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Optimization of Xylanase Production by *Thermomyces lanuginosus* in Solid State Fermentation

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Extracellular xylanase production by the thermophilic fungus *Thermomyces lanuginosus* 195 in solid state fermentation (SSF) was found to be significantly affected by fermentation temperature, duration, and inoculum volume ($p \leq 0.001$). Optimization of these parameters corresponded to a 21.7% increase in xylanase yield. Maximum activity (2,335 U/g of wheat bran) was obtained when 10 g of wheat bran was inoculated with 10 ml of liquid culture and cultivated at 45 °C for 40 h. The influence of supplemental carbon and nitrogen sources (3% w/v) on xylanase production was also assessed. Wheat bran, supplemented with glucose and cellulose, facilitated 10% and 7% increases in relative activity respectively. Ammonium based salts, nitrates, and a number of organic nitrogen sources served only to reduce xylanase production ($p \leq 0.005$) significantly. The enhanced xylanase titers achieved in the present study emphasize the need for optimizing growth conditions for maximum enzyme production in SSF.

Key words: xylanase; *Thermomyces lanuginosus*; optimization; solid state fermentation (SSF); medium supplementation

Solid state fermentation (SSF) involves the cultivation of microorganisms on a solid substrate in the near absence of free liquid.[1,2] The moisture involved in the fermentation process facilitates the growth and metabolic functions of the microorganism, and is typically retained within or complexed to the growth matrix.[1,2] The characteristics of filamentous fungi, most notably hyphal growth, tolerance of low water activity, protein secretion, and an ability to grow on a variety of low-value lignocellulosic materials provide a unique adaptability to SSF.[3,4] Additionally, SSF is generally regarded as a more appropriate process for enzyme production, considering the natural growth environment of most filamentous fungi.[5]

The range of enzymes produced during SSF is typically mediated by both the characteristics of the microorganism and the composition of the growth media.[6,7] Xylan is the major hemicellulose component of plant cell walls, and is typically comprised of a β-1,4-linked xylopyranose backbone.[8] Depending on its botanical origin, xylan can possess a number of side-linked groups, comprised of acetyl, arabinofuranosyl, and glucuronosyl residues.[9]

Thermophilic *Thermomyces lanuginosus* has demonstrated growth on a variety of carbon sources, with an optimum growth temperature of 40–50 °C,[10–12] and is particularly noted for its inability to produce cellulase.[13] Compared to those from mesophilic sources, enzymes from thermophiles tend to be more thermostable, a characteristic believed to be achieved through minor alterations in protein structure.[14] Consequently, thermophilic xylanases have numerous industrial and commercial applications, including uses in animal feed supplementation, Kraft bleaching, paper manufacturing, municipal waste treatment, and the treatment of lignocellulosic materials for bioethanol production.[14–16]

Certain environmental conditions can have a substantial influence on microbial growth, and, as a direct consequence, on protein formation. Such factors include fermentation duration, temperature, inoculum volume, and the concentration of available nutrients within the medium. In the present study, wheat bran was utilized as the primary fermentation medium. Its dry weight is comprised of approximately 25–40% xylan, making it an appropriate substrate for xylanase induction.[17,18] The present report details a stepwise approach to optimize a variety growth parameters associated with enzyme production. Due to the hyphal growth of filamentous fungi and its interaction with the substrate, it can be very difficult to quantify fungal mass in SSF directly.[19,20] To circumnavigate this, a number of indirect methods have been developed as alternative indicators of growth.[21] In the present study, the main aim was to maximize xylanase production specifically by *T. lanuginosus* 195 in SSF, and so, xylanase activity was quantified to assess the influence of optimization of the fermentation conditions. Having established a growth strategy for optimum xylanase production on wheat bran, the influence of supplemental carbon and nitrogen sources was assessed.

Materials and Methods

Materials. Media constituents and other reagents were of molecular biology grade or higher where appropriate, and were supplied by Sigma-Aldrich (St. Louis, MO). Wheat bran was supplied by Glasson Grain (Lancaster, UK).

Fungal strain and culture conditions. The fungal strain *T. lanuginosus* 195 was preserved at the Alltech Bioscience Centre, Dunboyne, County Meath, Ireland. The strain was grown on potato-dextrose-agar (PDA) medium at 45 °C for 4 d and stored at 4 °C.

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Spores were collected from PDA slants with 0.1% v/v Tween-20, washed, and diluted in sterile water. Liquid second seed medium was inoculated with 1×10^6 spores ml^{-1} and incubated for 3 d at 40 °C, 200 rpm. Liquid seed growth medium consisted of (g/l of deionised water): corn starch, 60; peptone, 18; glucose, 5; magnesium sulphate, 1.5; potassium phosphate, 1; potassium chloride, 0.5, and was sterilized at 105 °C for 30 min. Prior to optimization and for the purpose of an initial determination of xylanase activity, 10 g of sterilized wheat bran in 250-ml Erlenmeyer flasks was inoculated with 8 ml of liquid seed culture, diluted 1:4 with sterile water. Flasks, prepared in triplicate, were then fermented for 4 d at 40 °C at 80% relative humidity (RH) in a humidity chamber (Sheldon Manufacturing, Cornelius, OR).

Detection of xylanase activity. Fermented media were treated with 90 ml of deionised water for 90 min. The crude enzyme preparation was filtered through muslin cloth and centrifuged at 10,000 × *g* for 10 min at 4 °C. Enzyme preparations were examined for total xylanase activity using a modified version of the assay described by Bailey *et al.*,[22] using 2% w/v birchwood xylan as substrate. Sample absorbance was calculated at λ_{540nm} with a Shimadzu 160-A UV-Visible Spectrophotometer (Kyoto, Japan). One unit of xylanase (U) was defined as the amount of enzyme required to liberate 1 µmole of xylose per min under the assay conditions.

Optimization of growth parameters in SSF. The influence of fermentation temperature on xylanase production was investigated by incubating inoculated wheat bran at temperatures ranging from 25 to 45 °C, at 5 °C increments. The effect of the fermentation duration at each individual temperature was also assessed by analyzing SSF cultures for xylanase production at various time points. These optimized parameters were then employed to investigate the effect of inoculum volume on xylanase production. This was assessed by combining 10 g of sterilized wheat bran with varying volumes of inoculum, ranging from 4 ml to 10 ml.

Influence of supplemented carbon and nitrogen sources on xylanase production. Liquid mycelial inoculum was diluted 1:4 in the respective carbon and nitrogen sources, to a final concentration of 3% w/v, prior to the inoculation of wheat bran. Subsequent SSF was carried out according to the optimized growth parameters.

Statistical analysis. Data were analyzed using the Minitab statistical software package, version 15.0 (Coventry, UK). All statements of significance were calculated by One-Way ANOVA, based on a confidence level of 95%.

Results and Discussion

Initial screening of xylanase production in SSF

The initial screening for xylanase activity indicated that *T. lanuginosus* 195 was capable of producing approximately 1,919 U/g of wheat bran. Efforts were subsequently employed to enhance xylanase production by employing a step-by-step optimization procedure, altering the fermentation temperature, duration, and inoculum volume. The optimum conditions found for a specific parameter were retained in the process, and were used in subsequent determinations.

Effect of fermentation conditions on xylanase production

The results presented in Fig. 1 indicated that xylanase production was simultaneously influenced by incubation temperature and duration. High fermentation temperatures appeared to require a shorter fermentation time for optimal xylanase production (40 h at 45 °C). Conversely, lower fermentation temperatures typically required an extended incubation period for higher levels

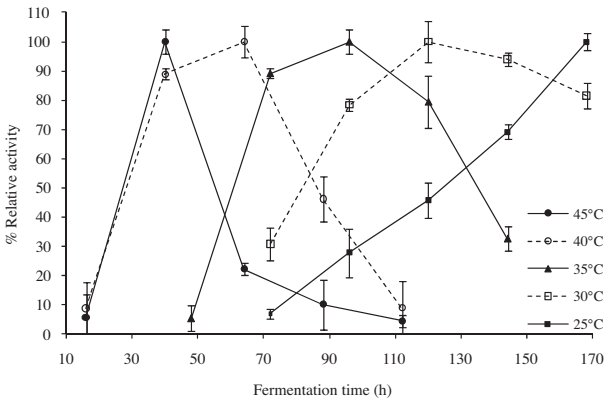

Fig. 1. Effect of Fermentation Duration on Xylanase Production at Various Temperatures.

Results are the mean of triplicate flasks with standard deviation represented by error bars. Each data series represents a fermentation carried out at a specific temperature, with closed circles (●) representing 45 °C, open circles (○) 40 °C, closed triangles (▲) 35 °C, open squares (□) 30 °C, and closed squares (■) 25 °C.

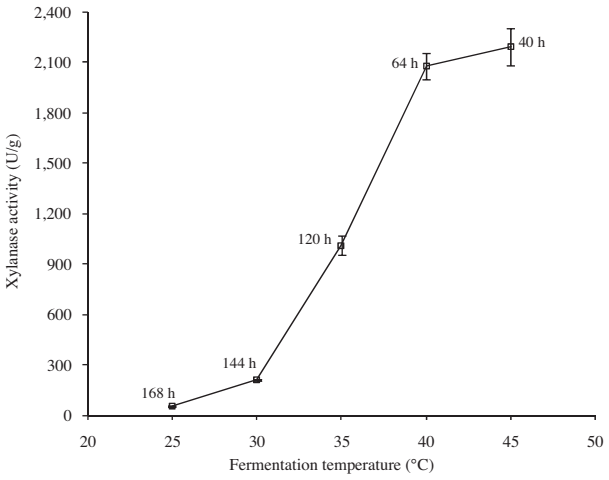

Fig. 2. Influence of Fermentation Temperature on Xylanase Production.

Results are the mean of triplicate flasks with standard deviation represented by error bars. Xylanase activity was detected following the optimal fermentation time for each temperature: 168 h at 25 °C, 144 h at 30 °C, 120 h at 35 °C, 64 h at 40 °C, and 40 h at 45 °C.

of xylanase production (168 h at 25 °C). Prolonged incubation beyond the optimal xylanase production period typically resulted in a steadily decreasing yield of enzyme activity, indicating the importance of optimizing the fermentation duration.

When incubated at 45 °C, *T. lanuginosus* 195 produced maximum xylanase after 40 h of fermentation. Comparatively, time course studies carried out on a number of *T. lanuginosus* strains by Christopher *et al.*[23] indicated that 4 d of fermentation was optimum for xylanase production at 45 °C on baggase pulp. Shorter incubation times are generally favored when considering the overall cost of fermentation in a large-scale process and the reduced potential of contamination.[24,25]

The effect of fermentation temperature on xylanase production was subsequently evaluated (Fig. 2). Data points represent the highest xylanase activity detected at each temperature following the optimum fermentation

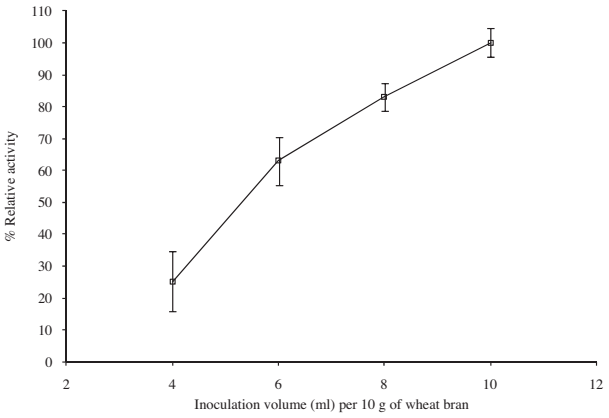

Fig. 3. Effect of Initial Inoculum Volume on Xylanase Production in SSF.

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

time. Fermentation temperature appeared to have an appreciable effect on xylanase production. *T. lanuginosus* 195 produced maximum xylanase at elevated temperatures (2,192 U/g at 45 °C) while displaying minimal activity at 25 °C (52 U/g) and at 30 °C (210 U/g).

Kamra and Satyanarayana[26] reported that 45 °C was optimal for xylanase production when *Thermomyces lanuginosus* TMD-3 was cultivated on wheat bran under SSF conditions. They also found a rapid reduction in xylanase activity for temperatures in excess of 50 °C. Smits *et al.*[27] concluded that the time required for enzyme production and maximum specific activity by filamentous fungi in SSF was largely mediated by the temperature of fermentation.

The influence of inoculum volume on xylanase production was assessed by altering the amount of inoculum added to the wheat bran substrate from a diluted mycelial culture. Triplicate flasks were incubated at the optimum fermentation temperature (45 °C) and duration (40 h), and were analyzed for xylanase activity (Fig. 3). The initial inoculum volume had a considerable effect on xylanase production from *T. lanuginosus* 195. Increased inoculum volume generated increased xylanase production, producing 2,335 U/g when 10 g of wheat bran was inoculated with 10 ml of liquid culture. Considering the inherent moisture content of the wheat bran, this volume of inoculum resulted in a moisture content of 62%. In turn, according to the relationship of moisture content to water activity (a_w) on wheat bran,[28] this equated to a water activity in a range of 98–99%.

Similar volumes have been reported for xylanase production from *Thermoascus aurantiacus* on wheat bran.[29] Lower inoculum volumes in SSF might not accommodate mycelial expansion and subsequent product formation.[30] Increased levels of inoculum typically improve growth-related activities,[31] but after a certain point, they serve to restrict gaseous exchange, reduce heat removal, and increase the demand for nutrients from the substrate.[32] Furthermore, a higher inoculum volume in SSF can increase the incidence of bacterial contamination.[2,33] The initial moisture content typically establishes the water activity of the process, determining the proportion of free water available in the solid matrix to facilitate biological and physiological activity.[34] A variety of hydrolytic enzymes have been optimally produced by SSF, with water activities ranging from 93 to 99%.[26,28,29,35] Reduced xylanase production from a strain of *Myceliophthora thermophila* was detected under similar conditions, corresponding to a 42% drop in relative activity when 10 ml of inoculum was used instead of the strain optimum of 6 ml per 10 g of wheat bran (equating to an approximate moisture content of 50% and an a_w of 96%) (data not shown).

Table 1. Summary of Optimized Cultivation Parameters in SSF

Optimized parameters	*Thermomyces lanuginosus* 195
Fermentation temperature (°C)	45
Fermentation time (h)	40
Inoculum volume (per 10 g of wheat bran)	10
Optimized xylanase activity (U/g)	2,335
Relative increase	21.7%
p-value	$p \leq 0.001$

Optimized growth parameters for xylanase production in SSF

Each of the optimized parameters was simultaneously employed in a single fermentation and compared to the originally screened enzyme activity of 1,919 U/g of wheat bran. The results indicated that xylanase production from *T. lanuginosus* 195 increased 21.7% under optimized conditions, yielding 2,335 U/g of wheat bran (Table 1). These findings indicate a significant effect of optimized fermentation parameters on xylanase production ($p \leq 0.001$).

Christopher *et al.*[23] reported varying levels of xylanase production from different strains of *T. lanuginosus*, ranging from 1,060 U/g to 5,098 U/g, when cultivated by SSF on bagasse pulp. Titers of 7,832 U/g of wheat bran,[26] 19,320 U/g of bagasse pulp,[36] and 20,180 U/g of milled corn cob[37] have also been achieved by SSF. Although xylanase activity in the present study reached 2,335 U/g, the overall improvement in xylanase production indicates the impact of specific parameters on enzyme production in SSF and the possibility of improving the process as a whole. A similar study, carried out by Sonia *et al.*,[38] yielded 39,726 U/g of sorghum straw, improving xylanase production 3.4-fold when specific culture conditions were optimized. Typically, the choice of SSF substrate is governed by its capacity to deliver the desired end-product.[30] Additionally, factors such as lignocellulosic composition, nutrient accessibility, potential contaminants or end-product inhibitors, particle size, cost, and availability must also be considered.[3,29,39] Such factors are particularly important for industrial-scale fermentation, whose set-up and geographical location may dictate substrate choice.

Effect of supplemented carbon source on xylanase production

The influence of supplemented carbon sources to the primary substrate (wheat bran) on xylanase production was assessed. The results are presented relative to the activities obtained in the absence of any supplemental carbon source (Fig. 4). Statistical analysis of the data

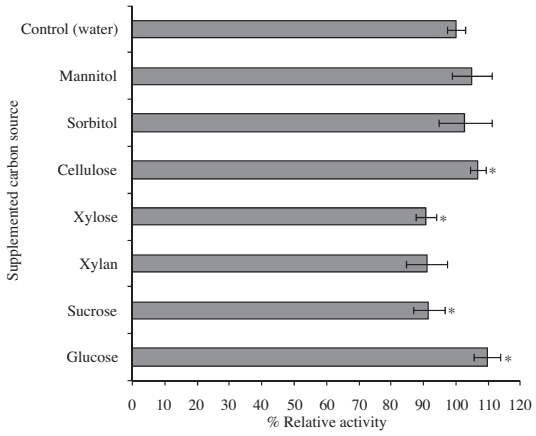

Fig. 4. Effect of Various Carbon Sources on Xylanase Production in SSF.

Results are the mean of triplicate flasks with standard deviation represented by error bars. Single asterisks denote a significant difference in xylanase activity as compared to the control, where $p \leq 0.05$.

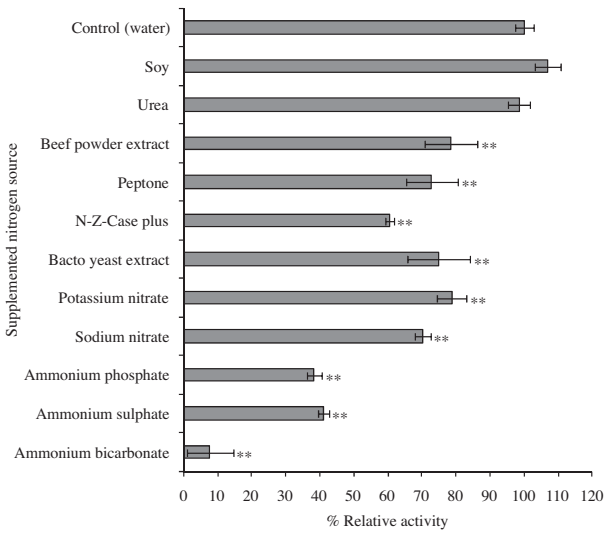

Fig. 5. Effect of Various Nitrogen Sources on Xylanase Production in SSF.

Results are the mean of triplicate flasks with standard deviation represented by error bars. Double asterisks denote a significant difference in xylanase activity as compared to the control, where $p \leq 0.005$.

indicated that xylanase production by *T. lanuginosus* 195 was significantly affected when wheat bran was supplemented with certain carbon sources ($p \leq 0.05$). Glucose and cellulose improved xylanase production, equating to a 10% and a 7% increase in relative activity (RA) respectively. Conversely, when added to wheat bran, xylose and sucrose reduced xylanase activity by approximately 9 and 8% respectively.

Xylanase production from *Humicola lanuginosa* was repressed by supplemental xylose in solid-state cultures.[26] Lemos and Pereira Junior[40] also found that sugar cane baggase, supplemented with xylose, reduced xylanase production by *A. awamori*. However, the same authors also documented reduced levels of xylanase activity following the addition of supplemental glucose, contradicting the results found in the present study. Botella *et al.*[41] concluded that although glucose significantly increased xylanase production by *A. awamori* on grape pomace, activity declined at supplemented levels of 8% w/w. De Souza *et al.*[42] reported similar findings, whereby increased concentrations of supplemented glucose reduced xylanase production from *Aspergillus tamarii*. The relevant literature would suggest that the primary substrate employed in an SSF process dictates the need for supplemented carbon sources.[26,34,42,43]

Effect of supplemented nitrogen source on xylanase production

The effect of nitrogen sources on xylanase production by *T. lanuginosus* 195 was determined relative to the results obtained in the absence of any supplemented nitrogen (Fig. 5). Xylanase activity was reduced by significant levels ($p \leq 0.005$) for a majority of the supplements listed in Fig. 5. Of particular note was the near complete elimination of xylanase activity following the incorporation of ammonium bicarbonate.

Roon *et al.*[44] concluded that ammonium has a propensity to inhibit protein formation and fundamentally impedes numerous cellular functions. This phenomenon has been found to occur in a number of fungal strains, including *Aspergillus* and *Fusarium* spp., whose growth in a solid state culture was completely inhibited in the presence of 1% w/w ammonium bicarbonate.[45] The relevant literature reports that organic sources of nitrogen typically increase xylanase production from filamentous fungi.[37,40,46,47] However, in the present study, none of the supplemented nitrogen sources increased xylanase production from *T. lanuginosus* 195 to a significant level ($p \leq 0.05$), suggesting that the influence of nitrogen supplementation is both strain and substrate dependent.

Increased environmental concerns have prompted the restricted use of highly toxic and mutagenic organochlorines, which are commonly used in the paper and pulping industry.[48] Incorporating xylanases in the pulping and bleaching processes has been found to reduce the need for such chlorinated compounds.[23,49] Xylanase preparations suited to such an application should retain activity at elevated temperatures and alkaline pH and be free of cellulases.[50–52] *Thermomyces lanuginosus* is a thermophilic fungus that is a cellulase-free, single-xylanase producer.[13] A single xylanase from *T. lanuginosus* 195 has been purified 9.3-fold from a crude koji extract, with a specific activity of 6,182 U/mg of protein.[53] Following cultivation by SSF, xylanase from *T. lanuginosus* 195 exhibited optimal activity at 80 °C (data not shown). Furthermore, the crude xylanase preparation was shown be exclusively xylanolytic, with stability demonstrated over a wide pH range (pH 2–10) and at elevated temperatures (30% RA at 100 °C).[53] Such characteristics indicate that the xylanase preparation from *T. lanuginosus* 195 can be used in the treatment of paper pulp, without the need for purification or excessive downstream processing.

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