

Amylase Production in Solid State Fermentation by the Thermophilic Fungus *Thermomyces lanuginosus*

Adinarayana Kunamneni,^{1*} Kugen Permaul,¹ and Suren Singh¹

Department of Biotechnology, Durban Institute of Technology, ML Sultan Campus,
P.O. Box 1334, Durban, South Africa¹

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The production of extracellular amylase by the thermophilic fungus *Thermomyces lanuginosus* was studied in solid state fermentation (SSF). Solid substrates such as wheat bran, molasses bran, rice bran, maize meal, millet cereal, wheat flakes, barley bran, crushed maize, corncobs and crushed wheat were studied for enzyme production. Growth on wheat bran gave the highest amylase activity. The maximum enzyme activity obtained was 534 U/g of wheat bran under optimum conditions of an incubation period of 120 h, an incubation temperature of 50°C, an initial moisture content of 90%, a pH of 6.0, an inoculum level of 10% (v/w), a salt solution concentration of 1.5:10 (v/w) and a ratio of substrate weight to flask volume of 1:100 with soluble starch (1% w/w) and peptone (1% w/w) as supplements.

[**Key words:** *Thermomyces lanuginosus*, amylase, solid state fermentation, wheat bran, optimization]

Amylases (a term that refers here to α -amylase, β -amylase and glucoamylase [GA]) are among the most important enzymes in present-day biotechnology. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Currently, a large number of microbial amylases are available commercially and they have almost completely replaced the chemical hydrolysis of starch in the starch processing industry (1). Amylases find potential application in a number of industrial processes such as in the food, fermentation, textile and paper industries.

Traditionally, amylase has been produced by submerged fermentation (SmF) and used in a one-way process in solution. In recent years, however, solid state fermentation (SSF) processes have been increasingly utilized for the production of this enzyme.

SSF holds tremendous potential for the production of enzymes (2). It can be of particular relevance in those processes where a crude fermented product may be used as an enzyme source (3). The selection of a particular strain, however, remains a tedious task, particularly when commercially significant enzyme yields are required. Agro-industrial residues are generally considered the best substrates for the SSF processes and enzyme production in SSF. The use of SSF for the production of enzymes and other products has many advantages over submerged fermentation (4) and these have been widely discussed in the literature (1, 4, 5).

SSF has gained renewed interest from researchers for the production of these enzymes in view of its economic and engineering advantages and is often employed to produce

amylases (2, 5). Selvakumar *et al.* (6) reviewed the microbial synthesis of starch-saccharifying enzymes in solid cultures. Since thermostability is a feature of most of the enzymes sold in bulk for industrial application, thermophilic microorganisms are of particular interest for the production of thermophilic amylases. Recent research on thermostable amylases has concentrated on the enzymes of thermophiles and extreme thermophiles.

The production of amylolytic enzymes from the thermophilic fungus *T. lanuginosus* has only been reported from submerged cultivations (7–9). *T. lanuginosus* grows on different carbon sources and produces a wide range of industrially important amylase enzymes (8). Reports on amylase production by SSF using wheat bran and thermophilic fungi are scanty. The purpose of the present study was to investigate the production of amylase under SSF conditions. In this paper, we report a number of factors that influence amylase production by *T. lanuginosus* through SSF.

MATERIALS AND METHODS

Microorganism and growth *T. lanuginosus* ATCC 58160 was grown at 50°C in submerged cultivation on a medium containing the following (g/l distilled water): soluble starch, 15.0; yeast extract, 5.0; K₂HPO₄, 5.0; MgSO₄, 5.0, with a pH of 6.0. All chemicals used were of analytical grade.

The experiments were conducted in 250-ml Erlenmeyer flasks containing 10 g of substrate and 1 ml of salt solution containing the following (g/l distilled water): K₂HPO₄, 5; MgSO₄·7H₂O, 5. Distilled water was added to achieve final substrate moisture content of 80%. After sterilization, the flasks were cooled and inoculated with a 10% (v/w) inoculum and incubated at 50°C for 96 h. After fermentation, the contents of the flasks were harvested and assayed.

Commercial quality wheat bran, molasses bran, rice bran, maize

* Corresponding author. e-mail: adikunamneni@rediffmail.com
phone: +27-31-3085351 fax: +27-31-3085321

meal, millet cereal, wheat flakes, barley bran, crushed maize, corn-cobs and crushed wheat were procured from the local market and used as solid substrates and their effect on the production of amylase was determined. The best solid substrate was selected and used in subsequent experiments.

Various process parameters were optimized by conventional methods for maximal enzyme production as follows: incubation period (24, 48, 72, 96, 120, 144 and 168 h), incubation temperature (40°C, 50°C, 60°C, 70°C and 80°C), initial total moisture content (50%, 60%, 70%, 80%, 90%, 100% and 110%), initial pH (4.0–8.0) and inoculum level (5%, 10%, 15%, 20%, 25% and 30% [v/w]). Wheat bran, the best solid substrate, was supplemented with different carbon sources (soluble starch, sucrose, lactose, maltose, dextrose, fructose and glucose) and nitrogen sources (peptone, tryptone, meat extract, ammonium sulphate, yeast extract, soybean meal, urea, ammonium sulphate and sodium nitrate) at 1% (w/w) and the ratios of salt solution concentration to substrate weight (v/w) (1.0:10, 1.5:10, 2.0:10 and 2.5:10) and substrate weight to flask volume (w/v) (1:50, 1:100, 1:150, 1:200, 1:250 and 1:300) were varied.

Production of amylase under optimum conditions On the basis of the results obtained with all the optimum parameters, namely viz, wheat bran containing soluble starch and peptone as supplements (1% w/w), pH of 6.0, moisture content of 90%, salt solution concentration of 1.5:10 (v/w), inoculum level of 10%, incubation temperature of 50°C and incubation period of 120 h. The extent of improvement in the optimized medium was evaluated using basal medium (10) as a control. The fermentation was carried out as described above.

Analytical methods At the end of fermentation, the solid biomass was treated with 50 ml of distilled water and agitated thoroughly on a magnetic stirrer for 30 min. The entire contents were filtered through muslin cloth and the residue was again treated with another 50 ml of distilled water in the same manner and filtered. The filtrates were pooled together and centrifuged (10,000 rpm, 15 min), and the clear supernatant was used as the enzyme source.

Amylase activity was determined at 50°C by mixing appropriately the diluted enzyme with 1% (w/v) soluble starch dissolved in 0.1 M sodium acetate buffer (pH, 6.0) and determining the reducing sugars, in the form of glucose, after 5 min (11). One unit of activity was defined as the amount of enzyme releasing 1 mg of glucose equivalents from the substrate per min at 50°C. All the experiments were conducted in triplicate and the mean of the three with standard deviation (SD) was represented as the number of units of enzyme produced per gram of wheat bran.

The moisture content of the wheat bran was estimated by drying 10 g of wheat bran to a constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, wheat bran was soaked with the desired quantity of water. After soaking, the sample was again dried as described above and the moisture content (%) was calculated as follows:

$$\text{percent moisture content (initial) of solid medium} \\ = (\text{wt. of the wheat bran} - \text{dry wt.}) \times 100/\text{dry wt.}$$

RESULTS AND DISCUSSION

In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. Here, all the substrates supported growth and enzyme formation by the culture, while wheat bran proved superior to the other substrates. A high titer of amylase activity (261 U/g) was obtained in a

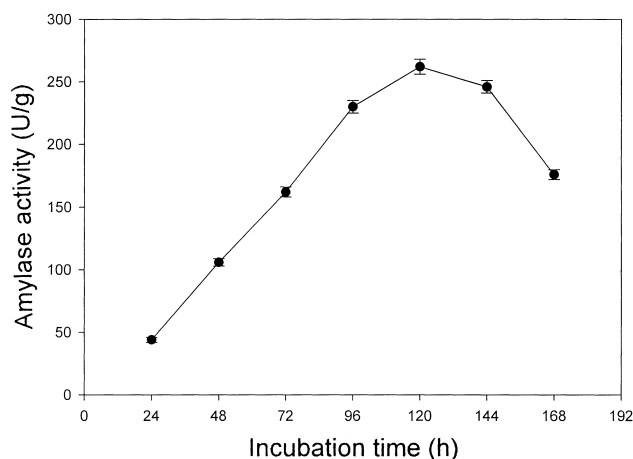


FIG. 1. Effect of incubation period on amylase production.

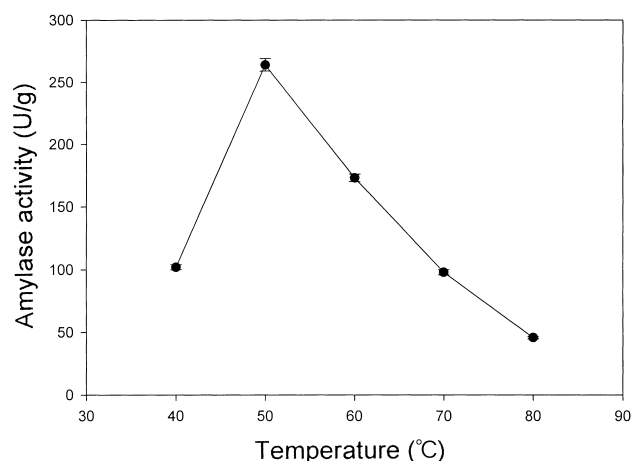


FIG. 2. Effect of incubation temperature on amylase production.

medium containing wheat bran alone as the substrate. The order of substrate suitability was wheat bran > millet cereal > crushed maize > crushed wheat > corn-cobs > wheat flakes > maize meal > barley bran > molasses bran > rice bran. Different solid substrates were found to effect the production of enzymes (12). It was previously reported that wheat bran was found to be the best substrate for glucoamylase production by an *Aspergillus* species (13) and suitable for necessary manipulation (13, 14). In subsequent experiments, therefore, wheat bran was used as the substrate for the production of amylase.

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The *T. lanuginosus* strain produced high titers of enzyme (262 U/g) at 120 h of incubation (Fig. 1). A similar result was reported by Ellaiah *et al.* (13). The optimal temperature for maximum amylase production (263 U/g) was found to be 50°C (Fig. 2). Previously, 30°C and 45°C were reported as optimum temperatures for amylase production by *Aspergillus flavus* and *Myceliophora thermophila*, respectively (15, 16).

A high enzyme titer (298 U/g) was attained when the initial moisture level was 90% (Fig. 3). The critical importance

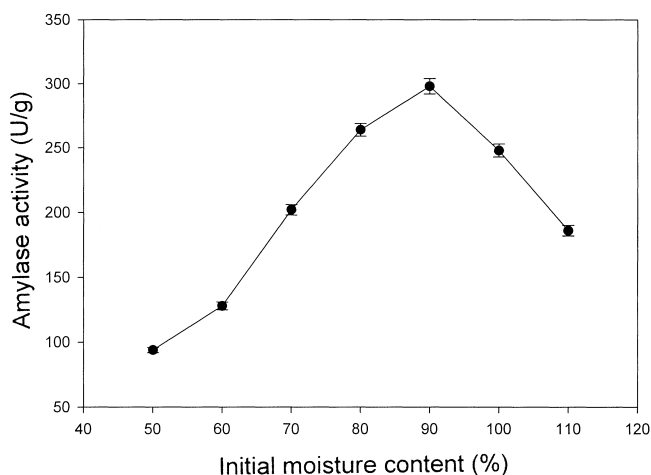


FIG. 3. Effect of initial moisture content on amylase production

of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. An increase in moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer (17). A low moisture content causes reduction in the solubility of nutrients of the substrate and a low degree of swelling (18).

The maximum amylase production (281 U/g) was obtained at a pH of 6.0. The inoculum level was also an important factor for the production of amylase. High inoculum levels are inhibitory in nature. The highest enzyme production (267 U/g) was obtained at an inoculum level of 10% (v/w).

The influence of supplementary carbon and nitrogen sources was studied. Of the carbon sources tested, soluble starch increased the amylase production (388 U/g) followed by sucrose (Table 1). Earlier workers reported soluble starch as the best carbon supplement for amylase production in *M. thermophila* D14 (16) and *A. fumigatus* (19).

Among the nitrogen sources, peptone increased amylase production (414 U/g) followed by tryptone and meat extract (Table 2). Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production in *A. fumigatus* (19), *A. niger* (20) and *A. oryzae* (21).

Maximum enzyme production (306 U/g) was obtained at a ratio of salt solution concentration to wheat bran weight of 1.5:10 (v/w), whilst further increases in salt concentration were found to inhibit enzyme activity.

The level of solid substrates is vital in SSF, particularly in tray processes (17). Also in flask experiments, the level and nature of substrates influence the porosity and aeration. The highest enzyme production (261 U/g) was observed when the ratio of substrate weight to flask volume was 1:100. Our finding is in agreement with the results of other investigators (12).

Production of amylase under optimized conditions

The maximum productivity of amylase (534 U/g) was achieved by utilizing wheat bran as the solid substrate for 120 h at 50°C, at an initial moisture content of 90%, a pH of 6.0, an inoculum level of 10% (v/w), a salt solution concen-

TABLE 1. Effect of various additional supplementary carbon sources on amylase production

Carbon source (1% w/w)	Amylase activity (U/g)
Soluble starch	388±9.0
Sucrose	362±8.5
Lactose	326±7.8
Maltose	294±7.2
Dextrose	278±6.7
Fructose	266±5.6
Glucose	192±5.0
Control	261±5.3

Values are means of three determinations with SD (±).

TABLE 2. Effect of various additional supplementary nitrogen sources on amylase production

Nitrogen source (1% w/w)	Amylase activity (U/g)
Peptone	414±11.6
Tryptone	356±10.4
Meat extract	338±9.0
Ammonium sulphate	310±8.5
Yeast extract	306±8.0
Soybean meal	294±7.3
Urea	280±6.7
Ammonium nitrate	272±6.0
Sodium nitrate	264±5.3
Control	261±5.4

Values are means of three determinations with SD (±).

tration of 1.5:10 (v/w) and a ratio of substrate weight to flask volume of 1:100 with soluble starch (1% w/w) and peptone (1% w/w) as supplements.

From the above-mentioned results, we conclude that a twofold increase in amylase production was achieved under the optimized fermentation conditions with 1% (w/w) soluble starch and 1% (w/w) peptone as supplements, as compared with the basal medium (control). Although the results of these investigations are based on experiments conducted in flasks, they provide valuable information for the production of amylase by SSF.

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