

## Alpha Amylase Production by *Bacillus cereus* MTCC 1305 Using Solid-State Fermentation

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### Summary

Production of  $\alpha$ -amylase under solid-state fermentation by *Bacillus cereus* MTCC 1305 has been investigated using wheat bran and rice flake manufacturing waste as substrates. With wheat bran, highest enzyme production expressed as units per mass of dry substrate ( $94 \pm 2$ ) U/g was observed. Production parameters were optimized as inoculum size 10 % (volume per mass) and substrate:moisture ratio 1:1. Among different carbon sources supplemented, glucose (0.04 g/g) showed enhanced enzyme production ( $122 \pm 5$ ) U/g. Supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production. Optimum  $\alpha$ -amylase enzyme activity was observed at 55 °C and pH=5. At 75 °C, enzyme showed 90 % activity compared to 55 °C.

*Key words:*  $\alpha$ -amylase, *Bacillus cereus*, solid-state fermentation, wheat bran, optimization

### Introduction

Amylases have been reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, namely  $\alpha$ -amylase and glucoamylase.  $\alpha$ -Amylases (endo-1,4- $\alpha$ -D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4- $\alpha$ -D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1,4- $\alpha$ -D-glucan glucohydrolase, E.C. 3.2.1.3) hydrolyzes single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner (1).

Among various extracellular enzymes,  $\alpha$ -amylase ranks first in terms of commercial exploitation (2). Spectrum of applications of  $\alpha$ -amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry (3).

The cost of enzyme production in submerged fermentation (SmF) is high, which necessitates reduction in production cost by alternative methods. The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products for the reduction of cost of the medium (4). The use of agricultural wastes makes solid-state fermentation (SSF) an attractive alternative method (5). Baysal *et al.* (6) have reported  $\alpha$ -amylase production in solid-state fermentation with wheat bran and rice husk as substrates. Ramachandran *et al.* (7) have checked the potential of coconut oil cake as substrate for the production of  $\alpha$ -amylase using *Aspergillus oryzae*, a GRAS strain. Ikram-ul-Haq *et al.* (4) have described the selection of a suitable low cost fermentation medium for the production of  $\alpha$ -amylase by using agricultural by-products. Glucoamylase production with an *Aspergillus* sp. has been reported using cheap rice flake manufacturing wastes as substrate (8).

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The most effective amylases are those that are thermostable (9). They are generally preferred as their application minimizes contamination risk and reduces reaction time, thus enabling considerable energy saving. Thermostable  $\alpha$ -amylases are used for the liquefaction of starch at high temperature and thermolabile  $\alpha$ -amylases are used for the saccharification of starch in baking (10). Babu and Satyanarayana (2) have reported production of  $\alpha$ -amylase by a thermophilic *Bacillus* sp. and optimization of culture conditions for maximum enzyme production. Suitability of thermophilic *Bacillus coagulans* for  $\alpha$ -amylase production by solid-state fermentation in flasks, reactor and trays has been reported (11).

In the present study  $\alpha$ -amylase production from *Bacillus cereus* MTCC 1305 using solid-state fermentation has been investigated and the enzyme is reported to show activity at high temperature.

## Material and Methods

### Microorganism

*Bacillus cereus* MTCC 1305 used in the present study was obtained from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on Bushnell Haas Agar (BHA) slants containing 1 % starch at 4 °C.

### Solid-state fermentation

Initial enzyme production was checked individually using wheat bran procured from local market and agro-industrial wastes generated during processing of rice to rice flakes. These two wastes were categorized as coarse and medium waste (8). Further optimization of process parameters was studied using wheat bran as substrate for solid-state fermentation.

### Development of the inoculum, enzyme production and extraction

For the development of inoculum, culture was transferred from stock to 100-mL nutrient broth and the inoculated flasks were incubated overnight at (35±2) °C and 150 rpm. Cells were harvested from the broth and their *A* was checked at 660 nm. Accordingly, cells with inoculum size of  $A_{660}=0.5$  (10 % inoculum (volume per mass)) per 5 g of substrate were harvested, washed and resuspended in sterile distilled water. Production media contained 5 g of solid substrate and 10 mL of Bushnell Haas (BH) mineral salt medium in 250-mL Erlenmeyer flasks and were inoculated with the above inoculum. Inoculated production media were incubated under static conditions at (35±2) °C and enzyme production was checked after every 24 h for 4 days. Enzyme was extracted in 50 mL of 0.1 M phosphate buffer (pH=7) on a rotary shaker at 250 rpm for 30 min. The content was filtered through muslin cloth, filtrate was centrifuged at 8325 × *g* for 10 min and clear brown supernatant was used as the enzyme source.

### $\alpha$ -Amylase enzyme assay

$\alpha$ -Amylase activity was determined by incubating a mixture of 0.5 mL of aliquote of each enzyme source

and 1 % soluble starch dissolved in 0.1 M phosphate buffer, pH=7, at 55 °C for 15 min (12). The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid, then followed by boiling for 10 min. The final volume was made up to 12 mL with distilled water and the reducing sugar released was measured at 540 nm (13). One unit (U) of  $\alpha$ -amylase activity was defined as the amount of enzyme that releases 1  $\mu$ mol of reducing sugar as glucose per minute, under assay conditions and expressed as U/g of dry substrate. All the experiments were performed in triplicates and the standard error has been reported.

### Optimization of cultural parameters

Inoculum size was varied as 10, 20, 30 and 40 % (volume per mass) of inoculum, where 1 % (volume per mass) corresponds to cells with  $A_{660}=1$  of inoculum size added to 100 g of substrate. Substrate:moisture ratio was maintained as 1:1, 1:1.5, 1:2 and 1:2.5 and the enzyme production was checked using wheat bran as substrate and BH mineral salt medium as moistening agent.

### Effect of additional nutrients

Carbon sources (0.04 g/g dry substrate) as glucose, soluble starch, maltose and sucrose, and nitrogen sources (0.02 g/g dry substrate) as casein, NH<sub>4</sub>Cl, yeast extract and urea were supplemented as individual components to the production media to check their effect on enzyme production.

### Effect of temperature and pH on enzyme activity

To determine temperature activity profile for an  $\alpha$ -amylase enzyme, assay was carried out at 35, 55 and 75 °C. For determination of suitable pH range for enzyme activity, pH of enzyme assay buffers (0.1 M) was varied as 5 (acetate buffer), 7 and 9 (phosphate buffer).

## Results and Discussion

*Bacillus* species are considered to be the most important sources of  $\alpha$ -amylase and have been used for enzyme production using SSF (1). Production of pullulanase and  $\beta$ -amylase using *Bacillus cereus* has been studied (1,9). Wheat bran and two waste products obtained while processing of rice to rice flakes, coarse waste and medium waste were evaluated for  $\alpha$ -amylase production by solid-state fermentation. Among the three substrates tested highest enzyme production was observed with wheat bran ((94±2) U/g) (Table 1). Maximum enzyme produc-

Table 1. Production of  $\alpha$ -amylase (U/g) by *Bacillus cereus* MTCC 1305 on different substrates by solid-state fermentation

Hours	Substrates		
	Wheat bran	Coarse rice waste	Medium rice waste
	Enzyme production/(U/g)		
24	20±2	7±2	13±2
48	70±2	25±2	15±2
72	94±2	34±2	19±1
96	55±3	16±3	7±2

tion was observed after 72 h, which decreased with further incubation. Among the two rice flake manufacturing wastes tested, coarse waste gave good enzyme production and further studies are required for efficient utilization of this waste generated in large quantities during the rice processing. Though rice flake manufacturing wastes were proved promising substitutes for wheat bran in glucoamylase production using fungal culture in our previous studies (8), the presence of husk particles along with broken rice in the wastes might have resulted in lower growth and enzyme production compared to wheat bran. Ikram-ul-Haq *et al.* (4) have reported wheat bran as the best substrate for  $\alpha$ -amylase production by *Bacillus licheniformis* using different agricultural by-products. In our subsequent optimization studies, wheat bran was used as the substrate for the production of  $\alpha$ -amylase.

Varying inoculum size of bacterial cells during the fermentation indicated 10 % (volume per mass) inoculum as optimum for the enzyme production (Fig. 1). Increase in inoculum size was found to adversely affect the enzyme production. As the moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities, adjusting the optimum moisture level of substrate during SSF is therefore most important (6). During solid-state fermentation, higher moisture level decreases porosity, changes wheat bran particle structure, promotes development of stickiness and lowers oxygen transfer (14,15), whereas lower moisture content causes reduction in the solubility of nutrients of the solid substrate, lower degree of swelling and higher water tension (15). In the present study, high enzyme titer was obtained when the substrate:moisture ratio was maintained as 1:2 in comparison with that of low or high moisture levels. Maximum  $\alpha$ -amylase enzyme production by thermophilic *B. coagulans* has been reported using a high level of moisture at 1:2.5 ratio of substrate:moisture content (11).

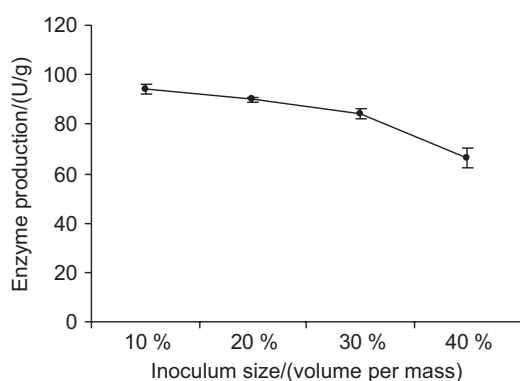


Fig. 1. Effect of inoculum size on the production of  $\alpha$ -amylase (U/g) by *Bacillus cereus* MTCC 1305 under solid-state fermentation using wheat bran as substrate

Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides resulted in marginal increase in  $\alpha$ -amylase production by *B. cereus* during solid-state fermentation using wheat bran. Highest production was observed with glucose ((122±5) U/g) (Fig. 2). *Bacillus thermooleovorans* is reported to prefer starch, glucose, lactose, maltose and maltodextrins as

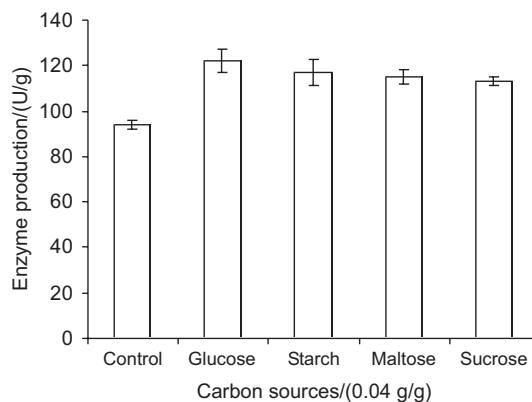


Fig. 2. Effect of carbon source (0.04 g/g) supplementation on  $\alpha$ -amylase (U/g) production by *Bacillus cereus* MTCC 1305 under solid-state fermentation using wheat bran as substrate

carbon sources for  $\alpha$ -amylase secretion (16). In contrast, carbon sources such as glucose, maltose and starch did not enhance  $\alpha$ -amylase production by thermophilic *B. coagulans* in solid-state fermentation using wheat bran (11). Addition of organic nitrogen sources such as casein, yeast extract and urea, and inorganic nitrogen source such as ammonium chloride to the medium resulted in considerable decrease in  $\alpha$ -amylase production by *B. cereus* (Fig. 3). Supplementation of additional nitrogen sources in general has been reported to be inhibitory for  $\alpha$ -amylase production by microorganisms. Presence of organic nitrogen sources, urea and peptone, has been reported to enhance  $\alpha$ -amylase enzyme production by *Aspergillus niger* in wheat bran containing solid substrate medium, but inorganic nitrogen source, ammonium chloride, repressed enzyme production (17). Decrease in  $\alpha$ -amylase enzyme production during solid-state fermentation when using organic nitrogen sources like casein, gelatin and soy meal as medium supplements has been reported (18).

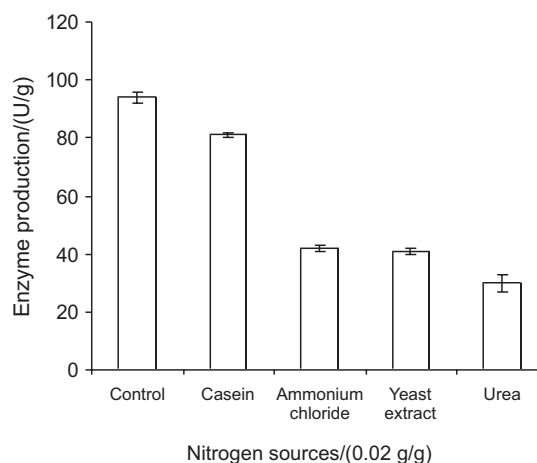


Fig. 3. Effect of nitrogen source (0.02 g/g) supplementation on  $\alpha$ -amylase (U/g) production by *Bacillus cereus* MTCC 1305 under solid-state fermentation using wheat bran as substrate

As starch liquefaction is generally carried out at higher temperatures of 70–90 °C, the thermostable  $\alpha$ -amylases are of great significance (19). In this study  $\alpha$ -amylase

produced by *B. cereus* showed considerable enzyme activity in the ranges from lower to higher temperature (Fig. 4). At 75 °C, 90 % activity was observed compared to the optimum enzyme activity at 55 °C. For  $\alpha$ -amylase produced by a laboratory *Bacillus* isolate AS-1, 88, 85 and 44 % of activity has been reported at 60, 70 and 80 °C, respectively (19). Thus, further studies on the thermal stability of  $\alpha$ -amylase enzyme produced by *B. cereus* MTCC 1305 have to be carried out to confirm its applications for starch liquefaction. Presence of metal ions like  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  known to improve the thermal stability may show promising results.

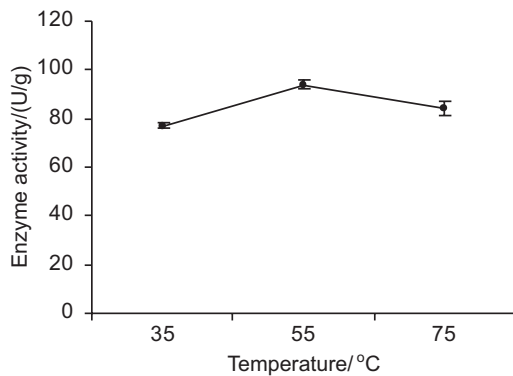


Fig. 4.  $\alpha$ -Amylase enzyme activity (U/g) of *Bacillus cereus* MTCC 1305 at three different temperatures

Maximum enzyme activity was observed at pH=5 ((96±2) U/g) (Fig. 5). The use of alkaline buffer (pH=9) for enzyme reaction resulted in a sharp decline in the enzyme activity.  $\alpha$ -Amylase of *Bacillus* sp. AS-1 is reported to have pH optimum at pH=6.5 and 98 % peak activity at pH=6 and 7. The same enzyme has also been shown efficient for liquefaction of gelatinized starch because of its thermal stability (19).

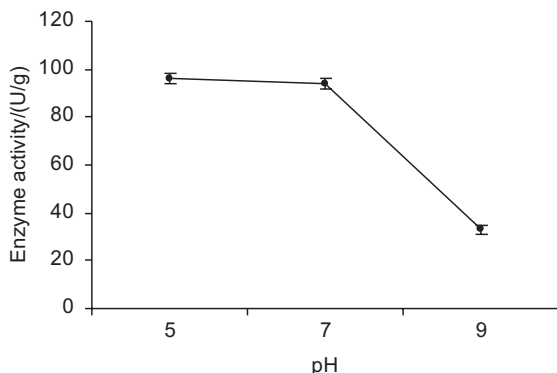


Fig. 5.  $\alpha$ -Amylase enzyme activity (U/g) of *Bacillus cereus* MTCC 1305 at three different pH values

## Conclusion

The results obtained in the present study indicated *Bacillus cereus* MTCC 1305 as a potential strain for  $\alpha$ -amylase production using solid-state fermentation with wheat

bran as substrate. Interesting observation was that it showed and retained 90 % enzyme activity at 75 °C compared to the optimum at 55 °C. Furthermore, the enzyme was found to show optimum activity under acidic condition (pH=5). This makes the  $\alpha$ -amylase of the organism useful for various industrial applications like starch liquefaction at increased temperatures.

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