Amylase production from *Aspergillus oryzae* LS1 by solid-state fermentation and its use for the hydrolysis of wheat flour

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Abstract

Nine *Aspergillus* and three of *Trichoderma* strains were grown on wheat bran (WB) medium under solid state fermentation (SSF) for amylase production. *Aspergillus oryzae* LS1 produced the highest level of the enzyme. The thermal stability profile of its crude enzyme revealed the half-life time of more than 2 h at 50 and 60ºC. The enzyme production was affected by strain type, incubation periods, level of moisture content and carbon source supplementation. Maximum enzyme production of about 14249 IU/g WB was obtained under optimum conditions with an incubation period of 120 h, an initial moisture content of 54.5% and in the presence of sucrose (1 g/100g WB) at 30ºC. Of substrates tested, soluble starch was the best one hydrolyzed by the crude enzyme. Corn starch, dextrin and potato starch were also hydrolyzed to a lesser extent. The enzyme exhibited maximum activity at 55ºC. Moreover, the enzyme was also able to hydrolyze wheat flour under optimized conditions with efficiency of 89%.

Keywords**:** Amylase; *Aspergillus oryzae;* solid state fermentation; production; application

INTRODUCTION

Amylases (a term that refers here to α-amylase, β-amylase and glucoamylase) are among the most important enzymes in present day biotechnology. Although amylases can be derived from several sources, microbial enzymes generally meet industrial demands. These enzymes, especially α-amylase (1, 4-α-D-glucan glucanohydrolase [EC 3.2.1.1]) have found wide applications in a number of industrial processes such as in the food, fermentation, textile and paper industries (Pandey *et al*., 2000 and Kunamneni *et al*., 2005). Industrially important enzymes including amylases have traditionally been obtained from submerged fermentation (SmF) because of ease of handling and greater control of environmental factors such as temperature and pH. Solid state fermentation (SSF) constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly (Ellaiah *et al*., 2002; Soni *et al*., 2003).

Solid-state fermentation presents a series of advantages over submerged fermentations (Hesseltine, 1972) and these have been widely discussed in the literature (Pandey, 1992; Pandey *et al*., 2000). The culture conditions are more similar to natural habitat of filamentous fungi, so that they are able to grow and excrete large quantities of biomaterials. Products concentration after extraction are usually higher than those obtained by submerged fermentation and the quantity of liquid waste generated during the fermentation process is lower (Castilho *et al*. 2000). SSF has gained renewed interest from researchers for enzymes production in view of its economic and engineering advantages and is often employed to produce amylases (Pandey *et al*., 1999).

Filamentous fungi have a number of properties which make them important both scientifically and industrially. They are also suitable microorganisms for SSF, especially because their morphology allows them to colonize and penetrate the solid substrate (Rahardjo *et al*., 2005). *Aspergillus oryzae* has been successfully

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used to express high levels of heterologous proteins (Huge-Jensen *et al*., 1989) and industrial enzymes (Barbesgaard *et al*., 1992).

The purpose of the present study was to optimize some of the critical factors affecting amylase production by a local strain of *Aspergillus oryzae* LS1 under SSF and study some of its properties.

MATERIALS AND METHODS

Microorganisms: Nine strains of *Aspergillus* sp and three strains of *Trichoderma* sp. were screened for their ability to produce amylase under solid state fermentation. Three local strains (LS) were identified as *A. oryzae LS1, A. niger LS1 and A. niger LS2* through the Microbial Resource Center (MIRCEN), Ain-Shams University, Cairo, Egypt. *Trichoderma harizanum* and *Trichoderma viridi* were kindly supplied by Dr. M. Fadel, National Research Center, Dokki, Egypt. The other strains of *Aspergillus awamori* NRLL 373*, Aspergillus awamori* NRLL 3112, *Aspergillus oryzae* NRRL 6586*, Aspergillus oryzae* NRRL 6584, *Aspergillus oryzae* NRRL 6583, *Aspergillus oryzae* NRRL 447 and *Trichoderma ressei* NRRL 6165 were obtained from Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA.

Inoculum preparation: The spore suspension was prepared from a 5 days old culture grown on starch casein agar slant by adding 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80 and suspending the spores with a sterile loop (Lingappa and Vivek Babu, 2005). Two ml of the spore suspension containing about 1×10^7 spores/ml counted by the dilution plate count method (Parkinson *et al*., 1971) was used as inoculum.

Solid state fermentation: Unless otherwise stated, only five grams of wheat bran in 250 ml Erlenmeyer flask were autoclaved at 120ºC and15 psi for 30 min. After sterilization, the flasks were cooled and inoculated with 2 ml spore suspension (~10^7 spores/ml). The appropriate amount of sterilized distilled water was added in such a way that the final substrate moisture content was 50%.

Optimization of SSF process: Factors like selection of strain, initial moisture content, incubation time and various carbon additives affecting the secretion of amylase by the selected strain under SSF were optimized. The strategy followed was to optimize each parameter independent of the others and subsequently optimal conditions were employed in all experiments. In a sequential order, the various process parameters were optimized for maximal enzyme production as follows: incubation period (24, 48, 72, 96, 120,144 and 168 h), initial total moisture content (9, 16, 28, 37.5, 44.4, 50, 54.5, 58.3, 61.5, 64.3 and 66.6%). Wheat bran was supplemented with different carbon sources (soluble starch, sucrose, lactose, maltose, glucose, corn starch, potato starch, rice starch and wheat flour) at 1% w/w to study their effect on amylase production. The fermentation was carried out at 30ºC keeping all other conditions at their optimum levels.

Analytical method

*Enzyme extraction***:** At the end of the fermentation process, the biomass was treated with 0.02 M phosphate buffer, pH = 6 (10 ml/g solid) and agitated thoroughly on a rotary shaker for 30 min. The whole contents were filtered and centrifuged at 4000 rpm and 4ºC for 10 min. The clear supernatant was stored at 4ºC and used as the enzyme source.

Determination of enzyme activity: Amylase activity was determined by the procedure of Jamieson *et al*. (1969) using soluble starch as a substrate. The reaction mixture containing 250 µl of 1% substrate (w/v) in 0.02 M phosphate buffer, pH = 6 and 250 µl of suitably diluted enzyme solution was incubated for 5min at 55ºC. The reaction was stopped by adding 500 µl of 3.5 dinitrosalicylic acid solution (1 mg/ml) followed by heating in a boiling water bath for 5 min , cooling at room temperature and then 10 ml of distilled water was added. Absorbance of each solution containing the brown reduction product was measured at 470 nm. One unit of enzymatic activity was defined as the amount of protein that produced 1 µmol of reducing sugar as maltose per min under the assay condition.

Application of crude enzyme preparation from A. oryzae LS1 in hydrolysis of wheat starch: The produced amylase from *A. oryzae* LS1 was evaluated for its capability to hydrolyze wheat flour according to Soni *et al*. (2003) with some modifications. 100 gm wheat flour was dispersed with constant stirring into 400 ml tap water in 1 L capacity flat bottom boiling flask. This was followed by the addition of 11.7 IU of fungal amylase preparation per mg solids and the temperature was maintained between 50 and 55ºC for pre-

Table 1. Screening for amylase production by different microorganisms under SSF.

Microorganism	crude extract pH	Protein (mg/g solid)	Amylase (IU/g solid)
A. oryzae LS1	7.28	43.5	1362.09
A. oryzae NRRL 6586	7.02	48.7	1490.62
A. oryzae NRRL 6584	7.00	39.1	1075.78
A. oryzae NRRL 6583	6.15	37.2	1133.04
A. oryzae NRRL 447	6.71	76.5	980.65
A. awamori NRRL 373	5.81	51.2	589.67
A. awamori NRRL 3112	6.09	47.0	381.34
A. niger LS1	6.16	51.2	0.0
A. niger LS2	6.73	52.9	467.84
T. ressei NRRL 6165	6.04	58.9	142.54
T. harzianum	6.28	52.5	1062.38
T. viridi	6.88	50.4	246.10

cooking, in a water bath, for 30 min. The mash was then cooked under pressure in an autoclave at 120ºC and 15 psi for 30 min. After gelatinization, the content of the flask were allowed to cool and another dose of amylase (11.7 IU/mg solids) was added while the temperature was maintained at 50-55ºC for another 30 min for starch liquefaction. The liberated reducing sugar was determined according to Jamieson *et al*. (1969) after 30, 60, 120 min and 24 h.

Conversion efficiency was calculated as follows:

Reducing sugars produced by hydrolysis ÷ Reducing sugars obtainable from starch in wheat X 100 /1/Sugars obtainable from starch hydrolysis as determined from stoichiometrical calculations were 1.11 g/g.

RESULTS

Optimization of solid state fermentation

Amylase levels of different strains: This experiment aimed to find out the most promising strain for amylase production. After sterilization of wheat bran medium the flasks were cooled, inoculated with 2 ml spore suspension of each organism separately and incubated at 28ºC for 96 h. Results in Table 1 showed that the levels of amylase are greatly dependent on strain type. *Aspergillus oryzae* LS1 and *Aspergillus oryzae NRRL 6586* produced the highest level of amylase. The other strains secreted lower levels of the enzyme during their growth on solid state media.

Results also indicated that no clear correlation is observed between the enzymes activities and the amount of extracellular protein released in the fungal solid cultures.

On the other hand, the selection of *A. oryzae* LS1 was also based on the stability of its amylase than that produced by *A. oryzae NRRL 6586* (Fig. 1). In this regard amylase from *A. oryzae* LS1 was optimally active at 50ºC and displayed about 94, 73, and 13 % of its peak activity at 50, 60, and 65ºC, respectively, after 90 min. The enzyme was also quite active even at the room temperature (30-40ºC).

Effect of incubation time: The pattern of amylase production as well as the protein content of the solid media was followed with time. *A. oryzae* LS1 was cultivated on wheat bran medium at 28-30ºC for 168 h in a rotary shaker at 200 rpm. Samples were taken every 24 h to follow up the amylase level and protein content of the growth medium. The results illustrated in Figure 2 showed that the production of amylase increased

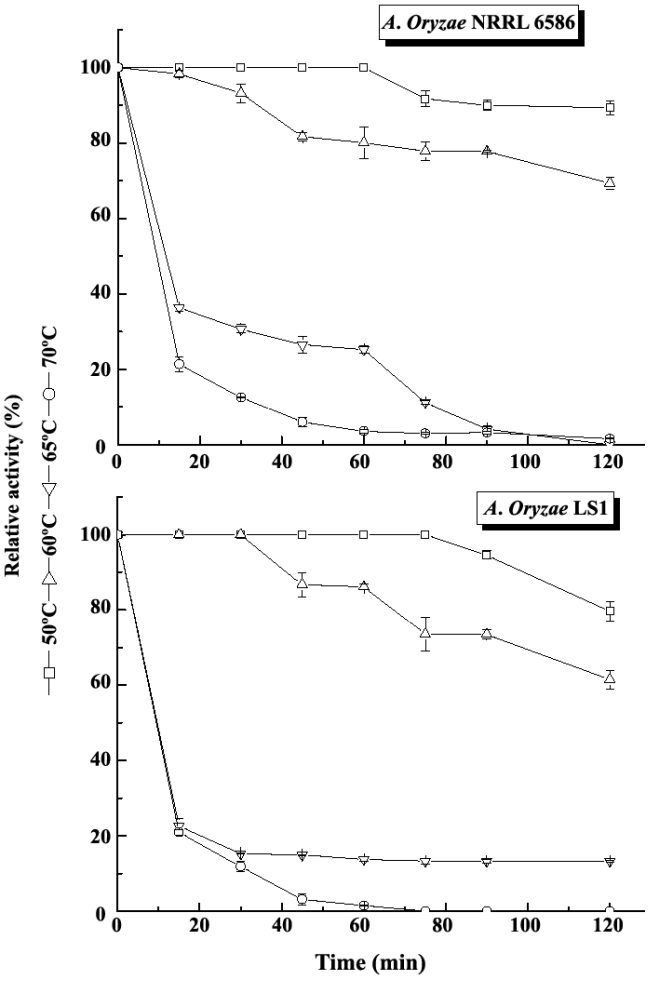

Figure 1. Thermal stability of crude amylase produced by *A. oryzae* LS1 and *A. oryzae* NRRL 6586 under solid state fermentation.

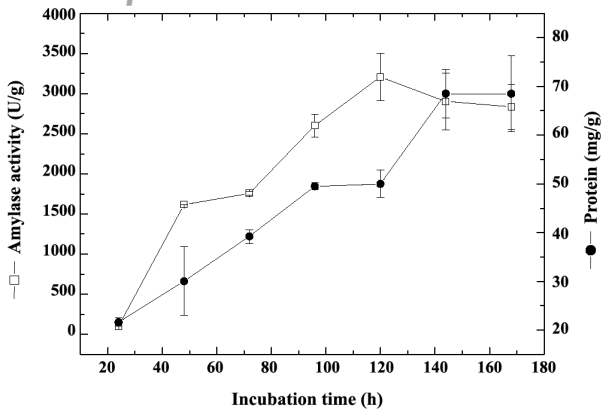

Figure 2. Effect of different incubation periods on amylase production by *A. oryzae* LS1 under SSF.

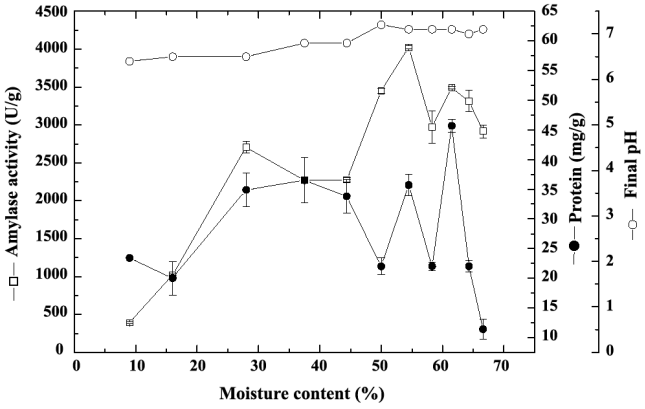

Figure 3. Effect of initial moisture content on amylase production by *A. oryzae* LS1 under SSF.

gradually from 24 h and reached its maximum value (3207 IU/g solids) at 120 h of incubation. After that the activity decreased. The results also showed that the protein content of the solid medium increased with the increase of the fermentation time.

*Effect of initial moisture content on enzyme productio*n: Figure 3 shows the effect of initial moisture level on amylase production by *Asp*. *oryzae* LS1 in SSF for wheat bran. 54.5% initial moisture content enhanced maximum enzyme production. The lowest moisture level tested in this work (10-30%) resulted in poor amylase formation. In addition, a reduction in enzyme level was also recorded at high initial moisture content.

Effect of various additional supplementary carbon sources: Supplementation of the wheat bran medium with various carbon sources such as glucose, lactose, maltose, sucrose, soluble starch, corn starch, potato starch, rice starch and wheat flour stimulated amylase production by *A*. *oryzae LS1* in the present study (Fig. 4). Of the carbon sources tested, maltose, sucrose, lactose and soluble starch increased the amylase production by about 180%. Slightly better amylase production resulted with the addition of corn starch, potato starch, rice starch and wheat flour to wheat bran media. Supplementation of the wheat bran medium with different concentrations of sucrose or maltose increased amylase activity. The highest yield of amylase (14249 IU/g solids) was obtained at 1% sucrose concentration (data not shown).

Characterization of amylase preparations

Substrate specificity: The crude enzyme was examined

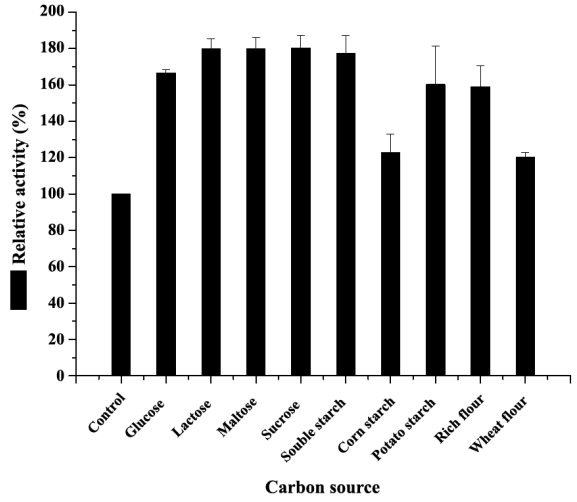

Figure 4. Effect of different additional supplementary carbon sources on amylase production by *A. oryzae* LS1 under SSF.

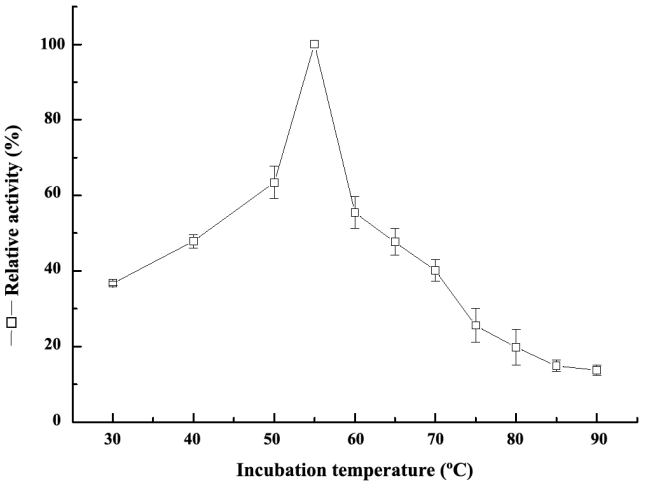

Figure 5. Effect of reaction temperature on the activity of amylase from *A. oryzae* LS.

Table 2. Substrate specificity of the crude amylase produced by *A. oryzae* LS1 under SSF.

Substrate	Relative activity (%)	Sd (yEr±)	SE (yEr±)
Soluble starch	100		
Potato starch	13	0.84853	0.60
Corn starch	64.16	1.18794	0.84
Yellow dextrin	29.02	2.85671	2.02
White dextrin	34.52	0.73539	0.52

for its ability to hydrolyze various carbohydrates (soluble starch, potato starch, corn starch, yellow dextrin and white dextrin) under the standard assay conditions as mentioned before. Of the substrate tested, soluble starch was the most hydrolyzed by the enzyme. Corn starch, white dextrin, yellow dextrin and potato starch were also hydrolyzed to a lesser extent (Table 2).

Effect of reaction temperature on the activity of α-*amylase*: The activity of amylase produced from *A. oryzae* LS1 was measured at various temperatures (30-90ºC) in phosphate buffer at pH 6 with soluble starch as a substrate. As shown in Fig. 5 it was found that the activity of produced amylase from *A. oryzae* LS1 gradually increased with the increase of the reaction temperature up to 55ºC whereby the maximal activity of the enzyme was recorded. However, further increasing in the reaction temperature above this degree resulted in a gradual decrease in the enzyme activity.

Effect of substrate concentration: The effect of soluble starch concentration on the activity of *A. oryzae* amylase was examined using different concentrations ranging from 250-2500 µg/ml (w/v). Results in Figure 6 show that an increase in starch concentration in the reaction mixture was accompanied by a proportional increase in the amounts of released reducing sugars. Maximum activity of the enzyme was recorded at substrate concentration of 1000 µg/ml. Increasing the substrate concentration above the aforementioned starch concentration exhibited no further noticeable released reducing sugar.

Effect of enzyme concentration: Different enzyme concentrations ranged from 2.87 to 57 µg/ml were tested with respect to their amylase activity. Figure 7 shows that the hydrolysis of starch as well as the activity of amylase of *A. oryzae LS1* under various enzyme concentrations. These results indicate that, at the same substrate level, increased concentrations of enzyme up to 50 µg/ml of the enzyme concentration were accompanied by a considerable increase in the starch hydrolysis. Higher enzyme levels (>50 µg/ml) appeared to be unfavorable for the hydrolysis process.

Application of amylase preparation from A. oryzae LS1 in the hydrolysis of wheat flour: The obtained amylase from *A. oryzae LS1* was evaluated for its ability to hydrolyze wheat flour. The enzyme worked well and revealed the overall conversion efficiencies of about 76, 78 and 89% after 1, 2 and 24 h, respectively (Table 3). The maximum conversion efficiency after 24 h at 55ºC was probably because amylase produced by *A. oryzae LS1* had temperature optima of 55ºC. The ability of the amylase preparation from *A. oryzae LS1* to bring about suitable application in hydrolysis of wheat starch with an overall conversion efficiency of about 89%.

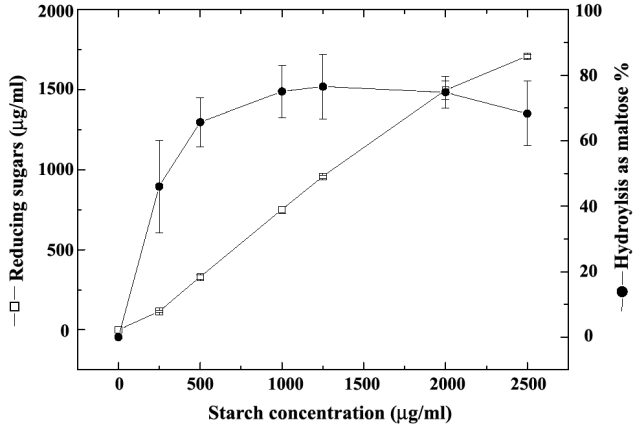

Figure 6. Effect of different starch concentrations on the activity of amylase from *A. oryzae* LS1.

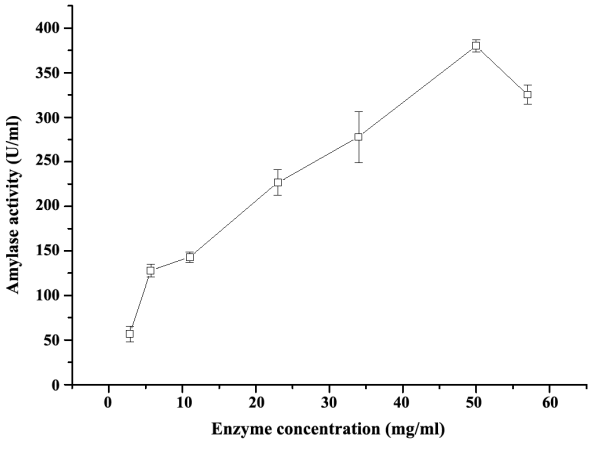

Figure 7. Effect of different enzyme concentrations on the activity of α-amylase from *A. oryzae* LS1.

DISCUSSION

There are several factors, which affect SSF process. Among these, selection of suitable strain, substrate and selection of process parameters (physical, chemical and biochemical) are crucial factors (Pandey *et al*., 2000). In the present work the selection of *Asp*. *spp*. was based on the fact that fungal amylases are produced mainly by *A*. *oryzae*, *A*. *niger* and *A*. *awamori*. It is also known that the amylase of *Aspergilli* species is known to produce more sugar than bacterial amylase (Nigam and Singh 1995). From the results of the screening process *A*. *oryzae* LS1 was the best for amylase production. Furthermore, the thermal stability profile of the crude amylase from the local stain revealed that its half life time is more than 2 h at 50 and 60ºC. This can also be a potential candidate for use in combination with culture of *Saccharomyces cerevisiae* for alcohol production from starchy materials, where the temperature of fermentation process is in the range of 30-40ºC (Farid *et al*., 2002).

The incubation time for achieving maximum enzyme yield is governed by the characteristics of the culture and based on growth rate and enzyme production (Kunamneni *et al*., 2005). Amylase production by the local strain reached its maximum level (3207 IU/g solids) after 120 h of incubation. On further incubation, the enzyme activity gradually decreased. This may be due to the depletion of essential nutrients required for the growth and enzyme production. Similar results were reported by Ellaiah *et al*. (2002). They mentioned that amylase production was found to be growth associated.

The critical importance of moisture level in SSF media and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles. Lonsane *et al*. (1985) and Sodhi *et al*. (2005) reported that higher moisture level decreases porosity, changes wheat bran particle structure, promotes development of stickiness, reduces gas volume and exchange and decreases diffusion, which results in lowered oxygen transfer and enhanced formation of aerial mycelium. On the other hand, lower moisture content reduces the solubility of nutrients present in solid substrate, decreases the degree of swelling and increases water tension (Feniksova *et al*., 1960). Acuna-Arguelles *et al*. (1994) and Pandey *et al*. (1994) reported that with low water-availability fungi suffer modification in their cell membranes leading to transport limitations and affecting microbial metabolism. Additionally, a reduction in enzyme production was recorded at high initial moisture content and this may be due to a reduction in substrate porosity, changes in the structure of substrate particles and reduction of gas volume. Based on the present results, moisture contents between 54-60% seems to result in a compromise among water availability, substrate swelling and oxygen diffusion effect, favoring amylase production by *A*. *oryzae LS1*.

The nature of solid substrate is the most important factor in SSF. However, nutrients supplies to the culture also serve as an anchorage for the microbial cells (Lonsane *et al*., 1985). Both natural as well as synthetic substrates can be used in SSF. The selection of a substrate for SSF process depends upon several factors mainly related with cost and availability. It would be necessary to supplement them externally. Wheat bran has been the prime substrate among many processes, which have been developed and have utilized this as a raw material for the production of bulk chemicals and fine products (Pandey *et al*., 2001). Commercial wheat bran contains about 8.5% starch and 9.5% protein (Fisher 1973) in addition to various minerals. It may not provide all the nutrients needed by the organism for maximum α-amylase production during SSF. Hence, the exogenous addition of various carbon sources to the solid medium may improve the growth of the produced microorganism and thus the product yield (Sodhi *et al*., 2005). In the present work, addition of different sugars resulted in better amylase production with maltose, sucrose and soluble starch. These results are in agreement with that reported by many authors. Earlier workers reported soluble starch as the best carbon supplement for amylase production in *Myceliaphthora thermophila* D14, (Sadhukhan *et al*., 1990), *Aspergillus fumigatus* (Goto *et al*., 1998) and *Thermomyces lanuginosus* (Kunamneni *et al*., 2005).

Table 3. Enzymatic hydrolysis of wheat flour by *A. oryzae* LS1 amylase produced under SSF.

Parameter	Conversion efficiency %	Sd (yEr±)	SE (yEr±)
Pre-cooking			
30 min	53.5	2.82843	2
Post-cooking			
30 min	58.3	0.98995	0.7
60 min	76.2	0.56569	0.4
120 min	78.5	3011127	2.2
24 h	89.3	0.70711	0.5

The reaction was carried out using 11.7 IU/mg solid at 55ºC, in 0.02 M phosphate buffer, pH = 6.

On the other hand, Tada *et al*. (1991) and Lachmund *et al*. (1993) reported that the α-amylase production in *A. oryzae* is induced by carbohydrates having α-1, 4 glucosidic bonds, e.g., maltose, starch.

The present study has revealed that the amylase produced by *A. oryzae* LS1 has a maximum activity at 55ºC using soluble starch as substrate at 1000 µg/ml and enzyme concentration of 50 µg/ml. These results are in agreement with that reported by Elegado and Fujio (1993). The authors reported that wheat and cassava starches are the most favored substrates by all strains while potato and corn starches were the least. Ghose *et al*. (1990) reported also similar finding on starch hydrolysis by *A. terrus* amylase. They indicated that wheat starch was the best substrate followed by rice starch, potato starch, parley, flour, sago and finally topica starch.

The ability of amylase preparation from *A. oryzae* lS1 to bring about significant hydrolysis of wheat flour with an overall conversion efficiency of about 89% after 24 h suggests that this enzyme can be employed for the hydrolysis of wheat flour and can also be used in combination with *Saccharomyces cerevisiae* for production of ethyl alcohol.

CONCLUSIONS

A two fold increase in a thermo stable amylase production by a new local strain (*Aspergillus oryzae LS1*) was achieved under solid state fermentation. Supplementation of wheat bran medium with different moisture and carbon sources affect the enzyme production. The produced enzyme showed maximum activity when soluble starch was used as a substrate at 55ºC. The production process can be further improved by optimizing the fermentation and culture conditions in order to achieve better yields and reduce the cost. The produced enzyme obtained in this style has some advantages for further studies such as stabilization and immobilization.

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