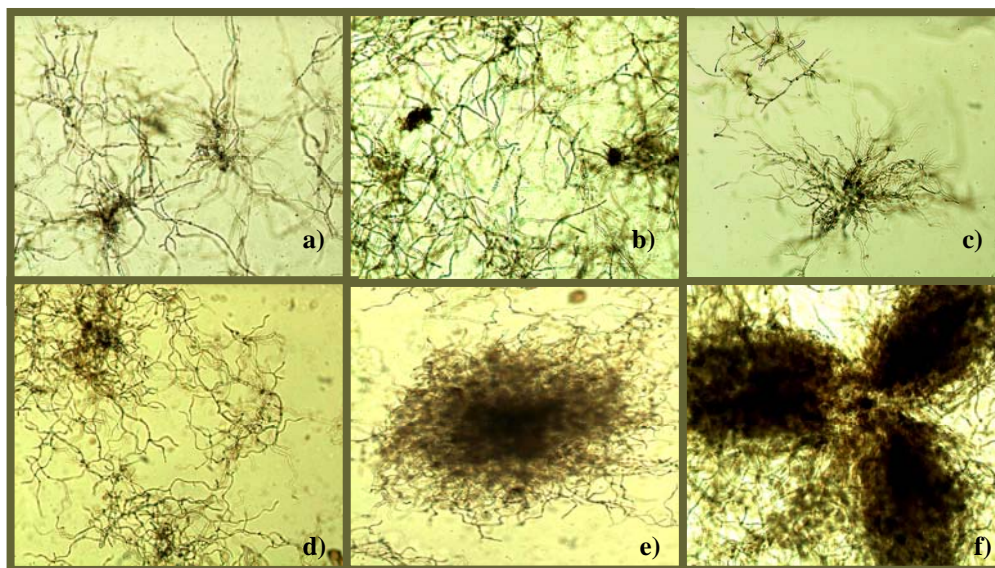


Figure 3.4 Morphological development of the *A. niger* Phy-A mycelium

a) Tapioca flour, **b)** oatmeal, **c)** pectin and **d)** cornstarch media showed free mycelial trees and clumps; **e)** and **f)** are images from the carrageenan medium where large pellets were visualised. All the images were observed at 100x optical magnification, following 72 hours of fermentation.

3.3.1.2 Determination of optimal thickening agent concentration

In an effort to determine the optimal concentration of the selected thickening agents, experiments were conducted whereby various concentrations of cornstarch, tapioca flour and oatmeal were added to the liquid seed medium and subsequently correlated with viscosity and phytase production in SSF (Figures 3.5 to 3.10). The concentration of sugars present in the different media after fermentation such as, glucose, xylose and maltose, were also calculated and are presented in Tables 3.2 to 3.4. To accomplish this, triplicate flasks of the liquid seed medium with differing treatment concentrations were inoculated as described in Section 2.2.1.4. Extra flasks were prepared for all treatments and used to determine the viscosity prior to fermentation (Section 2.2.4). Solid state fermentations were carried out in triplicate flasks of 10 g sterile wheat bran (Section 2.2.1.5) and cell free extracts prepared as outlined in Section 2.2.1.7 were analysed for total phytase activity (Section 2.2.2.1). A fraction (1.0 mL) of the liquid media was prepared as outlined in Section 2.2.1.6 to obtain cell free extracts, from which carbohydrate concentrations were determined by HPLC (Section 2.2.1.8).

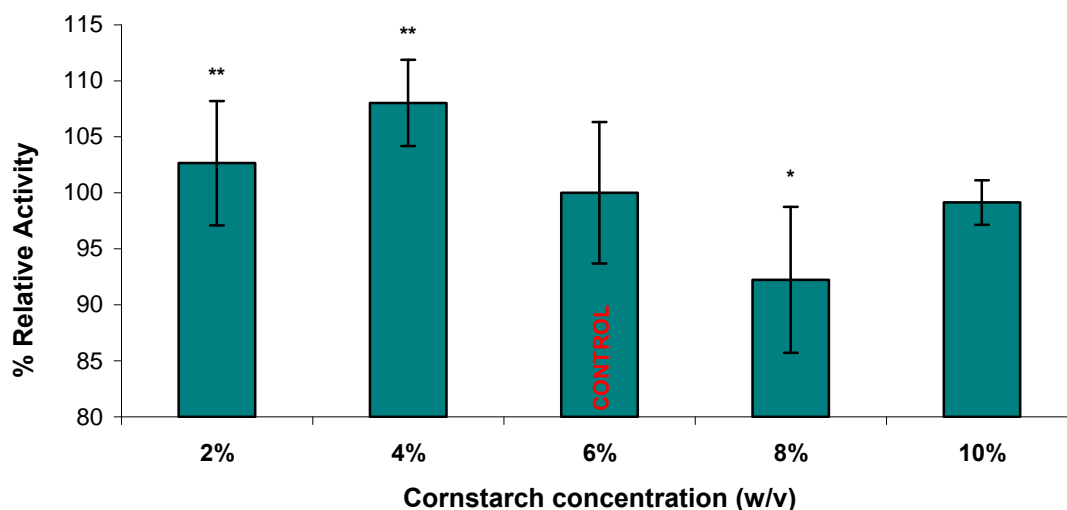


Figure 3.5 Effect of cornstarch inclusion on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%.

Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

Table 3.2 HPLC analysis of sugar profile post cornstarch liquid fermentation

% Reducing sugar	Cornstarch concentration (w/v)				
	2%	4%	6%	8%	10%
Glucose	0.59	2.37	4.31	6.13	6.86
Maltose	0.00	0.11	0.28	0.47	0.49
Xylose	0.04	0.07	0.15	0.24	0.26

Data represent MEAN value of three fermentation extracts.

As can be appreciated from Figure 3.5, the concentration of cornstarch in the inoculum influenced subsequent phytase production in SSF. The optimal cornstarch concentration was 4% (w/v) and yielded 952 ± 34 SPU/g representing a significant 8% increase in phytase activity ($p \leq 0.05$) compared to the previously used concentration (6% (w/v)). As the concentration of the cornstarch increased from 4 to 8% (w/v), a 16% reduction in phytase activity was observed. This is potentially related to the effect of glucose-induced catabolite repression, since glucose is the final product of starch hydrolysis. The repressive effect of glucose in enzyme production by *Aspergilli* has been well documented in the literature (Solis-Pereira *et al.*, 1993; Ebune *et al.*, 1995b; Nandakumar *et al.*, 1999). The sugar concentrations in the media after 72 hours of fermentation are outlined in Table 3.2. With increasing concentration of cornstarch more unutilised glucose remained in the media. At a cornstarch concentration of 2% (w/v), glucose at 0.59% (w/v) was present in the liquid culture. In terms of the optimal concentration of cornstarch, an inclusion rate of 2% (w/v) represents the best compromise since the addition of an additional 2% (w/v) only yielded a 3% increase in

phytase activity and would not be economically viable. Of note also is the hyperbolic increase in viscosity (Figure 3.6) a factor which potentially impacted negatively on mycelial growth.

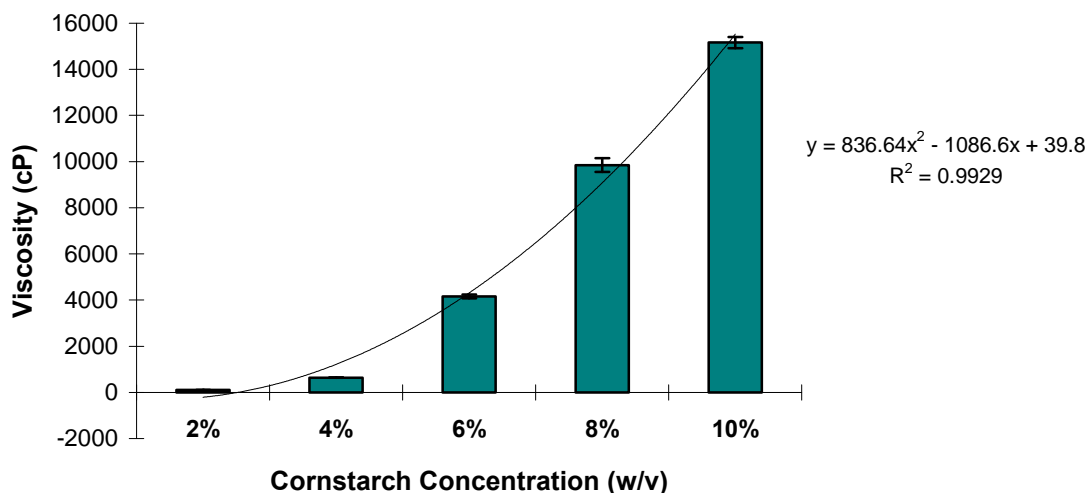


Figure 3.6 Viscosity measurements of liquid media supplemented with corn starch

Data represent MEAN value \pm SD of twenty readings over a 5 minutes period.

The data presented in Figures 3.7 and 3.8 along with Table 3.3 for the tapioca flour treatment are comparable to the previous results obtained for the cornstarch (Figures 3.5, 3.6, Table 3.2). These similarities can be explained in part by the fact that both tapioca flour and cornstarch are starches. For commercial use, starches can be derived from a variety of cereals like rice, wheat, corn and tubers like potato, tapioca, etc. The cassava plant is cultivated in most equatorial regions and is known by many names, such as tapioca and mandioca. Corn flour, cassava starch or rice flour; have been shown to be good phytase inducers in some microorganisms (Wodzinski and Ullah, 1996). Hong *et al.* (2001) showed that cassava dregs could be employed for phytase production after the addition of a nitrogen source and mineral salts. Gomes *et al.* (2005) also reported that cassava starch was shown to be a better substrate than corn starch for glucoamylase production by *Aspergillus flavus*.

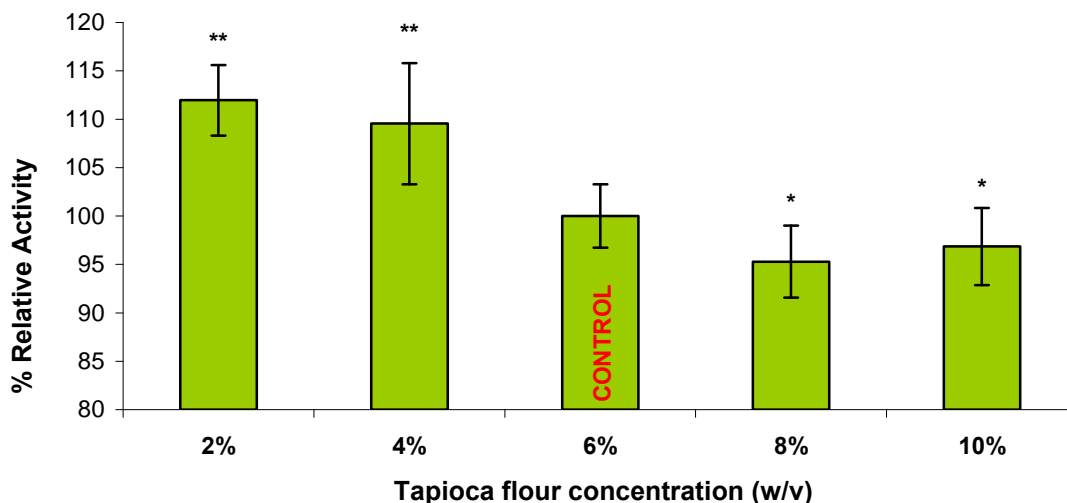


Figure 3.7 Effect of tapioca flour inclusion on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%.

Probability values were determined by One way-ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

Table 3.3 HPLC analysis of sugar profile post tapioca flour liquid fermentation

% Reducing sugar	Tapioca flour concentration (w/v)				
	2%	4%	6%	8%	10%
Glucose	0.68	2.62	3.89	5.69	6.42
Maltose	0.05	0.10	0.22	0.38	0.41
Xylose	0.05	0.06	0.11	0.20	0.22

Data represent MEAN value of three fermentation extracts.

Examination of the results presented in Figure 3.7 indicates that the addition of tapioca flour to the liquid seed media had a pronounced effect on phytase production. The phytase yield declined significantly ($p \leq 0.05$) with concentrations higher than 4% (w/v). The optimal tapioca flour inclusion level was 2% (w/v) which yielded 973 ± 32 SPU/g, representing a 12% increase in phytase production relative to the control. The carbon repression theory can also be applied in this case and the results show a relationship with the concentration of glucose in the fermentation media (Table 3.3). Tapioca flour at a concentration of 2% (w/v) represented the most efficient fermentation. Comparing Tables 3.2 and 3.3, the parallels between both starches was again illustrated from the products of their hydrolysis.

The direct comparison between cornstarch and tapioca flour on the basis of viscosity (Figures 3.6 and 3.8) is difficult as it would require a very precise optimisation of the concentrations of both. Differences in sources of starch determine different degrees of modification on the starch that influence the viscosity profile (Luallen, 2007).

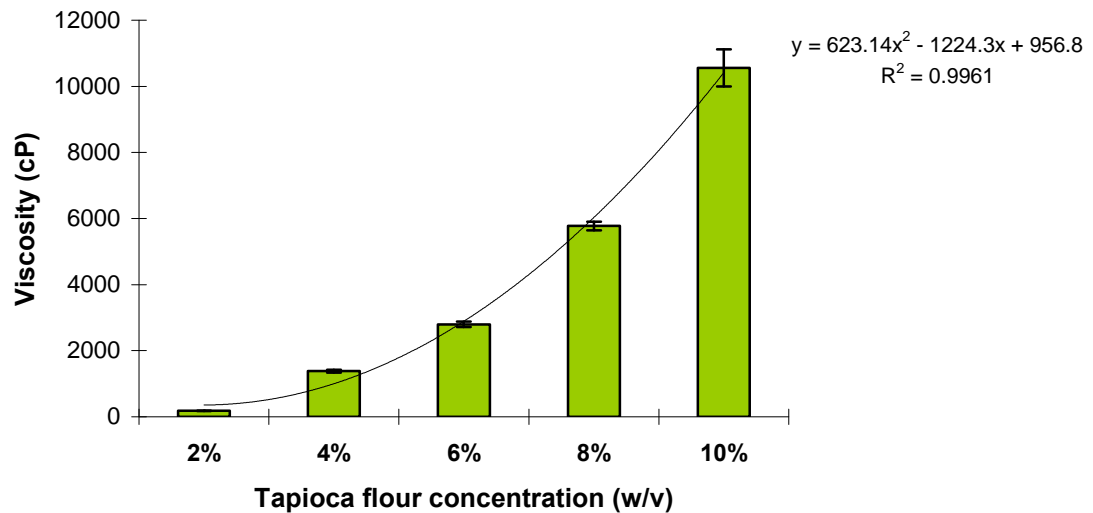


Figure 3.8 Viscosity measurements of liquid media supplemented with tapioca flour
Data represent MEAN value \pm SD of twenty readings over a 5 minutes period.

In common with other grains, starch is the most abundant component in oats where it constitutes about 60% of the dry matter of the entire oat grain (Zhou *et al.*, 1998). Oats are also nutritious and rich in minerals, but principally contain large amounts of phytate (Haefner *et al.*, 2005), the substrate for phytase hydrolysis. Li *et al.* (2008) reported promising results when attempting to optimise the cultural conditions for maximizing phytase production from marine yeast in an inexpensive oatmeal medium.

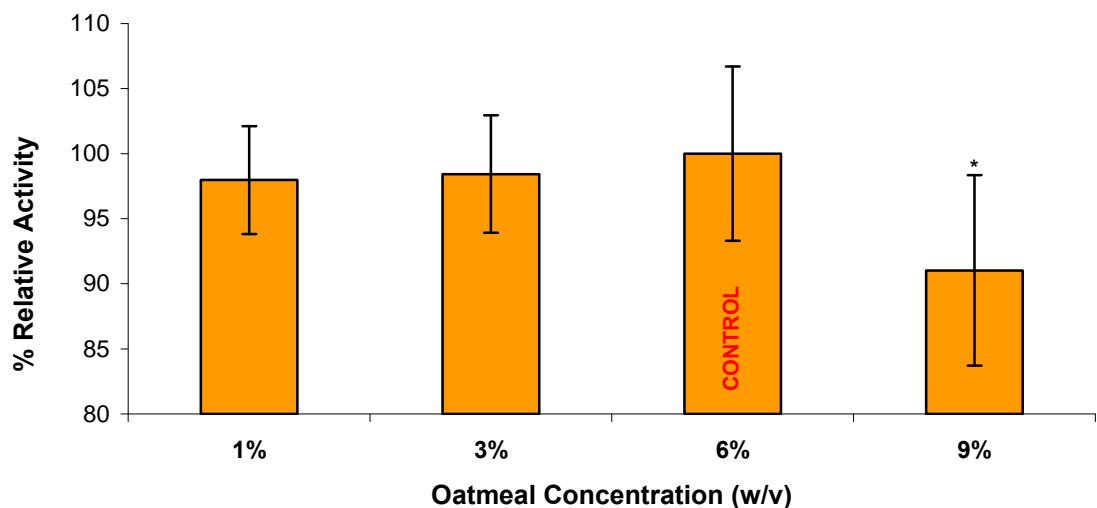


Figure 3.9 Effect of oatmeal inclusion on phytase production

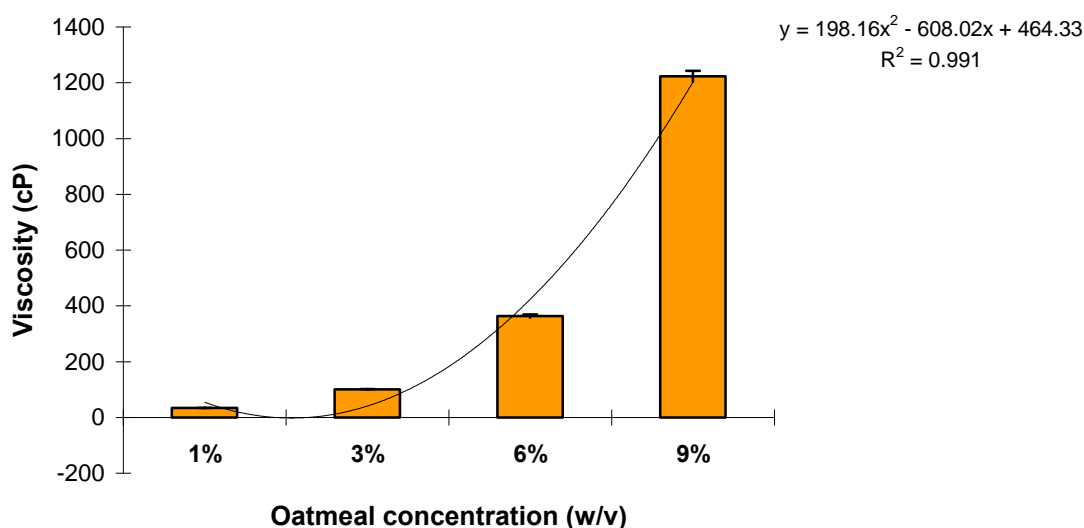
Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

Table 3.4 HPLC analysis of sugar profile post oatmeal liquid fermentation

% Reducing sugar	Oatmeal concentration (w/v)			
	1%	3%	6%	9%
Glucose	0.05	0.65	2.53	4.00
Maltose	0.00	0.05	0.15	0.33
Xylose	0.05	0.11	0.11	0.11

Data represent MEAN value of three fermentation extracts with less than 5% of variation.

It is evident from Figure 3.9 that the concentration of oatmeal did not influence the subsequent production of phytase in this study. With 1, 3 and 6% (w/v) oatmeal inclusion there was no significant difference in phytase activity ($p \leq 0.05$), however, with the inclusion of 9% (w/v) oatmeal the phytase production decreased significantly. Oatmeal inclusion at 1% (w/v) was the most cost-efficient fermentation (Table 3.4) and while a 2% relative decrease in phytase activity was noted, this was achieved with significantly less inclusion relative to the control (Figure 3.9).

**Figure 3.10 Viscosity measurements of liquid media supplemented with oatmeal**

Data represent MEAN value \pm SD of twenty readings over a 5 minutes period.

As can be appreciated from Figure 3.10, the viscosities achieved by oatmeal inclusion in the liquid media were much lower than the viscosities achieved by the other starches (Figures 3.6 and 3.8).

These series of experiments were unable to demonstrate the extent to which the thickening property of the treatments in the liquid seed media affected phytase production in subsequent SSF. In view of the fact that no clear correlation was observed between viscosity and phytase production, the thickening agents possibly acted mainly as a carbon source. However, the mycelial growth changed with the

addition of more than 6% (w/v) of starch (cornstarch and tapioca flour), as the relative amount of pellets and clumps increased in the media.

3.3.1.3 Effect of starch hydrolysates and starch on phytase production

A study was carried out in which all the liquid seed media treatments were directly compared in a single experiment. The concentrations selected from the former results (Section 3.3.1.2) were 4% (w/v) cornstarch, 2% (w/v) tapioca flour and 1% (w/v) oatmeal. These selections were based principally on the phytase yield but also took into consideration the cost benefits of the system. Because the initial liquid seed medium was cornstarch based, this thickening agent remained as the control. For this study lodex5 and glucose were also added to the media in the same concentration as the control (4% (w/v)), on the basis of functioning entirely as carbon sources and not to confer bulkiness to the media. Lodex5 is a low dextrose equivalent (DE) maltodextrin. Low DE maltodextrins (DE<5) are soluble in cold water and have low viscosity in solution (Marchal *et al.*, 1999).

This experiment was carried out in the same way as described in Section 3.2.2, through which, chosen concentrations of each treatment were supplemented to the liquid seed medium and the subsequent effect on phytase production was determined. The results are presented in Figure 3.11.

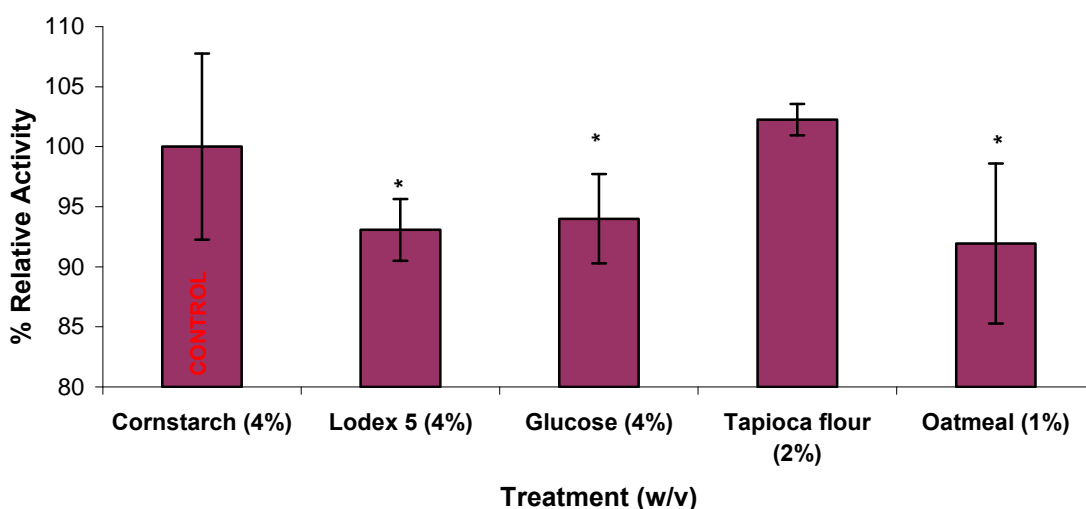


Figure 3.11 Effect of different treatments on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

Table 3.5 HPLC analysis of sugar profile post starch hydrolysates and starch liquid fermentation

% Reducing sugar	Treatment concentration (w/v)				
	Cornstarch (4%)	Lodex5 (4%)	Glucose (4%)	Tapioca flour (2%)	Oatmeal (1%)
Glucose	1.99	2.42	2.37	1.13	0.13
Maltose	0.10	0.09	0.09	0.07	0.00
Xylose	0.08	0.09	0.09	0.09	0.14

Data represent MEAN value of three fermentation extracts.

Figure 3.11 illustrates that the inclusion of lodex5 and glucose significantly decreased phytase production ($p \leq 0.05$). Addition of tapioca flour resulted in the highest enzyme yield (1022 ± 13 SPU/g) however; this effect was not significant when compared to the control (1000 ± 48 SPU/g). These results are consistent with the study of Gomes *et al.* (2005) who reported no statistical difference in the amounts of enzyme synthesized by *Thermomyces lanuginosus* when cultivated with different starches; although, it was observed that enzyme activity was higher in the medium containing cassava starch. Furthermore, in the present study the tapioca flour was required at only half the concentration needed for cornstarch and as such has an economic benefit. In addition, phytase activities produced by clonal isolates grown in this media were less variable for this treatment ($SD = \pm 13$ SPU/g). For these reasons, 2% (w/v) tapioca flour was selected as the optimal treatment. A striking observation to emerge from the data analysis was the repressing effect that lodex5 and glucose exerted on phytase production. From the HPLC analysis of the sugar profiles post liquid fermentation presented in Table 3.5, it can be seen that the media with lodex5 and glucose inclusion at 4% exhibited higher levels of unutilised glucose. These results suggest that when glucose and maltodextrin are readily available in higher concentrations in the growth media, they potentially act as catabolic-repressors of phytase production in subsequent SSF, possibly through carryover onto the solid substrate. For phytase production, the *A. niger* Phy-A strain appears to have a preference for a slow release of sugars through the progressive hydrolysis of starch instead of a readily available source. This observation is in agreement with Shieh and Ware (1968) whose findings showed that when simple sugars, such as glucose and sucrose were used as the sole source of carbon, low yields of phytase were produced. Furthermore, Roopesh *et al.* (2006) found that starch enhanced phytase production in the SSF medium compared to other sugars, such as glucose and lactose.

In summation, the preceding studies did not provide enough evidence to suggest that thickening agents acted as inducers of phytase activity; rather the results suggest that starches were a preferred carbon source for subsequent induction of phytase production in this system and also promoted an open mycelial growth. Fungi such as *Mucor*, *Aspergillus* and *Rhizopus* sp. can produce phytase and accessory enzymes by solid state fermentation (Pandey *et al.*, 2001a). Therefore the accessory enzyme production could have resulted in better utilisation of starch, which enhanced the phytase production (Roopesh *et al.*, 2006). In addition, during the dilution of the culture media, any carryover of glucose from the liquid inoculum could have potentially been the cause of the enzyme inhibition in SSF. This is supported by the fact that for each of the thickening agents, optimal phytase production was produced with a low level of inclusion and thus a low level of residual sugar as evidenced through HPLC profiling.

3.3.2 Effect of supplemental carbon on phytase production

Type, nature and concentration of carbon sources are important factors for any fermentation process, since the adequate supply of carbon as an energy source affects the growth as well as the metabolism of the organism. The extent of enzyme induction is usually related to carbon source and with the products from its utilisation. This phenomenon is known as carbon catabolite repression, i.e. suppression of enzyme synthesis by easily metabolisable sugars (Archana and Satyanarayana, 1997), such as glucose.

To study the effect of various carbon sources on phytase production, liquid seed media were prepared with 2% (w/v) tapioca flour supplemented with either 0.5% (w/v) or 3% (w/v) glucose, sucrose, xylose, mannose, sorbitol, corn steep liquor and wheat germ oil. The medium with 0.5% (w/v) glucose served as control and a medium with tapioca flour solely as carbon source was also prepared. For each carbon source tested, triplicate flasks containing 100 mL of liquid seed medium were cultivated as detailed in Section 2.2.1.4. Solid state fermentation was conducted in triplicate flasks of sterile wheat bran (10 g) as described in section 2.2.1.5 and cell free extracts were obtained (Section 2.2.1.7) and analysed for phytase activity by the method outlined in Section 2.2.2.1.

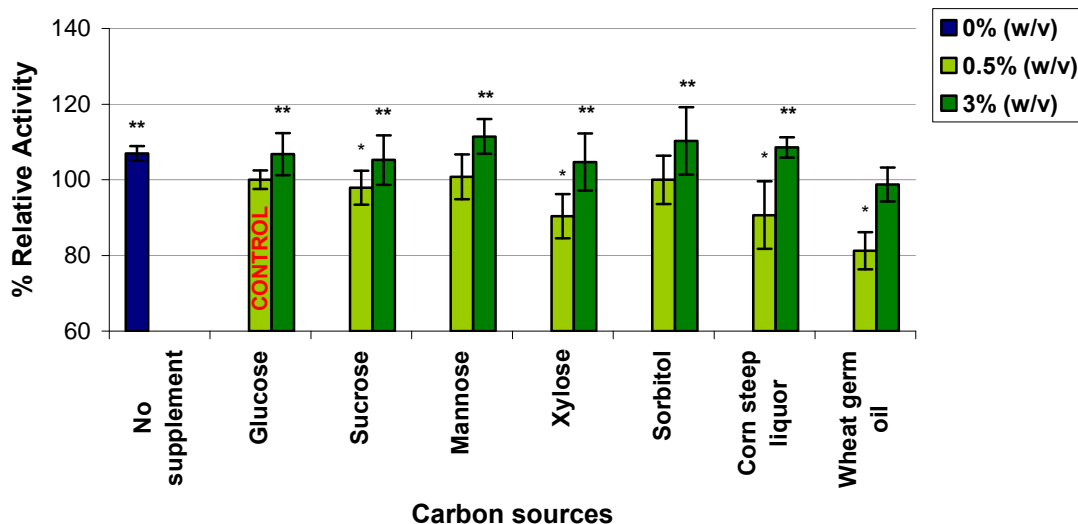


Figure 3.12 Effect of various carbon sources on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

Statistical analysis of the data shown in Figure 3.12 indicates that supplementation of the liquid seed medium with 3% (w/v) of all carbon sources except wheat germ oil, significantly increased phytase production ($p \leq 0.05$). In contrast, at 0.5% (w/v) inclusion, phytase production was significantly reduced relative to the control for most of the treatments ($p \leq 0.05$). On the other hand, supplementation with just 2% (w/v) tapioca flour as a carbon source also produced a significant increase of 7% in the phytase yield ($p \leq 0.05$). A review of the data suggests that none of the carbon sources studied enhanced phytase production at levels which would be economically or commercially beneficial to invest in. Therefore from an economical point of view the optimal carbon source was considered to be 2% (w/v) tapioca flour, which yielded 989 ± 18 SPU/g.

In the context of the research, four treatments at a concentration of 3% (w/v) had a positive effect that merits further discussion; mannose, sorbitol, glucose and corn steep liquor (CSL). Sorbitol is a sugar alcohol, mannose and glucose are hexose sugars that differ in the orientation of the hydroxyl groups (-OH), and CSL is a by-product of the wet milling process of the corn-starch industry (Lawford and Rousseau, 1997). Supplemental mannose, sorbitol, CSL and glucose significantly improved phytase production, yielding an 11, 10, 9 and 7% increase in relative activity ($p \leq 0.05$), respectively. The findings of this study differ from the findings of Gunashree and Venkateswaran (2008), where mannose and sorbitol were found to be inefficient carbon

sources for phytase production by *Aspergillus niger*. Corn steep liquor was reported by Kona *et al.* (2001) as an economical nutrient source for glucose oxidase production by *A. niger*. Glucose has been shown to increase phytase production by *A. niger* in submerged and/or solid state fermentation (Vats and Banerjee 2002, 2004); however, as discussed in Section 3.3.1.3, supplemental or residual glucose resulted in a decrease in phytase production. Therefore, determination of the optimal supplemental glucose level is an important factor to consider when optimising a media, since different levels of this sugar can induce or repress phytase production by *A. niger* in both submerged or solid state fermentation (Ebune *et al.*, 1995a; El-Batal *et al.*, 2001; Martin *et al.*, 2003; Soni and Khire, 2007).

3.3.3 Effect of supplemental nitrogen on phytase production

The mechanisms that regulate the formation of extracellular enzymes are influenced by the availability of precursors for protein synthesis (Kulkarni *et al.*, 1999). Fungi cannot fix nitrogen and need to be supplied with nitrogen-containing compounds, either in organic form such as amino acids, or inorganic form such as ammonium salts (Walker and White, 2005). For the purpose of analysing of the effect of various nitrogen sources on phytase production, the liquid seed medium consisting of 2% (w/v) tapioca flour was supplemented with 1.8% (w/v) or 5% (w/v) organic nitrogen sources, including peptone, yeast extract, beef extract, whey, soy flour, green algae and red algae, as well as inorganic nitrogen sources in the form of ammonium salts, such as ammonium phosphate, sulphate, acetate and chloride. The medium with 1.8% (w/v) peptone was the control. For each supplemental nitrogen source, triplicate flasks with 100 mL of liquid seed medium were prepared (Section 2.2.1.4). Solid state fermentations were performed in triplicate flasks of sterile wheat bran (10 g) as described in Section 2.2.1.5 and cell free extracts (Section 2.2.1.7) were analysed for phytase activity by the method outlined in Section 2.2.2.1.

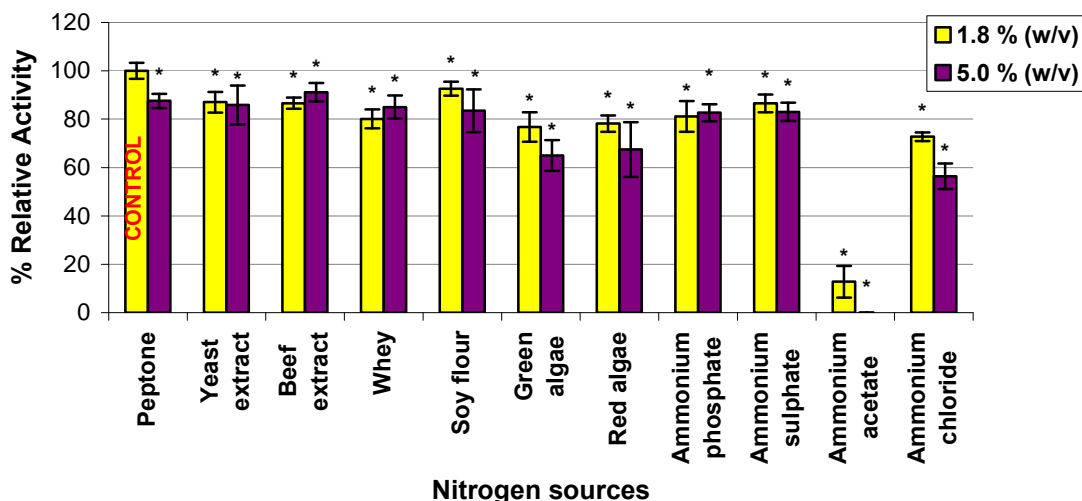


Figure 3.13 Effect of various organic and inorganic nitrogen sources on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

As evident in Figure 3.13, among the various organic and inorganic nitrogen sources added to the liquid seed medium, peptone (control) at a concentration of 1.8% (w/v) was the optimal nitrogen source yielding 1125 ± 37 SPU/g in subsequent SSF. This is comparable to reports where the significant role of nitrogen in growth and phytase production in *Aspergillus niger* was studied and maximal phytase production was achieved with supplemental peptone (Vats and Banerjee, 2002). Recently, Gunashree and Venkateswaran (2008) verified that peptone in the submerged fermentation medium increased phytase production by *A. niger*. Figure 3.13 also illustrates that phytase production was significantly reduced through supplementation with the other organic sources analysed ($p \leq 0.05$). Additionally, every ammonium based salt significantly reduced the enzyme yield ($p \leq 0.05$). This strong repression of enzyme production observed upon supplementation of the growth media with nitrogen sources could have been due to an imbalance in the C/N ratio required for enzyme production (Roopesh *et al.*, 2006). This ratio has been known to influence phytase production (Vohra and Satyanarayana, 2002). One unanticipated finding was the complete phytase inhibition when ammonium acetate was present. It was reported by Arnthong *et al.* (2010) that a combination of cassava starch as a carbon source and ammonium acetate as nitrogen source were optimal for glucoamylase production by *Rhizopus microsporus*. However, ammonium sulphate is the frequently used nitrogen source for phytase production in several investigations (Vats and Banerjee, 2002;

Nampoothiri *et al.*, 2004; Soni and Khire, 2007).

The inclusion of peptone at a concentration of 1.8% (w/v) was found to enhance phytase production, however at the higher 5% (w/v) concentration, enzyme synthesis decreased. Therefore, an additional experiment was conducted to evaluate the effect of supplemental peptone concentration and to determine its optimum supplementation level in the liquid seed medium for phytase production (Figure 3.14).

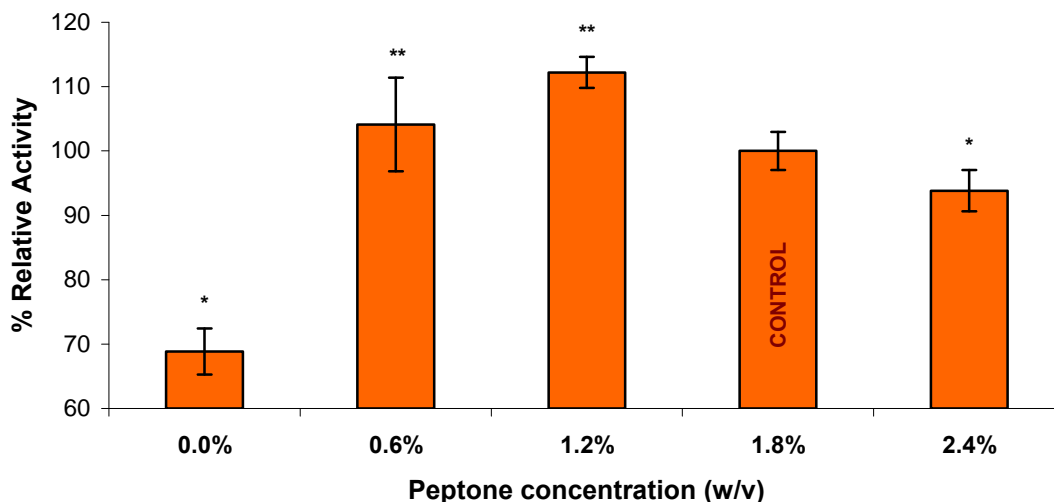


Figure 3.14 Effect of peptone concentration on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

Peptone supplementation from 0 to 3% (w/v) was assessed. The corresponding effects on phytase activity are plotted in Figure 3.14 where the highest level of phytase activity in SSF was observed in cultures inoculated with 1.2% (w/v) concentration in the liquid medium and represented a significant 12% increase relative to the control ($p \leq 0.05$). Without any peptone supplementation the phytase production significantly declined ($p \leq 0.05$). This confirmed what was previously indicated by personal communication from Paul Kilgallen (Alltech Inc.), that a minor amount of peptone was required for stable growth and phytase production by the *A. niger* Phy-A strain. Peptone inclusion was reduced from 1.8% (w/v) to 1.2% (w/v), thus lowering the effective cost of peptone.

Finally, it is evident that the optimisation of the liquid seed media was of crucial importance for phytase production by the *A. niger* Phy-A strain and involved changes in its composition that not only affected product yield, but also potential cost-savings. The inclusion of tapioca flour at 2% (w/v) reduced the amount of thickening agent, since

initially an inclusion of 6% (w/v) cornstarch was used; however it also replaced the requirement for 0.5% (w/v) glucose inclusion as a readily available sugar source in the medium. Peptone is an expensive nitrogen source and although it could not be successfully replaced by a cheaper nitrogen source, its inclusion concentration was still reduced by 0.6% (w/v).

In conclusion, the optimised liquid seed medium was determined to be (g/L deionised water): tapioca, 20.0; wheat bran, 20.0; peptone, 12.0; magnesium sulphate, 1.5; potassium phosphate, 1.0; potassium chloride, 0.5.

3.4 Optimisation of the Solid State Fermentation System

As outlined in Section 1.3.2, there are several important aspects, which should be considered for the development of any bioprocess in SSF. These include the selection of a suitable microorganism and substrate as well as the optimisation of the process parameters for product yield (Pandey, 2003). Enzyme synthesis is directly mediated by the metabolic processes of the microorganism, implying that its optimum growth on an appropriate substrate associated with the temperature, oxygen supply, quality and size of inoculum, will return maximal enzyme production (Tunga *et al.*, 1998). The selection of an adequate substrate is a key aspect of SSF acting as both physical support and source of nutrients (Pandey, 2003).

In initial studies (Section 3.2.2), the reduction in the SSF flask enzyme variability of the phytase producing *A. niger* Phy-A strain, as well as the optimisation of the liquid seed media composition (Sections 3.3.1 to 3.3.3) were achieved. Additional research involved studies to determine the best substrate, inoculum age and size, the effect of different nutritional ingredients such as carbon, nitrogen and phosphorous, as well as fermentation temperature and duration on the production of phytase enzyme by *A. niger* Phy-A in solid state fermentation. All process parameters influencing the phytase synthesis in SSF continued to be evaluated by single parameter optimisation studies.

3.4.1 Determination of the optimum substrate for SSF

Various substrates have been evaluated for phytase production in SSF (Table 1.4), however as previously discussed in Section 1.3.2.2, wheat bran is the most frequently used. The selection of a substrate for SSF depends upon several factors, including cost, availability, reliability, stability, ease of handling and above all,

the effect on the productivity process (Ahmed, 2008). Thus, the choice of a suitable solid substrate involves the screening of a number of agro-industrial materials for microbial growth and product formation (Ellaiah *et al.*, 2002). The ideal solid substrate is one that provides all the necessary nutrients for the microorganism (Kaur and Satyanarayana, 2004).

The objective of the present study was to establish the effect of different substrates and substrate combinations on phytase production by the *A. niger* phy-A strain in SSF. In an initial experiment, different sources of wheat bran (Europe, Mexico and USA), wheat middlings (midds), wheat germ, cotton seeds hulls and corn cob were used as substrates for phytase production in SSF. The *A. niger* Phy-A strain was cultivated on wheat germ agar slants at 30°C for 12 days as optimised in Section 3.2.3. Triplicate flasks of the optimised liquid seed medium were inoculated as detailed in Section 2.2.1.4. Solid state fermentations were carried out in triplicate flasks with 10 g of each substrate tested (Section 2.2.1.5). Cell free extracts were prepared (Section 2.2.1.7) and assayed for phytase activity as described in Section 2.2.2.1. The results are shown in Figure 3.15, with phytase activity expressed relative to the phytase yield produced by the control (wheat bran Europe).

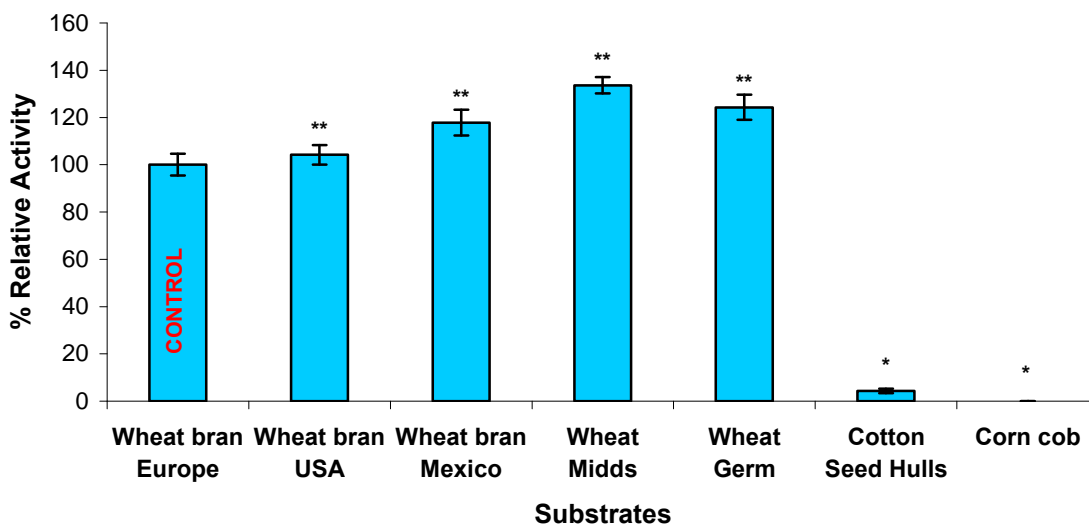


Figure 3.15 Phytase production on different substrates

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

It is evident from Figure 3.15 that using wheat midds as substrate, a significant 34% increase in phytase synthesis by *A. niger* Phy-A was noted, yielding 1205 ± 31 SPU/g ($p \leq 0.05$). A similarly impressive increase of 24% was also recorded when

wheat germ was used equating to 1121 ± 48 SPU/g ($p \leq 0.05$). Surprisingly, enzyme production on wheat bran was extremely variable depending on the source (location) of the substrate. Solid state fermentation using corn cob resulted in no growth. Additionally, on cotton seed hulls growth of the strain and enzyme production were minimal. Corn cob and cotton seed hulls were tested as alternative sources primarily due to their status as low cost agricultural waste by-products.

Based on the previous results, it was decided to investigate the reasons, if any, behind the differences noted between the phytase activities produced during SSF on the by-products of the wheat milling process i.e., the middlings and bran; and the source location of the wheat bran. It is known that the nutrient content of cereals such as wheat can be influenced by type and variety as well as environmental factors experienced during production and storage of the wheat crop (Blasi *et al.*, 1998). Phosphorous is one of these nutrients. In wheat endosperm, phytate is almost nonexistent however aleurone layers of the kernel and the bran contain considerable amounts of this element (De Boland *et al.*, 1975). Wheat midds consist of fine particles of wheat bran, wheat shorts, wheat germ and wheat flour (Blasi *et al.*, 1998) and are also smaller than the wheat bran in terms of particle size. For these reasons, an experiment was carried out in which wheat midds (WM), wheat bran from Europe (WBEU), wheat bran from the USA (WBUSA) and wheat bran from Mexico (WBMEX), were sieved through a standard mesh of two particle sizes (PS), $PS \geq 1$ mm or $PS < 1$ mm, and used for SSF. In this study the cell free extracts (Section 2.2.1.7) were not only assayed for phytase activity (Section 2.2.2.1) but also for inorganic phosphorous as determined by an adaptation of the procedure of Engelen *et al.* (1994) described in Section 2.2.3.

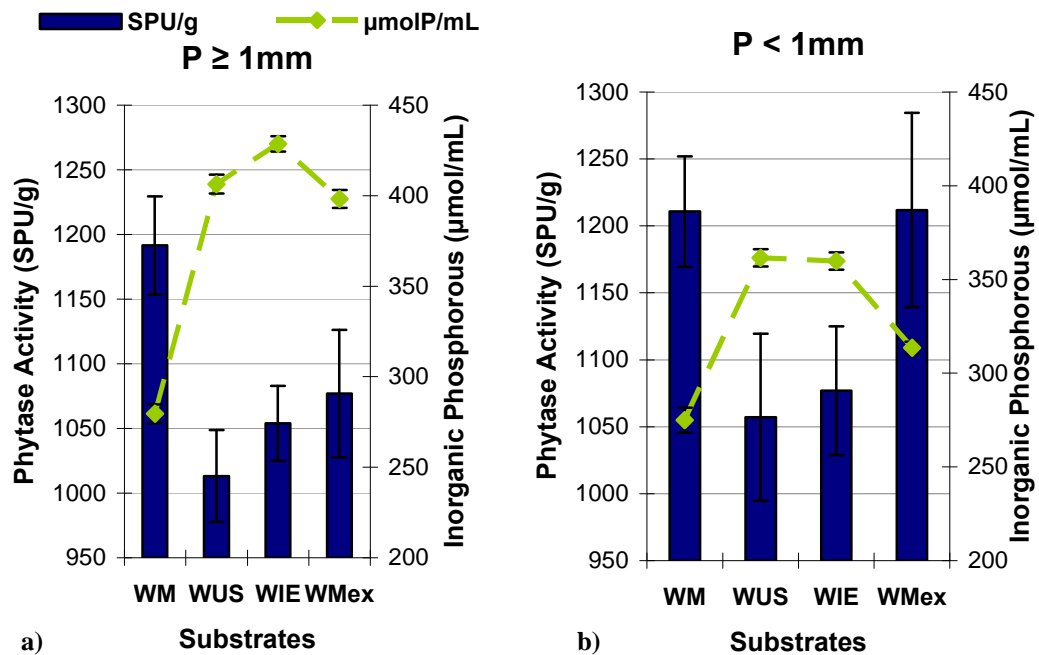


Figure 3.16 Phytase production and inorganic phosphorous content (extracted) of different particle sizes of wheat substrates

Data represent MEAN value \pm SD of twenty seven measurements.

a) Particle sizes bigger than 1mm ($P \geq 1$ mm); b) Particle sizes smaller than 1mm ($P < 1$ mm).

The phosphorous content determined in this experiment was the soluble P and is not a representation of the total P content of the sources.

From Figures 3.16 a) and b) it can be observed that phytase production was correlated not only with the particle size of the substrate, but also with the phosphorous content. In general, higher phosphorous contents corresponded to lower phytase activities. As outlined in Section 1.4.2.3, a factor that could contribute to low phytase activity is inhibition by inorganic phosphate. Hence, this might explain why substrates with higher phosphorous content resulted in lower phytase activity. This fact also correlates with the results of Spier *et al.* (2008) in which phytase production during SSF was well correlated with the initial concentration of inorganic phosphate in the substrates used. In Figure 3.16 b), the phosphorous contents decreased with a reduced particle size ($PS < 1$ mm), but they also varied with wheat source. In parallel, the phytase activity changed with type of constituent and with the origins of the wheat (Figures 3.16 a) and b)). This observations seem to be consistent with the conclusions of Dintzis *et al.* (1992) which showed that for three varieties of wheat, the phytate content of bran from kernels in the smallest size distribution was significantly lower than that for kernels in the larger distributions and also that the phytate content of the bran is affected mainly by environmental influences. Particle size can also influence enzyme production in SSF as outlined in Section 1.3.2.2.

Moloney *et al.* (1984) reported that substrates with finer particles showed improved degradation due to an increase in surface area. Thus, phosphorous content and particle size seem associated and both influence phytase yield in SSF.

When compared to wheat bran, wheat midds are mainly composed of small particles and have lower phosphorous content; bran also contains 53% more phosphorous than wheat midds (Blasi *et al.*, 1998). In the wheat midds sample 84.3% of the particles were < 1mm in size, in contrast with the wheat bran samples from the US and Europe where most of the particles were ≥ 1 mm in size (65.3% and 74.8%, respectively, of the total sample). In the wheat bran from Mexico, 64.6% of the particles were < 1 mm in size; this wheat bran also had a reduced phosphorous content and resulted in higher phytase activity.

In an attempt to optimise the substrate further, wheat midds (WM) and wheat germ (WG) were combined in different ratios (8:2, 6:4, 4:6, 2:8 w/w) and utilised for phytase production. A substrate of wheat midds on its own was used as control. The results are illustrated in Figure 3.17.

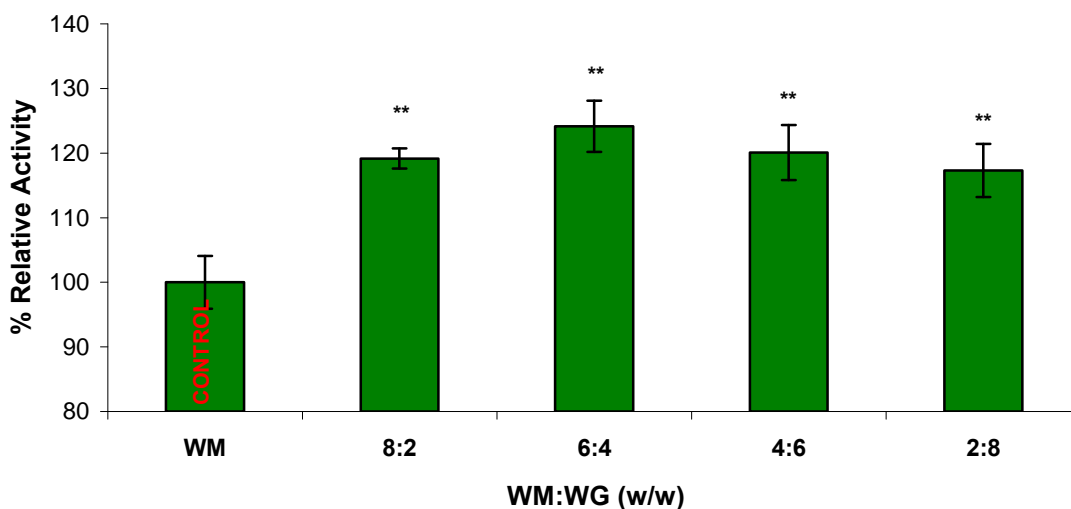


Figure 3.17 Phytase production using a mix of wheat midds and wheat germ

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

It can be observed that the mixed substrate fermentation using WM and WG resulted in a significant increase in phytase production in comparison to the individual WM substrate ($p \leq 0.05$). Increases in yield of 19, 24, 20 and 17% of enzyme through the use of mixed substrates at 8:2, 6:4, 4:6, and 2:8 ratios were noted. This corresponded to 1410 ± 18 , 1469 ± 47 , 1421 ± 50 and 1388 ± 49 SPU/g respectively,

in comparison to the use of WM on its own (1183 ± 48 SPU/g). The optimal mixed ratio was 6:4 (w/w) WM:WG; however wheat germ is a more expensive substrate, therefore its application should be minimal. Consequently it is more economical to use the 8:2 (w/w) WM:WG ratio. Hence, in all subsequent experiments, a mixed substrate consisting of WM and WG (8:2 w/w) was used.

3.4.2 Influence of liquid inoculum age and volume on phytase production in SSF

As considered in Section 1.4.2.4, the form, age and ratio of inoculum are of critical importance in SSF systems. For example, an appropriate inoculum ratio can control contamination by limiting available moisture (Lonsane *et al.*, 1992). The inoculum age and density also influence the productivity and economics of the fermentation (Sen and Swaminathan, 2004). The degree of influence of these factors depends on the specific physiological features of the microorganisms and it must be determined on a strictly individual basis (Gargova and Sariyska, 2003). The size of the vegetative inoculum correlates directly with moisture content as well as the water activity in the SSF substrate (Section 1.3.2.1). Therefore, studies on the effect of physical parameters such as inoculum age and inoculum volume on phytase production by *A. niger* Phy-A in SSF were carried out.

3.4.2.1 Effect of liquid inoculum age

In this study, the optimum inoculum age for phytase production in the solid state system was determined. This was accomplished by utilising liquid cultures which were incubated for different time periods (24, 48, 72, 96, 120, 144 and 168 hours) to inoculate triplicate SSF flasks containing 10 g of mixed substrate. The flasks were incubated at 30°C and 80% RH for 4 days. Subsequently cell free extracts (Section 2.2.1.7) were assessed for phytase activity as outlined in Section 2.2.2.1.

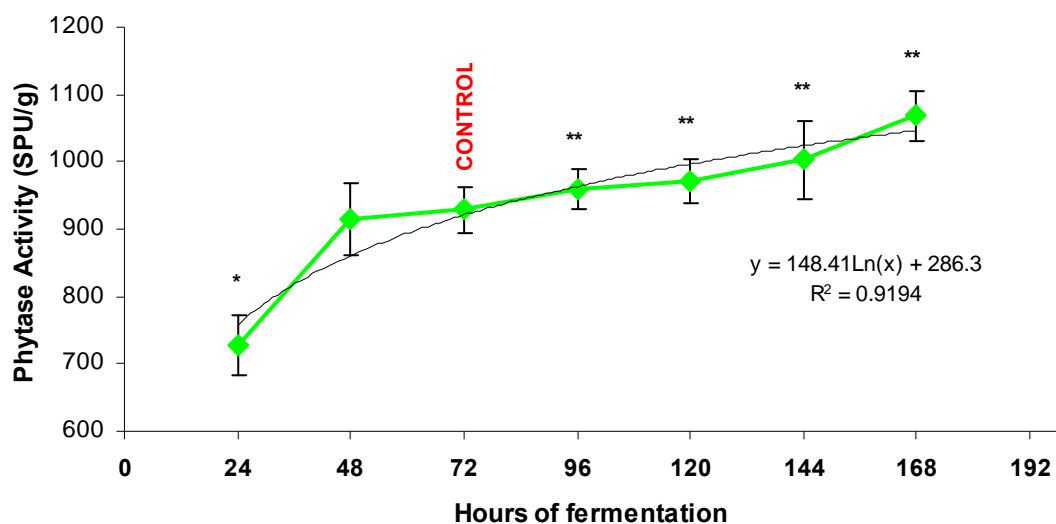


Figure 3.18 Effect of inoculum age on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

As shown in Figure 3.18, phytase production significantly increased with increasing inoculum age ($p \leq 0.05$). Maximal productivity was achieved (1068 ± 38 SPU/g) when a 168 hours culture was used as inoculum and a 1.15 fold increase in yield was verified compared to the control (72 hours). These results are similar to the findings of Ebune *et al.*, (1995b) who found that the age of inoculum used in the solid state culture affected the amount of enzyme produced. They concluded that the older the inoculum, the higher the phytase yield. Other researchers have also considered phytase production by *Aspergillus niger* to be growth associated (Krishna *et al.*, 2001; Papagianni *et al.*, 2001).

It was decided to continue the optimisation process using a 72 hours inoculum, since older cultures are more susceptible to contamination and a 1.15 fold increase in phytase production is not high enough to justify the cost implications of extending inoculum incubation period (Dr. Richard Murphy, personal communication).

3.4.2.2 Optimisation of liquid inoculum volume

Microbial growth in the solid substrate generally depends on the initial moisture level and it indirectly affects the production titres (John *et al.*, 2006). The influence of inoculum volume on phytase production was also investigated by John *et al.* (2006) in the solid state system.

The effects on enzyme yield were studied by altering the volume of inoculum added to the substrate from a diluted liquid culture. Triplicate flasks containing a

mixture of 10g of WM:WG (8:2 w/w) were inoculated with volumes ranging from 6 to 12 mL. Following thorough mixing, flasks were incubated at 30°C and 80% RH for 4 days, after which, cell free extracts (Section 2.2.1.7) were assessed for phytase activity as outlined in Section 2.2.2.1. The effect of increasing inoculum volume on enzyme production is represented in Figure 3.19, with activity expressed as a percentage of the highest yield recorded.

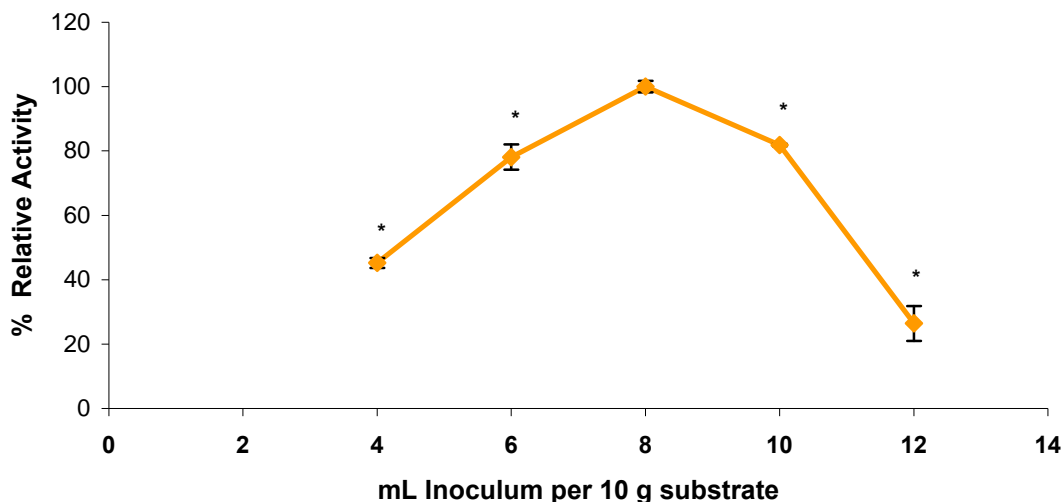


Figure 3.19 Effect of initial moisture content on phytase production

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%. Probability values were determined by One-way ANOVA against the highest yield detected. * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

As is evidenced from Figure 3.19, the optimal volume of inoculum for maximal phytase production by the *A. niger* Phy-A strain was 8 mL per 10 g of mixed substrate. In these SSF cultures, phytase activities of 1564 ± 28 SPU/g were recorded. An increase or decrease in initial moisture content beyond this value led to a significant decrease in enzyme activity ($p \leq 0.05$). If the inherent moisture content present in the substrate is taken into account, the optimal initial moisture content for phytase produced by *A. niger* Phy-A was approximately 48.5% (w/w). The optimum moisture content depends on the microbial species and fermentation system (Tunga *et al.*, 1998). Gautam *et al.* (2002) verified that the optimal moisture content for maximum phytase yield by *Aspergillus niger* (synonym *A. ficuum*) and *Rhizopus oligosporus* was 58.3%. For lower and higher moisture levels, enzyme production was reduced. A substrate moisture content of 52% was the optimum moisture level for phytase production by *Rhizopus spp.* (Ramachandran *et al.*, 2005). Increasing the moisture content results in the decrease of enzyme activity and may be attributed to the phenomenon of flooding of

inter-particle space of the substrate (Ramachandran *et al.*, 2005). The evidence suggests that water availability and oxygen availability are acting as limiting conditions in lower and upper moisture contents, respectively (Tunga *et al.*, 1998).

3.4.3 Solid state substrate supplementation

As previously outlined in Section 1.3.2.2, the ideal substrate should provide all the required nutrients to the organism. However, some of the nutrients may not be available or may only be available in sub-optimal concentrations. Consequently external supplementation to the substrate may be necessary (Pandey *et al.*, 1999). Depending on their nature and concentration, carbon and nitrogen sources may repress or induce enzyme production (Wang *et al.*, 2005). Hence, the chemical composition of the different feed ingredients, the quality of the carbon and nitrogen sources and the phosphate content may regulate phytase production in solid state fermentation (Bogar *et al.*, 2003a).

As reviewed in Section 1.3.1 there are inherent differences between submerged and solid state fermentation processes thus it was important to study the influence of supplementation of SSF cultures on phytase production.

3.4.3.1 Effect of supplemental carbon source on phytase production under solid state fermentation conditions

The influence on enzyme production of supplementing the fermentation medium with various carbon sources was evaluated. This was carried out by incorporating tapioca flour, glucose, sucrose, xylose, mannose, sorbitol, corn steep liquor and wheat germ oil into the inoculation solution at a final concentration of either 0.5% (w/v) or 3% (w/v). Having considered the previous effects of this supplementation on the inoculum (Section 3.3.2), these concentrations were considered appropriate to study any induction or repression effects. For each carbon source tested, triplicate flasks containing 10 g of the mixed substrate were inoculated with 8 mL of diluted liquid culture as described in Section 2.2.1.5; the only modification being that a solution of the respective carbon source (0.5 or 3% (w/v) final concentration) was used to dilute the liquid culture instead of distilled water. Triplicate flasks without any supplementation served as control. Following incubation for 4 days at 30°C and 80% RH, cell free extracts were prepared as outlined in Section 2.2.1.7 and assayed for phytase activity (Section 2.2.2.1).

The results are illustrated in Figure 3.20 with phytase activity being expressed as a percentage relative to the activity in the control.

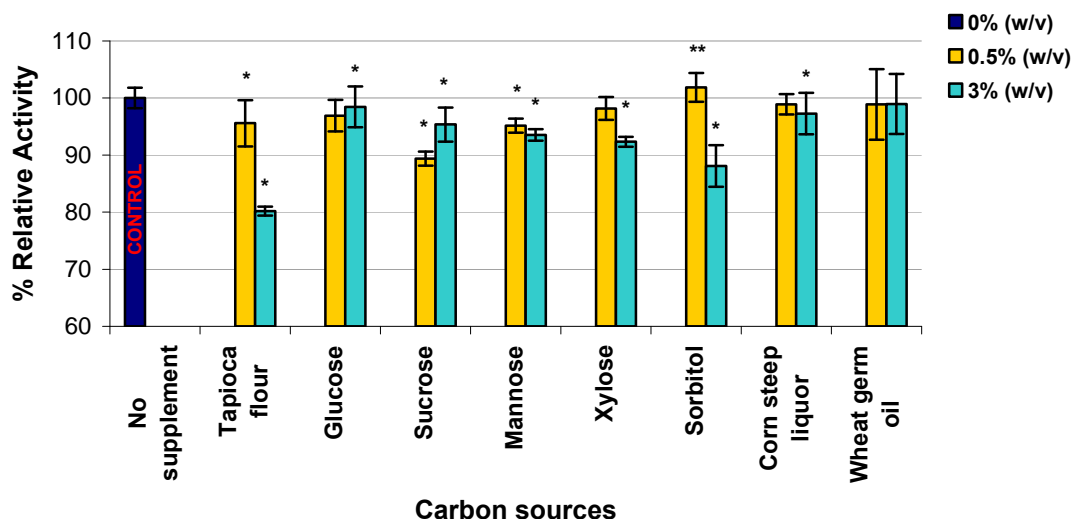


Figure 3.20 Effect of various carbon sources on phytase production in SSF

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%.

Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

It is clear from Figure 3.20 that the *A. niger* Phy-A strain was able to grow and produce phytase in the presence of all the carbon sources tested. A number of the carbon sources promoted a significant effect on the enzyme yield ($p \leq 0.05$); however the phytase activity was only slightly enhanced or repressed when compared to the control (1380 ± 25 SPU/g).

The ability of SSF systems to minimise catabolite repression has been described for the microbial production of different hydrolytic enzymes (Ramesh and Lonsane, 1991; Nandakumar *et al.*, 1999; Viniegra-González and Favela-Torres, 2006). Ramesh and Lonsane (1991) suggested that this ability was conferred by various physico-chemical factors and culture conditions specific to the system.

Sorbitol at 0.5% (w/v) concentration had a positive effect on phytase production yielding a 2% increase in relative activity. It is clear that this enhancement in enzyme yield, even though statistically significant ($p \leq 0.05$), was not to a level which would be economically viable. However, it is interesting to note that sorbitol was the only carbon source that enhanced phytase activity when supplemented both the liquid seed media and solid state substrate. All the other carbon sources, particularly at 3% (w/v) concentration, repressed phytase production with or without significance. Contrary to expectations, supplemental tapioca flour repressed phytase production, particularly at a 3% (w/v) concentration with a 20% decrease in yield. Once again, this could be a result of carbon-repression promoted by the excess glucose in the fermented media subsequent

to the hydrolysis of starch. Glucose was also not effective in enhancing phytase activity.

Supplementation of the SSF substrate for phytase production by *Aspergillus niger* has been studied by other researchers. Gautam *et al.* (2002) reported similar findings when supplementing an inert substrate of polystyrene with various carbon sources and none promoted phytase production by *A. ficuum*. In contrast, Bogar *et al.* (2003a) demonstrated using Plackett-Burman design that supplementing wheat bran with 3% (w/w) concentration of starch promoted maximal phytase production. Recently, Gunashree and Venkateswaran (2008) showed that sucrose supplemented wheat bran substrate enhanced phytase yield in both submerged and solid state fermentations.

This study successfully demonstrated that the mixed substrate of wheat midds and wheat germ was adequate for growth and phytase production by the *A. niger* Phy-A strain. Supplementation of the fermentation substrate can be expensive and generally contributes to an increase of the overall production costs (Choi and Lee, 1999; Naveen *et al.*, 2006). However, it is important to reiterate the possibility of glucose or hydrolysed starch carryover from the tapioca flour based liquid inoculum, since it could potentially lead to inhibition in SSF.

3.4.3.2 Effect of supplemental nitrogen source on phytase production under solid state fermentation conditions

Various nitrogen sources were incorporated into the inoculum solution, to a final concentration of either 1.8 or 5% (w/v) to establish the effect of supplemental nitrogen on phytase production in SSF. For each organic and inorganic nitrogen source tested, triplicate flasks (10 g) of mixed substrate WM:WG (8:2 w/w) were inoculated as outlined in Section 2.2.1.5; the only modification being that a solution of the respective nitrogen source (1.8 or 5% (w/v) final concentration) was used to dilute the mycelial suspension instead of distilled water. After the incubation period, cell free extracts (Section 2.2.1.7) were assessed for phytase activity by the method described in Section 2.2.2.1.

The results are presented in Figure 3.21 with phytase activity being expressed as a percentage of the activity in the control.

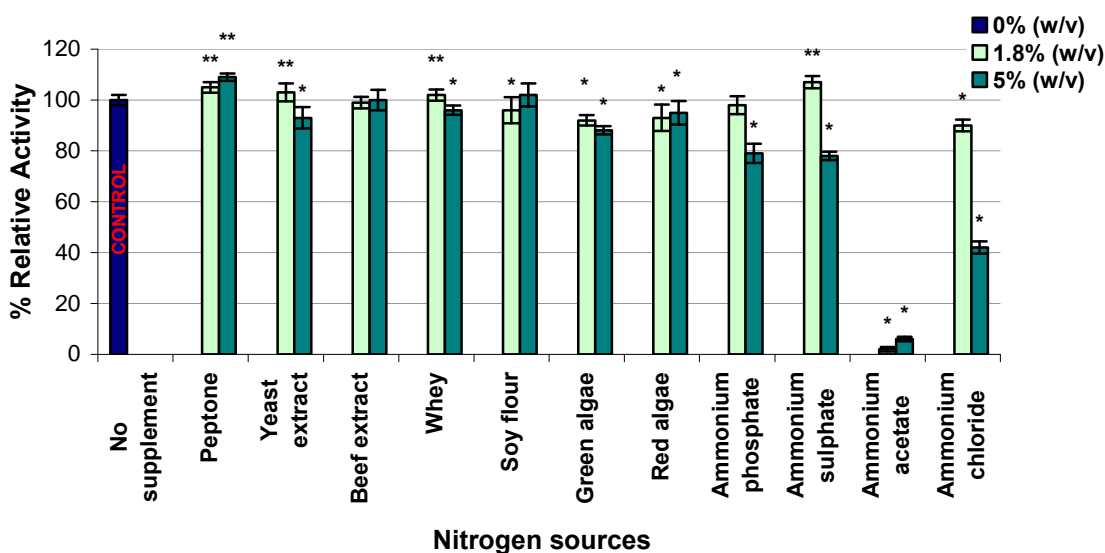


Figure 3.21 Effect of various nitrogen sources on phytase production in SSF

Data represent MEAN value \pm SD of twenty seven measurements. Confidence level set at 95%.

Probability values were determined by One-way ANOVA against the control. ** denotes a statistically significant increase in phytase activity at $p \leq 0.05$; * denotes a statistically significant decrease in phytase activity at $p \leq 0.05$.

It can be observed that among the various organic and inorganic nitrogen sources added to the solid substrate, peptone at 5% (w/v) inclusion was the optimum nitrogen source yielding 1621 ± 21 SPU/g in SSF. Gunashree and Venkateswaran (2008) verified that peptone increased phytase production by *Aspergillus niger* in submerged fermentation medium, however equivalent results were not achieved under solid state fermentation conditions. Although, in the present study peptone significantly enhanced enzyme production in the solid state system for both concentration levels tested. On the other hand, supplementation of the liquid seed media with peptone at a concentration of 5% (w/v) repressed enzyme synthesis (Section 3.3.3). These contrasting results suggest that the requirements for nitrogen are different in submerged and solid state fermentation systems. Otherwise, the positive influence of peptone could be related to the balance in the C/N ratio required for enzyme production as previously discussed in Section 3.3.3. Also contrasting with the results obtained for the liquid seed media supplementation (Section 3.3.3), inclusion of ammonium sulphate at 1.8% (w/v) resulted in a 7% increase in phytase production. This fact was in agreement with the findings of Singh and Satyanarayana (2008b), whose supplementation of sesame oil cake with ammonium sulphate further enhanced phytase yield by *Sporotrichum thermophile*. Moreover, Spier *et al.* (2008) reported that addition of ammonium sulphate resulted in a significant increase in phytase production by *A. niger* when supplemented to citric pulp. In contrast, Ramachandran *et al.* (2005) showed that

ammonium sulphate supplementation inhibited enzyme formation by the fungal culture in a mixed substrate fermentation using oilcakes.

Once again ammonium acetate promoted a complete inhibition of phytase production in SSF; analogous to what was observed for the liquid seed media (Section 3.3.3).

A review of the data suggests that none of the nitrogen sources enhanced phytase production at levels which would be economically or commercially beneficial. Therefore from an economic point of view, a mixture of wheat midds and wheat germ (8:2 w/w) continues to be the ideal cost-effective substrate.

3.4.4 Determination of the optimal incubation period and temperature for phytase production under solid state fermentation conditions

The duration of fermentation influences the success of any bioprocess and represents the time requirement for the complete utilisation of the source material and the corresponding accumulation of the desired product (John *et al.*, 2006). In addition, the viability of the culture depends on the incubation temperature, however throughout the solid state fermentation process metabolic heat will also be generated as a result of the dynamic growth of the fungi and it may positively or negatively affect the culture (Cen and Xia, 1999). Therefore, in order to determine the optimal incubation period and temperature for maximal phytase production from SSF cultures of *A. niger* Phy-A, flasks containing 10 g of mixed WM:WG (8:2 w/w) were inoculated (Section 2.2.1.5) and incubated for a period up to a maximum of 6 days at various temperatures (25, 30 and 33°C) and 80% RH. For each temperature, triplicate flasks were incubated for time periods from 72 hours through 144 hours, subsequently cell free extracts were prepared as described in Section 2.2.1.7 and were assessed for phytase activity by the method outlined in Section 2.2.2.1.

The results obtained for each temperature during the designated incubation period are individually plotted in Figure 3.22, with phytase activity expressed relative to the highest activity attained.

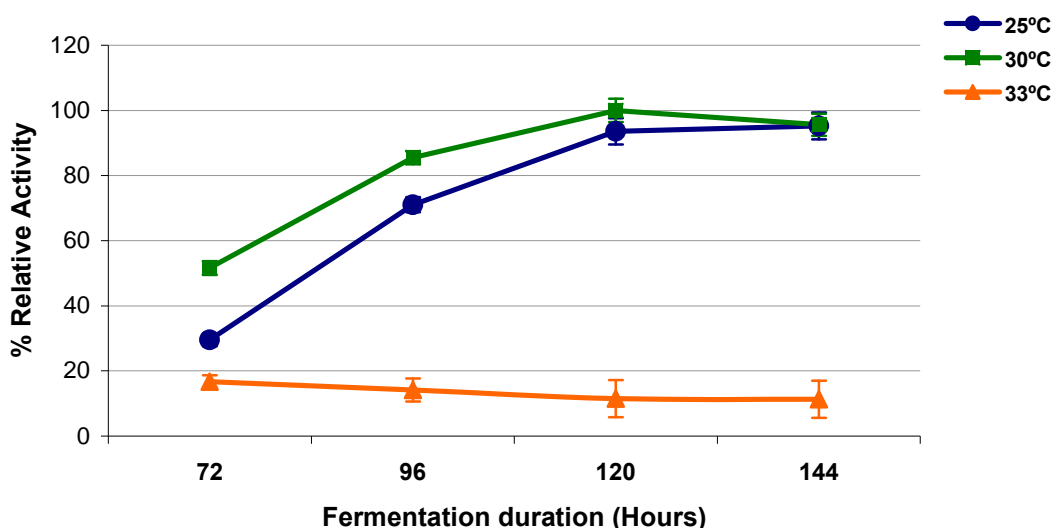


Figure 3.22 Time course of phytase production by *A. niger* Phy-A relative to incubation temperature

Data represent MEAN value \pm SD of twenty seven measurements.

In Figure 3.22 a specific trend can be seen demonstrating that phytase production was simultaneously influenced by incubation temperature and duration of fermentation. Incubation at 25°C resulted in an increased incubation time (144 hours) required for maximal enzyme activity (1760 ± 76 SPU/g). Incubation at higher temperature (33°C) affected fungal growth and impacted negatively on the enzyme synthesis whereby maximal activity of 309 ± 37 SPU/g was achieved after 72 hours fermentation. Because enzymes are produced during the exponential growth phase, incubation at high temperatures could lead to deficient growth and consequently reduced enzyme production (Sabu *et al.*, 2002). Furthermore, the centre of solid substrate can overheat as the heat transfer in the solid layer is quite poor, which is unfavourable for spore germination, mycelial growth and enzyme production (Cen *et al.*, 1999).

The optimal temperature for *A. niger* Phy-A growth and phytase production was 30°C with an optimised incubation period of 5 days (120 hours) corresponding to a 15% increase in phytase yield in comparison with the 4 day (96 hours) incubation period used throughout this research. Shorter incubation periods can potentially reduce the cost of enzyme production, therefore in terms of industrial production it would have to be decided if the increase in phytase production with an extra 24 hours of incubation would justify the related costs. *Aspergillus niger* phytase production was optimised at 30°C for 96 hours by Gautam *et al.* (2002) and Spier *et al.* (2008). Mandviwala and Khire (2000) verified that *A. niger* grew rapidly on wheat bran particles with a corresponding increase in phytase activity until the seventh day of SSF at an optimum

temperature of 30°C. Krishna and Nokes (2001a) verified that the duration of SSF could be shortened to 144 hours for a maximum phytase yield using 72 hours old liquid inoculum from 7- to 14 day plates. This is comparable to the present findings, in which maximal enzyme production was achieved after 120 hours at 30°C using liquid cultures inoculated with 12 days old *A. niger* Phy-A slants and incubated for 72 hours.

3.4.5 Overall summary assessment of the optimisation studies

According to the results of the stabilisation and optimisation work outlined in Sections 3.2.2 to 3.4.4, phytase production by the *A. niger* Phy-A strain was successfully stabilised in replicated SSF flasks as well as optimised. The ultimate goal of the optimisation was to create the ideal conditions for maximal phytase production using cost-effective materials and straightforward procedures that would be feasible for implementing the process on an industrial scale. A summary of the relevant optimised parameters is shown in Table 3.6.

Table 3.6 Summary of optimised fermentation parameters

Optimised parameters			Standard Deviation	% Increase in activity relative to the control
Slant	Medium	WGA	SD < 50 SPU/g	---
	Incubation period	12 days		---
Liquid seed medium	Medium composition	2% (w/v) tapioca flour 1.2% (w/v) peptone	SD < 50 SPU/g	7% 12%
	SSF	Substrate	WM	SD ≈ 50 SPU/g
	Mixed substrate	WM:WG (8:2 w/w)		19%
	Inoculum age	72 hours		---
	Inoculum volume	8 mL		---
	Incubation temperature	30°C		15%
	Incubation period	120 hours (5 days)		

WGA = wheat germ agar; WM = wheat middlings; WG = wheat germ

In the subsequent experiment, the interaction between all optimised variables was assessed. In order to compare phytase production by the *A. niger* Phy-A strain before and after optimisation, two set of cultures were prepared in parallel, one according to the growth conditions outlined in Section 2.2.1 (before optimisation) and

the other combining all the optimisation parameters. Subsequently, flasks containing 10 g of wheat bran or mixed WM:WG (8:2 w/w) were inoculated with the respective cultures (Section 2.2.1.5) and incubated for a period up to a maximum of 6 days at 30°C and 80% of relative humidity. For either the non-optimised or optimised culture, triplicate flasks were incubated for a period from 72 hours up to 144 hours fermentation, after which cell free extracts (Section 2.2.1.7) were measured for phytase activity (Section 2.2.2.1).

A plot of phytase production over the course of the fermentation before and after optimisation is presented in Figure 3.23.

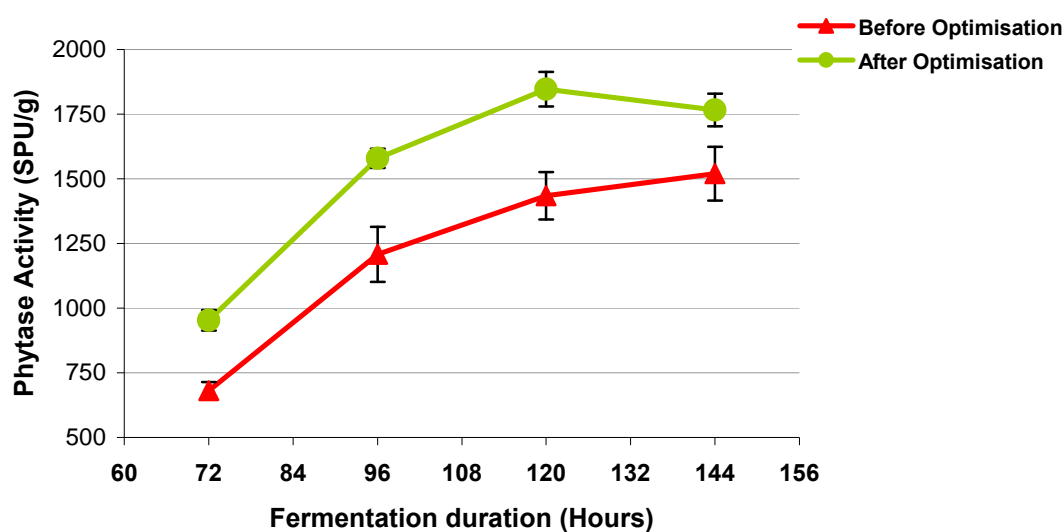


Figure 3.23 Phytase production by *A. niger* Phy-A before and after optimisation

Data represent MEAN value \pm SD of twenty seven measurements.

Overall, a 29% improvement in phytase production was achieved using the optimised fermentation conditions yielding 1847 ± 63 SPU/g, in contrast to 1434 ± 92 SPU/g produced under unoptimised conditions. It was interesting to verify that prior to optimisation the fungus needed an extra day of fermentation to achieve its optimal phytase yield (1520 ± 104 SPU/g). It also has to be considered that throughout this study, a parallel was established between maintaining low variability of the clonal isolates and optimal enzyme production. It can be observed in Figure 3.23 that the standard deviations before optimisation continue to be considerably higher than after optimisation, attesting to the improved stability attributed to the optimised media. However, the SD had a slight increase during the SSF optimisation, possibly explained by the use of a mixture of substrates that can increase the variability in nutrient availability.

In summation, increased phytase production was achieved through optimisation of the solid state fermentation, in particular through optimisation of growth conditions on the solid substrate. Phytase production was found to be well correlated with the initial concentration of inorganic phosphate in the substrates used, and was also influenced by the particle size of the substrate. Phytase production was higher in substrates with a low concentration of phosphorous and reduced particle size (<1 mm), such as wheat midds. However, a combination of wheat midds and wheat germ (8:2 w/w) resulted in higher phytase production compared to wheat midds individually. Comparatively, the effects of supplementing the fermentation medium with various carbon and nitrogen sources did not increase enzyme yield to the degree that would be economical on an industrial scale. Moreover, the mixed substrate of WM:WG (8:2 w/w) fulfilled all the carbon and nitrogen requirements required by the *A. niger* Phy-A strain for phytase production. The size of the vegetative inoculum was representative of the initial moisture content of the solid substrate. SSF systems depend on the use of an appropriate inoculum ratio for suitable growth and enzyme production as well as to control contamination. Phytase production by this strain appears to be growth associated as the enzyme yield increased with the age of inoculum. The optimal temperature for phytase production under SSF conditions was 30°C for 5 days (120 hours).

3.4.6 Ancillary enzyme production by the *A. niger* Phy-A strain

The assorted nature of the substrates used in SSF, whose macromolecular structure is composed of starch, lignocellulose, pectin and other polysaccharides (Raimbault, 1998), enables the production of a range of enzymes from a single substrate. Thus, the fungal SSF product usually contains not only phytase, but also accessory enzymes, fungal protein and organic acids all of which increase feed digestibility and access to the phytic acid component (Pandey *et al.*, 2001a). Solid state fermentation is widely known for its ability to produce a wide range of hydrolytic enzymes, including protease, cellulase, β -glucanase and xylanase, therefore it was decided worthwhile to determine if the *A. niger* Phy-A strain produced these enzymes in the SSF system. The cell free extracts were assessed for protease, cellulase, β -glucanase and xylanase activities as outlined in Section 2.2.2 and the results are illustrated in Table 3.7. These enzyme activities were determined using the cell free extracts from the SSF cultures obtained in the previous experiment (Section 2.2.1.7).

Table 3.7 Enzymes Activities before and after optimisation

Enzymes		Non-Optimised Activities	Optimised Activities	Relative Activity % \pm SD
Phytase	(SPU/g)	1434 \pm 92	1847 \pm 67	129 \pm 5
Protease	(HUT/g)	11584 \pm 3025	10925 \pm 1790	94 \pm 16
Cellulase	(CMCU/g)	178 \pm 3	164 \pm 5	92 \pm 3
β -Glucanase	(BGU/g)	749 \pm 50	591 \pm 31	79 \pm 4
Xylanase	(XU/g)	520 \pm 26	326 \pm 19	63 \pm 4

Data represent MEAN value \pm SD of twenty seven measurements. The relative activity was determined comparatively to the non-optimised activities accessed for each enzyme.

As previously established, phytase activity increased by 29% with optimisation; however for all the other enzymes tested, the activity was reduced. Couri *et al.* (2000) had similar findings when they verified that in a mixture containing polygalacturonase, cellulase, xylanase and protease produced by *Aspergillus niger*, each enzyme was differently influenced via medium composition on solid-state fermentation, concluding that for the production of each enzyme there is a standard condition.

3.5 Stability Studies

As outlined in Section 1.5, several researchers have investigated different phytases in relation to their potential benefits in animal nutrition (Matsui *et al.*, 2000; Simon and Igbasan, 2002; Augspurger *et al.*, 2003; Park *et al.*, 2003). These studies have highlighted differences in performance amongst phytases, particularly with respect to thermal stability and resistance against proteolytic degradation. These factors are analysed in the following sections.

3.5.1 Thermostability Analysis

As discussed in Section 1.5.5, a commercially successful phytase should be able to withstand brief elevated temperatures (60–90°C) prior to encountering an animal's digestive tract with a temperature of 37°C (Berka *et al.*, 1998). For this reason, a comparison of the thermostabilities of the optimised phytase and three commercially available phytases were assessed. The thermostability of the enzyme was determined by incubating enzyme solutions at 80°C for periods up to 5 minutes and immediately cooled on ice (Section 2.2.5.1). The residual phytase activities were measured as outlined in Section 2.2.2.1. The results are presented in Figure 3.24.

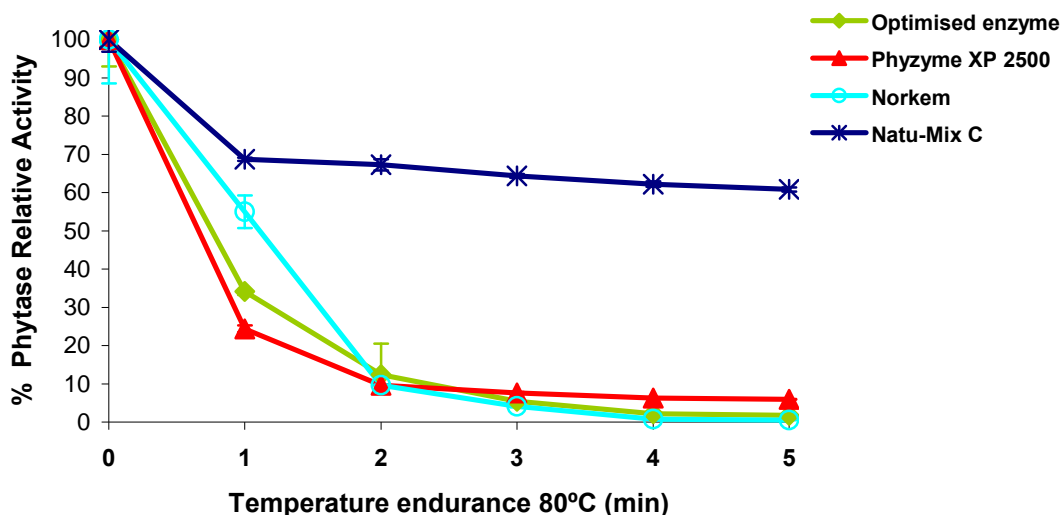


Figure 3.24 Thermostability of commercially available phytases and *A. niger* Phy-A phytase

Residual activity = % activity remaining after incubating to 80°C for the indicated time, relative to the activity of an unincubated enzyme sample. Data represent MEAN value \pm SD of nine measurements.

Thermostability profiling observed in Figure 3.24 showed that the enzyme preparations assessed lost between 39.2% and 99.5% of their original activities following incubation for 5 minutes at 80°C. The most considerable decrease in phytase activity occurred during the first minute in which the greatest decrease was observed with Phyzyme XP2500. A clear stability ranking is evident whereby Phyzyme XP2500 > *A. niger* Phy-A > Norkem > Natu-mix C with respective activity losses of 75.6% > 65.9% > 45.1% > 31.3%. Natu-mix C was the most thermostable enzyme preparation retaining 60.8% of its residual activity after treatment at 80°C for 5 minutes. Dvoráková *et al.* (1997) and Wyss *et al.* (1998) showed that *Aspergillus niger* phytase lost 70 to 80% of its activity following incubation at 80°C. Wyss *et al.* (1998) attributed the destruction of activity to an irreversible conformational change of the enzyme.

The thermostability results presented in Figure 3.24 cannot be generalised and must be cautiously interpreted since interactions with feed components were not studied in this experiment. It is documented that feed constituents can have a potential stabilising influence by providing thermoprotection to the enzymes (Bedford *et al.*, 2001). Therefore, measuring phytase activity in pelleted feed or *in vivo* animal trials will provide a more accurate assessment of its degree of inactivation.

3.5.2 Analysis of Proteolytic Stability Using Simulated *in vitro*

Gastrointestinal Digestion

Series of *in vitro* studies were carried out to assess the relative stability of the *A. niger* Phy-A phytase and three commercial phytase products when exposed to simulated conditions found in the monogastric digestive tract. The experiments were carried out as outlined in Section 2.2.5.2 after which the pH of the samples was adjusted to pH 5.5 before the determination of phytase activity (Section 2.2.2.1). The results obtained are shown in Table 3.8.

Table 3.8 Effect of simulated *in vitro* monogastric digestion conditions on phytase activity

Enzyme preparation	Relative Activity \pm SD	
	pH 2.5 & Pepsin (2H)	pH 2.5 only (2H)
Optimised <i>A. niger</i> Phy-A	97 \pm 1	99 \pm 2
Phyzyme XP2500	90 \pm 3	91 \pm 4
Norkem Phytase	82 \pm 4	86 \pm 5
Natu-mix C	60 \pm 3	76 \pm 8
Enzymes preparation	Relative Activity \pm SD	
	pH 6.8 & Pancreatin (4H)	pH 6.8 only (4H)
Optimised <i>A. niger</i> Phy-A	4 \pm 0.3	74 \pm 3
Phyzyme XP2500	38 \pm 3	67 \pm 1
Norkem Phytase	37 \pm 2	87 \pm 7
Natu-mix C	0	57 \pm 1
Enzyme preparation	Relative Activity \pm SD	
	pH 2.5 & Pepsin (2H) + pH 6.8 & Pancreatin (4H)	pH 2.5 only (2H) + pH 6.8 only (2H)
Optimised <i>A. niger</i> Phy-A	6 \pm 0.2	54 \pm 17
Phyzyme XP2500	36 \pm 1	50 \pm 3
Norkem Phytase	29 \pm 1	73 \pm 1
Natu-mix C	0	44 \pm 1

Data expressed as a percentage of original activity (activity of a sample maintained at pH 5.5 and not exposed to pepsin or pancreatin or both). Each value represents mean \pm SD of nine measurements.

Total simulated gastric digestion (pH 2.5 with pepsin) resulted in a considerable decrease in the activity of two commercial enzymes, i.e. Norkem and Natu-mix C, relative to their corresponding controls. However, the *A. niger* Phy-A phytase and Phyzyme XP2500 retained almost all their original activity with 97% and 90%, respectively. Omission of pepsin had no effect on the enzymes, indicating that loss of activity was almost entirely due to instability at low pH. Simulated small intestinal digestion resulted in a very significant decrease in enzyme activity in all 4 cases, with nearly complete inactivation of the *A. niger* Phy-A and Natu-mix C phytases. All of the enzyme preparations lost a considerable portion of phytase activity when incubated at pH 6.8 in the absence of pancreatin; however in this case it was the proteolytic ability of

pancreatin that contributed the most. When a total gastrointestinal digestion was mimicked, only Phyzyme XP2500 and Norkem phytases displayed any residual activities with 36 and 29% respectively.

These series of experiments had limitations, given that yet again, actual feed interactions were not taken into account. The inactive stomach pH is in the region of 2.5, but feed ingestion would have a considerable buffering effect, raising the pH to values towards 6–6.5, pH at which the phytase enzyme would be significantly more stable and catalytically active (Boyce and Walsh, 2006). Feed ingestion, on the other hand, activates gastric acid secretion that lowers the pH of the stomach. Additionally, dietary phytic acid may bind to positively charged amino acids on the pepsinogen protein in the stomach, leading to reduced pepsin activity and hence increased pepsin and HCl secretions via negative feedback mechanisms (Woyengo *et al.*, 2009).

In summary, phytases might display higher stability to proteolysis *in vivo*, therefore their commercial viability would ideally be assessed by performing animal trials.

3.5.3 Effect of Ruminal Fluid on Phytase Stability

Ruminal microbial populations produce a remarkable range of catalytic activities in ruminant animals; however supplementation with exogenous enzymes may be beneficial under certain circumstances (Walsh *et al.*, 1994). Recently, Kincaid *et al.* (2005) compared different diets and the effect of the addition of exogenous phytase on phosphorous digestibility in dairy cows. They concluded that exogenous dietary phytase might improve phosphorous digestibility in dairy cows depending on the formulation of the diet, without detriment to dry matter intake and milk efficiency. However, the author found little or no information about the stability of exogenous phytases in the rumen. For this reason, it was decided worthwhile to assess phytase stability in presence of rumen fluid using the method described in Section 2.2.5.3.

Table 3.9 displays the results obtained for the residual phytase activities of the four enzyme preparations after a period of 6 or 24 hours incubation with ruminal fluid.

Table 3.9 Effect of ruminal fluid on phytase stability

Enzyme preparation	Relative Activity \pm SD	
	Ruminal Fluid (6H)	pH 6 only (6H)
Optimised <i>A. niger</i> Phy-A	90 \pm 2	95 \pm 1
Phyzyme XP2500	75 \pm 4	99 \pm 1
Norkem Phytase	80 \pm 2	98 \pm 3
Natu-mix C	87 \pm 2	95 \pm 1

Enzyme preparation	Relative Activity \pm SD	
	Ruminal Fluid (24H)	pH 6 only (24H)
Optimised <i>A. niger</i> Phy-A	88 \pm 2	86 \pm 2
Phyzyme XP2500	47 \pm 2	82 \pm 5
Norkem Phytase	66 \pm 2	88 \pm 3
Natu-mix C	78 \pm 6	79 \pm 2

Data expressed as a percentage of original activity (activity of a sample maintained at pH 5.5 and not exposed to ruminal fluid). Each value represents mean \pm SD of nine measurements.

It is apparent from Table 3.9 that all 4 enzymes were resistant to inactivation in the ruminal fluid, especially in comparison with their resistance to proteolysis in the simulated gastrointestinal tract (Section 3.6). Interestingly, *A. niger* Phy-A phytase was the most stable enzyme, retaining 90 and 88% of its activity after 6 and 24 hours of incubation, respectively.

This study suggests that *A. niger* Phy-A phytase is highly stable in the presence of ruminal fluid and that it could potentially be used successfully for exogenous supplementation of ruminant diets.

Conclusion

4. Conclusion

Phytases are eco-friendly enzymes that are widespread in nature. Their supplementation to animal feed is an effective way to increase the availability of phosphorous to animals, thus improving their performance and reducing manure-borne phosphorous pollution. In addition to its major application in animal nutrition, phytase is also used for processing of human food.

Traditionally, phytases for industrial applications were produced in submerged cultivations; however, there is an increasing interest in phytase production by solid state fermentation. Studies on the production of fungal enzymes under solid state versus submerged fermentation conditions have shown that SSF production offers higher enzyme titres, lower level of catabolic repression and increased stability of the secreted enzymes. It is also widely reported that the productivity in SSF is much higher than in SmF.

The objective of this study was the stabilisation and optimisation of phytase production by a non-genetically modified strain of *Aspergillus niger* using solid state fermentation, for possible application in the animal feed industry. A successful decrease in the variability of phytase productivities between replicate SSF cultures produced by clonal isolates of the *A. niger* Phy-A strain was achieved through optimisation of the slant medium composition; however the variability in enzyme productivity noted between individual slants remained. The use of wheat germ agar (WGA) slants reduced the standard deviation between SSF cultures and increased phytase activity (1094 ± 25 SPU/g). An incubation period of 12 days was determined as optimal for WGA slants, resulting in increased phytase titres (1258 ± 48 SPU/g).

The *A. niger* Phy-A strain does not produce significant levels of spores thus a traditional submerged fermentation system was used to generate a liquid culture. The liquid seed medium was successfully optimised for phytase production by manipulation of its composition. The results did not furnish the author with adequate support to the theory that thickening agents acted as inducers of phytase activity. No clear correlation between the viscosity conferred by the thickening agent and phytase production was noted; however, the morphology of fungal growth changed depending on the concentration and the type of thickening agent added to the liquid seed medium.

The *A. niger* Phy-A strain also appears to preferentially utilise the slow release sugars through the hydrolysis of starch instead of readily available sugar sources. This observation was in agreement with other researchers (Shieh and Ware, 1968; Vats and Banerjee, 2002; Roopest *et al.*, 2006). Tapioca flour at 2% (w/v) inclusion was

determined as the most suitable starch and carbon source, resulting in a significant 7% increase in phytase yield relative to the standard medium ($p \leq 0.05$). Peptone as a supplemental nitrogen source to the liquid seed medium was deemed essential to induce phytase production by this system in subsequent SSF. Inclusion at 1.2% (w/v) represented a significant 12 % increase relative to the control ($p \leq 0.05$). Optimisation of the liquid seed medium not only achieved increased product yield, but could potentially reduce production costs.

Phytase production by *A. niger* Phy-A was significantly enhanced through optimisation of solid state fermentation conditions. Production was correlated with the concentration of inorganic phosphate in the substrates and also influenced by the particle size. Wheat midds was the optimal substrate for phytase production as it had low phosphorous content and was mainly constituted of particles of less than 1 mm. However, in a further optimisation of the substrate, a mixture of wheat midds and wheat germ (8:2 w/w) increased phytase production by 19% compared to wheat midds on its own. The extra supplementation of the substrate with carbon and nitrogen sources did not increase enzyme production to levels that would justify investment on an industrial scale. The optimum inoculum size was 8 mL per 10 g of WM:WG (8:2 w/w) and the optimised incubation parameters were temperature at 30°C for 5 days of incubation corresponding to a 15% increase in phytase production. Overall, a 29% increase in phytase production by for *A. niger* Phy-A was achieved through optimisation of the SSF growth conditions.

In general, fungal SSF products contain other ancillary enzymes apart from phytase which helps to increase feed digestibility. The *A. niger* Phy-A strain produces reasonable levels of enzymes such as cellulase, xylanase, β -glucanase and protease. These accessory hydrolytic enzymes are an additional benefit that makes this SSF preparation a potential competitor for the best commercial phytase preparations currently available.

The biochemical characteristics of an ideal phytase for application into animal feed are still largely unknown, although ideally the enzyme should be thermostable at temperatures up to 80°C and resist gastrointestinal proteolysis. Unfortunately, the *A. niger* Phy-A phytase displayed very little activity following incubation at 80 °C, however, it was stable in presence of pepsin during simulation of *in vitro* gastric digestion at pH 2.5 retaining 97% of its initial activity. The equivalent did not occur in the simulation of the small intestinal digestion and resulted in near complete inactivation of the *A. niger* phytase Phy-A.

The results obtained for the thermostability and proteolytic stability studies must be interpreted with caution and cannot be generalised. It is possible that the *A. niger* Phy-A phytase could display increased resistance to pelleting conditions when properly mixed with other feed ingredients, as well as better proteolytic resistance to pancreatin. Studies *in vivo* to attest the validity of these facts should be carried out in pelleted feed by performing animal trials.

A final, novel study was performed to assess the effect of ruminal fluid on phytase stability for the potential exogenous supplementation of phytase to ruminant diets. The results obtained with the *A. niger* Phy-A phytase indicated that this enzyme is highly stable in ruminal fluid (pH 6.0) over a 24 hour period. This enzyme could potentially be used with success for supplementation of ruminant diets; however *in vivo* trials should be also performed.

In conclusion, there is the potential for the *A. niger* Phy-A phytase to be applied as a feed supplement with the added benefit of a possible reduction in the cost of production in SSF.

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