



# Production of thermostable extracellular $\alpha$ -amylase by a moderate thermophilic *Bacillus licheniformis*-AZ2 isolated from Qinarje Hot spring (Ardebil prov. of Iran)

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**List of nonstandard abbreviations:**

**16S rRNA:** 16S ribosomal RNA  
**Agro-wastes:** Agricultural- wastes  
**DNS Reagent:** 3,5- di nitro salicylic acid reagent  
**LB medium:** Luria-Bertani medium  
**OFAT method:** one-factor-at-a-time method  
**PEG 4000:** Polyethylene Glycol MW 4000  
**PEG 6000:** Polyethylene Glycol MW 6000  
**RSM:** Response surface method  
**SDS:** Sodium dodecyl sulfate  
**SmF:** Submerged fermentation  
**SSF:** Solid state fermentation

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## Abstract

**Background and Purpose:** Amylases are the most important enzymes that share about 30% of the world enzyme market in food, animal feed, fermentation, textile and detergent industries. This study aimed at screening of some bacterial strains isolated from Qinarje Hot spring for amylolytic activity, and optimization of submerged fermentation conditions for maximum enzyme production by the higher  $\alpha$ -amylase producer.

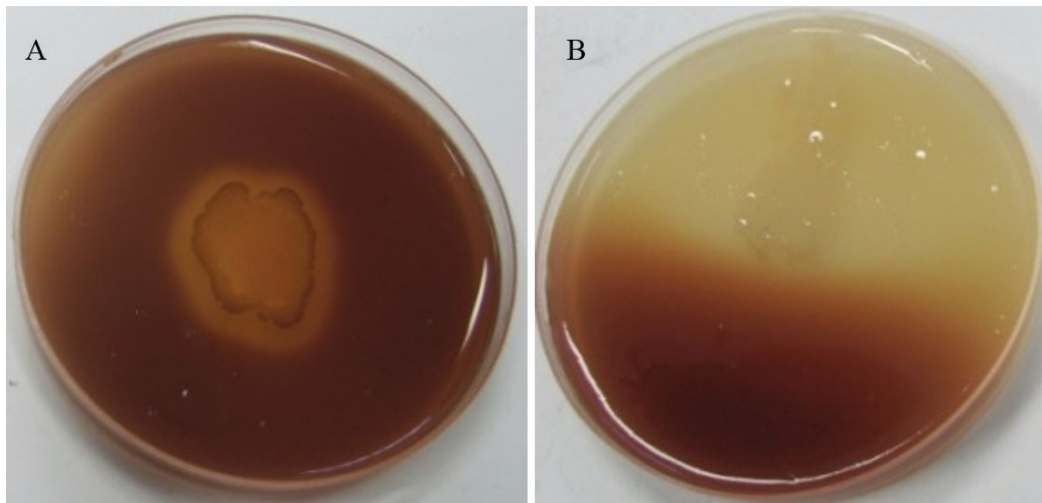
**Material and Methods:** Among 3 bacterial strains, *Bacillus licheniformis*-AZ2 showed the highest amylolytic activity after starch hydrolysis test and Gram's iodine staining. Then, effect of some physicochemical and nutritional parameters were studied on bacterial growth and  $\alpha$ -amylase production using turbidimetric and DNS method, respectively. One-factor-at-a-time method was followed to select the optimum level of each parameter. Partially purified extracellular enzyme from *B. licheniformis*-AZ2 was used to study the effects of pH and temperature on  $\alpha$ -amylase activity and stability.

**Results:** Compared to the basal medium, enzyme production increased 2.4 fold in optimized medium containing 1% (w/v) of tryptone (as a nitrogen source), 1% (w/v) of rice husk (as a carbon source), 0.3 g/L of  $\text{CaCl}_2$  and 2% (w/v) of Tween 80 with 2% (v/v) inoculum after 84 hours of incubation at 40°C and pH 9.0. The partially purified enzyme showed maximum activity at pH 7.0 and 80°C. It was also observed that  $\alpha$ -amylase is stable over a temperature range of 30°C to 80°C and pH of 6.0 to 10.0.

**Conclusion:** Our results show that *B. licheniformis*-AZ2 strain has an ability to produce the thermostable  $\alpha$ -amylase which could be suitable in starch processing and detergent industries.

## INTRODUCTION

The enzymes belonging to amylase family ( $\alpha$ -amylase,  $\beta$ -amylase and glucoamylase) are very important due to their wide range of industrial applications such as food, fermentation, textile, paper and detergents. Though they can originate from plants, animals and microorganisms, fungal and bacterial amylases are mainly used for the industrial production, because of higher production rate, thermostability, less time and space for production and easier process modification and optimization (1-3). Alpha amylase (1,4- $\alpha$ -D-glucan glucanohydrolase; E.C.3.2.1.1) is an enzyme that cleaves the  $\alpha$ -1,4-glucosidic linkages in starch, generating maltose and malto-oligosaccharides (4). A



**Figure 1.** Detection of amylase activity after starch digestion by *B. licheniformis*-Q (A) and *B. licheniformis*-AZ2 (B). Starch agar plates were inoculated with bacterial spots and flooded with Gram's iodine solution after 24 h.

significant increase in amylase production and utilization occurred in the early 1960s when the *Bacillus subtilis*  $\alpha$ -amylases and *Aspergillus niger* glucoamylases were used to replace acid catalysis in industrial production of dextrose from starch (5). Bacterial amylases are generally preferred to fungal amylases due to several characteristics and advantages they offers (1). Almost all members of the *Bacillus* genus can synthesize  $\alpha$ -amylase. Therefore, *Bacillus* spp. are considered to be the most important sources of  $\alpha$ -amylase and have been used extensively for this purpose (6). Despite similarities in the pattern of growth and the enzyme profiles of *Bacillus* spp., the optimized conditions for maximum enzyme production differ widely, depending upon strain (3). Thus, optimization of nutritional and physicochemical parameters is of utmost importance to perform the industrial process cost-effective and economically viable (7). The production of bacterial thermostable  $\alpha$ -amylases has been thoroughly investigated in submerged fermentation (SmF) and solid state fermentation (SSF). However, because of the greater control of environmental factors and ease of handling, SmF is more common and is affected by a variety of physicochemical parameters such as composition of growth medium, inoculum age, pH, temperature, nitrogen source and carbon source (8, 9). Enzyme production in fermentation process is usually measured by enzyme activity to determine the amount of enzyme present under defined conditions (10). OFAT approach (changing one-factor-at-a-time) and keeping the others at a constant level is the main strategy used for selecting optimal operating condition. In spite of some limitations due to OFAT approach, it continues to be used widely in preliminary optimization studies to identify the most important parameters, and these limitations subsequently can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the parameters in advanced studies (11, 12). Keeping all these facts in

mind, in the present study three bacterial strains isolated from Qinarje Hot spring, were screened for amylase production through starch hydrolysis test. The strain showing maximal amylolytic activity was further optimized for  $\alpha$ -amylase production on fermentation with different nitrogen and carbon sources including other physicochemical parameters using OFAT method.

## MATERIALS AND METHODS

### Chemicals and reagents

The DNS reagent and dialysis tubing cellulose membrane were purchased from Sigma-Aldrich (USA). Potato soluble starch from Carl Roth (Germany); tryptone, peptone and bacteriological agar powder from HiMedia (India); sucrose, lactose, maltose and glucose from Scharlau (Spain) were also used in experiments. All other chemicals were purchased from Merck (Germany).

### Microorganisms

The strains of *Anoxybacillus contaminans*-AZ1, *B. licheniformis*-Q and *B. licheniformis*-AZ2 used in the present study were obtained from Microbial Collection of Agricultural Biotechnology Department, at Bu-Ali Sina University, Hamedan, Iran. These strains have recently been isolated from Qinarje Hot springs (water sample of 82°C and pH 6.5) and identified based on 16S rRNA gene sequence analysis.

### Screening of potent $\alpha$ -amylase producing bacteria

Bacterial strains were screened for amylolytic activity by starch hydrolysis test on starch agar plates [LB agar medium containing 1% (w/v) potato starch at pH=7.0]. The bacterial strains were spot inoculated on the starch

agar plates and incubated at 40°C for 24 h. After incubation, a 1% iodine solution [2% (w/v) KI+1% (w/v) I<sub>2</sub>] (freshly prepared) was flooded on the starch agar plate. Presence of blue reddish color around the colonies was considered as negative result and a clear zone of hydrolysis surrounding the colonies indicated as positive result (Figure 1). Each colony producing largest clear halo of hydrolysis was considered a potential  $\alpha$ -amylase producer. Such colony was selected for further investigations.

### Inoculum preparation

The strain of *B. licheniformis*-AZ2 was streaked on LB agar plate and incubated for 24 h at 40°C. Then, a loopful of freshly grown bacterial cells were transferred into 100 mL of LB liquid medium [tryptone 10; NaCl 10; yeast extract 5 (g/L)] and incubated at 40°C. Inoculum was taken from the early exponential phase of growth after 24 h.

### Submerged fermentation for $\alpha$ -amylase production

$\alpha$ -amylase production was carried out in 250 mL Erlenmeyer flasks in 100 mL of basal medium consisting of (g/L): starch 10, yeast extract 3, peptone 5, NaCl 3, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 (pH=7). The aliquot of 1% (v/v) of the total basal medium was taken as inoculum. The flasks were kept at 40°C on a rotary shaker at 120 rpm for 120 h. The  $\alpha$ -amylase production was determined by 3,5-dinitrosalicylic acid method (13). At regular intervals (6 h), samples were harvested and the cell debris were removed by centrifugation (10,000 ×g for 10 min at 4°C) in a refrigerated centrifuge (Eppendorf 5810R, Germany). The cell-free supernatant was used for enzyme assay. In each series of experiments for optimization of basal medium (BM),  $\alpha$ -amylase produced under such conditions served as a control.

### $\alpha$ -amylase assay

$\alpha$ -amylase activity was determined by spectrophotometric method as described by Rick and Stegbauer (14). According to the procedure  $\alpha$ -amylase activity was assayed by adding 1 mL of enzyme (crude extract/fermented broth cell-free supernatant) to 1 mL of 1% (w/v) soluble starch in 50 mM Tris-HCl buffer (pH=7.0) in a test tube. The test tubes were covered and incubated for 5 min at 65°C in a water bath. Then 2 mL DNS reagent was added to each tube to stop the reaction and placed in boiling water bath exactly for 5 min. After cooling the samples in a cold water bath, the absorbance was read at 540 nm in spectrophotometer (Varian CARY 100 UV-vis spectrophotometer, Australia). An  $\alpha$ -amylase activity unit was defined as the amount of  $\alpha$ -amylase required to catalyze the liberation of reducing sugar equivalent to one milligram of maltose per minute under the assay conditions.

### Optimization of the process parameters in submerged fermentation using OFAT method

The basal medium was optimized with various factors influencing the  $\alpha$ -amylase production using OFAT methodology. The best choice in each experiment was used for the optimization of the next factor. Bacterial growth was also determined by measuring optical density of culture broth at 600 nm in each experiment.

### Optimization of physicochemical parameters

The various physicochemical parameters of fermentation were optimized which included temperature, initial pH, inoculum size and incubation time. Optimal temperature and pH were obtained by varying the temperature range from 10°C to 70°C and pH from 4.0 to 11.0. The effect of inoculum size on  $\alpha$ -amylase production was also investigated by inoculating the basal medium with different concentrations of inoculum, from 1% to 7% (v/v). In addition, production of  $\alpha$ -amylase and bacterial growth were monitored during 120 h by taking samples every 6 h intervals at the optimal temperature, pH and inoculum size.

### Effect of nitrogen and carbon sources

To investigate the effect of different nitrogen sources on  $\alpha$ -amylase production, peptone and yeast extract as the only nitrogen sources in the basal medium were replaced by different nitrogen sources such as tryptone, beef extract, yeast extract, peptone, ammonium hydrogen phosphate, sodium nitrate, potassium nitrate and urea at 1% (w/v) concentrations. Similarly, effect of various sugars and agricultural-wastes such as xylose, fructose, sucrose, glucose, lactose, sorbitol, *myo* inositol, mannitol, arabinose, wheat bran and rice husk at 1% (w/v) concentrations were examined by replacing starch as the only carbon source in the basal medium.

### Effect of inorganic salts and surfactants

Effect of different concentrations of inorganic salts such as NaCl (1 to 9 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 to 3 g/L), K<sub>2</sub>HPO<sub>4</sub> (2 to 8 g/L) and CaCl<sub>2</sub> (0.1 to 0.4 g/L) were investigated separately by replacing NaCl and MgSO<sub>4</sub>·7H<sub>2</sub>O in the basal medium. Effect of various surfactants on  $\alpha$ -amylase production also were evaluated by adding SDS, PEG 4000, PEG 6000, Glycerol, Tween 80 and Triton X-100 at 2% (w/v) into the finally optimized medium.

### Partial purification of enzyme

1 L of cell-free supernatant from optimized medium was fractionated into four fractions with ammonium sul-

fate (0-30%, 30-60%, 60-90% and 90-100%). Precipitated proteins were collected by centrifugation at 10,000  $\times$ g for 10 min at 4°C and pellet obtained from each fraction was dissolved in 5 mL of 50 mM Tris-HCl buffer (pH 7.0). Then, resuspended protein precipitates were dialysed overnight against 1 L (three changes) of the same buffer at 4°C with continuous stirring to completely remove ammonium sulfate.  $\alpha$ -amylase activity was measured in each fraction applying DNS method: 0.5 ml of dialysed partially purified enzyme was added to 0.5 mL soluble starch 1% (w/v) in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM  $\text{CaCl}_2$ , test tubes were covered and incubated for 5 min at 65°C in a water bath. Then, 1 mL DNS reagent was added to each tube to stop the reaction and placed in boiling water bath for 5 min, after cooling the samples in a cold water bath, the absorbance was read at 540 nm.

### Effect of pH on $\alpha$ -amylase activity and stability

The effect of pH on  $\alpha$ -amylase activity of partially purified enzyme was evaluated by performing the  $\alpha$ -amylase assay in the following buffer systems: 50 mM sodium acetate (pH 3.0-5.0), 50 mM potassium phosphate (pH 6.0-7.0), 50 mM Tris-HCl (pH 8.0-9.0) and 50 mM glycine-NaOH (pH 9.0-11.0). To study the influence of pH on the stability of  $\alpha$ -amylase, partially purified enzyme was mixed with the selected buffer systems at a ratio of 1:2 and incubated at 30°C for 24 h, then the residual activity (%) was determined under standard assay conditions.

### Effect of temperature on $\alpha$ -amylase activity and stability

To determine the optimum temperature, activity of the partially purified enzyme was measured at temperatures ranging from 30°C to 100°C for 5 min at 50 mM Tris-HCl buffer (pH 7.0). For thermal stability, the partially purified enzyme was preincubated for 1 h at various temperatures (30-100°C) before enzyme assay, and promptly cooled on ice and residual activity (%) was determined under standard assay conditions.

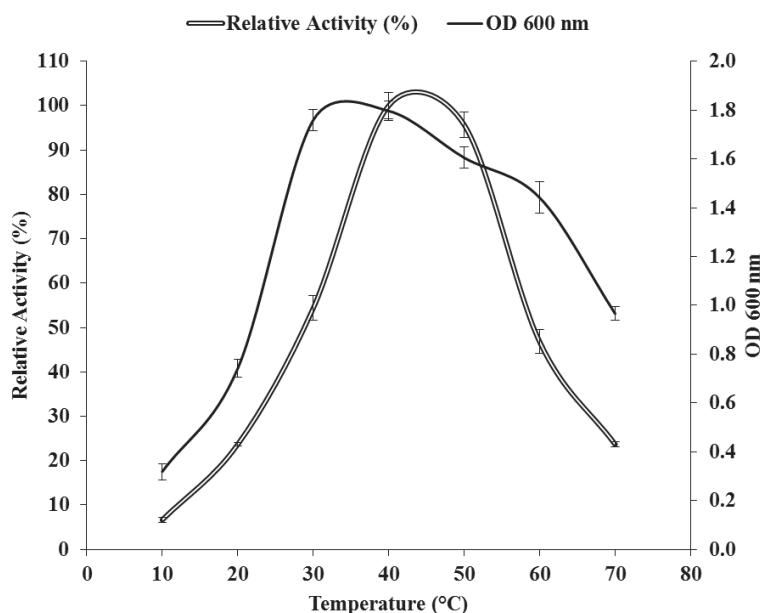
### Statistical analysis

All experiments were carried out in three replications based on a completely randomized design (CRD). Mean values and standard errors were calculated from the replications for each treatments. Statistical analysis was performed using SPSS V 16.0 software (SPSS inc. Polar Engineering and Consulting, USA) based on single factor analysis of variance (ANOVA). Duncan's multiple range test was used for mean comparison of the treatments at a  $p$  value of 0.05.

## RESULTS AND DISCUSSION

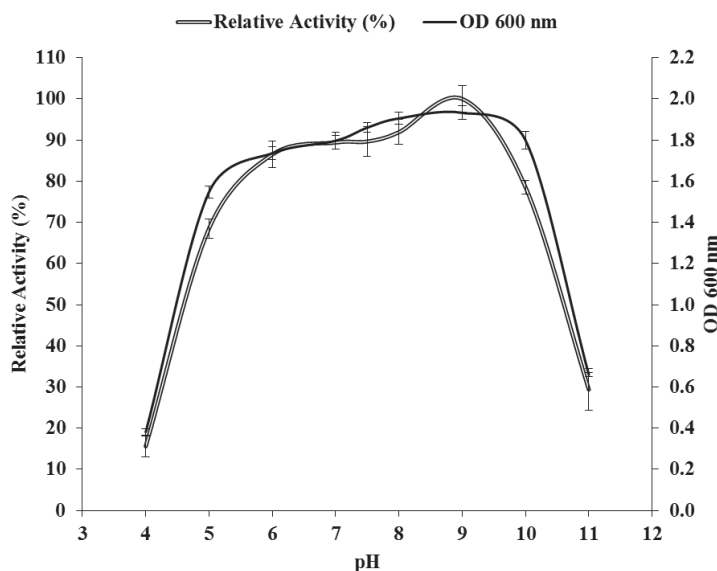
### Isolation of $\alpha$ -amylase producing bacteria

Among 3 bacterial strains isolated from Qinarje Hot spring, two of them i.e, Q and AZ2 hydrolysed starch on LB agar plate containing 1% (w/v) potato starch at pH



**Figure 2.** Effect of temperature on  $\alpha$ -amylase production and bacterial growth in basal medium, at pH 7.0 and 1% (v/v) inoculum after 120 h. Relative activity was defined as the percentage of maximal activity detected at standard assay conditions. Mean  $\pm$  S.E.M = Mean values  $\pm$  Standard error of means of three replications.





**Figure 3.** Effect of pH on  $\alpha$ -amylase production and bacterial growth in basal medium at optimal temperature 40°C and 1% (v/v) inoculum after 120 h.

Relative activity was defined as the percentage of maximal activity detected at standard assay conditions.

Mean  $\pm$  S.E.M = Mean values  $\pm$  Standard error of means of three replications.

7.0 and 40°C (Figure 1). AZ2 strain produced a larger clear zone compared to the Q strain, which indicates higher amylolytic activity. Therefore it was selected for further studies on  $\alpha$ -amylase production. The higher  $\alpha$ -amylase production of strain AZ2 against strain Q was also confirmed using DNS method in the LB liquid medium containing 1% (w/v) potato starch at pH 7.0 and 40°C (data not shown).

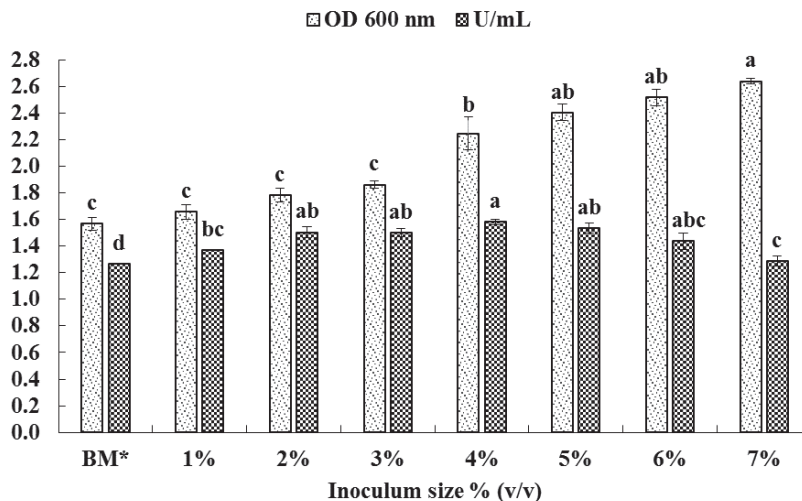
### Effect of temperature

The bacterial growth and  $\alpha$ -amylase production were studied at different temperatures ranging from 10°C to 70°C, at pH 7.0 in basal medium with 1% (v/v) inoculum after 120 h. The bacteria could grow at all temperatures and enzyme synthesis occurred from 20°C to 70°C. However, the maximal  $\alpha$ -amylase production in basal medium was observed at 40°C (Figure 2). Above the 50°C both, the growth and  $\alpha$ -amylase production, were decreased, indicates that the optimum temperature for maximal bacterial growth and  $\alpha$ -amylase production are similar. The influence of temperature on  $\alpha$ -amylase production is related to the growth of the organism. A wide range of temperature (35–80°C) has been reported for optimal growth and  $\alpha$ -amylase production in *Bacillus* spp. (15). Konsula and Liakopoulou- Kyriakides reported that a thermophilic *B. subtilis* strain, isolated from fresh sheep's milk, produced maximal extracellular thermostable  $\alpha$ -amylase at 40°C in a medium containing low starch concentration (16). Furthermore, *B. licheniformis*-AZ2 shows similar optimal temperature (40°C) for  $\alpha$ -amylase production and growth of *B. licheniformis* SPT27 (17), *Bacillus* sp. Marini (18), *B. megaterium* (5), *B. licheniformis*

BS1, *B. licheniformis* FS1, *B. licheniformis* GS1 (19) and *B. subtilis* GCBM-25 (4).

### Effect of pH

Enzyme synthesis and bacterial growth of *B. licheniformis*-AZ2 were studied at pH values from 4.0 to 11.0 in basal medium with 1% (v/v) inoculum at 40°C after 120 h. Optimal pH for  $\alpha$ -amylase production was found to be slightly alkaline at the pH 9.0. As the pH was increased, a sudden decrease in bacterial growth and enzyme production was detected (Figure 3). No satisfactory growth rate was observed for the bacteria in the basal medium adjusted to pH below 5.0 and above 10.0. Among the physical parameters of the growth medium, pH plays an important role on enzyme secretion from the strain by inducing morphological changes such as cell size, cell shape, cell water content and cell complexity based on surface to volume ratio (20, 21). Enzymes are also affected by pH. Changes in pH can lead to breaking of the ionic bonds that hold the tertiary structure of the enzyme in place and causes the enzyme to lose its functional shape, particularly the shape of the active site. pH changes also shifts charges of the active-site amino acids leading to enzyme-substrate complex disruption (22). Most of the *Bacillus* strains used commercially for the production of  $\alpha$ -amylase, optimally grow and secrete  $\alpha$ -amylase at pH ranging from 6.0 to 9.0 (23). The pH range 5.0 to 9.0 was found to be optimal for  $\alpha$ -amylase production of our strain as previously reported for *B. subtilis* CM3 (24), *B. licheniformis* CUMC305 (25), *B. thermoolovorans* NP54 (26), *B. subtilis* AX20 (27) and *B. licheniformis* (28).



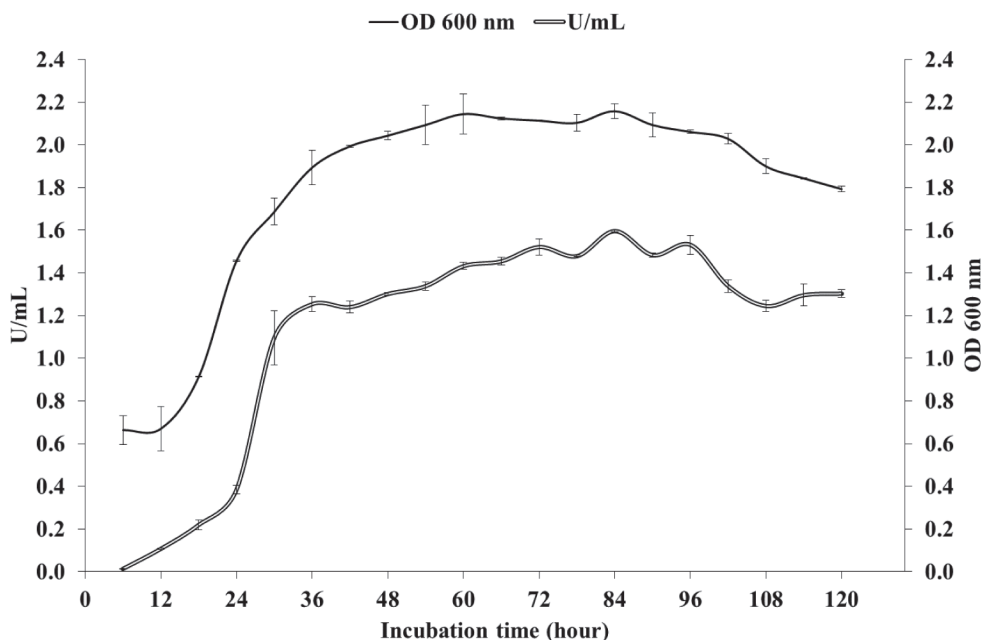
**Figure 4.** Effect of different inocula on  $\alpha$ -amylase production and bacterial growth in basal medium at pH 9.0 and temperature 40°C after 120 h. Each letter denotes the mean of three replications and bar represents standard error. Means with different letters indicate significant differences between treatments ( $P < 0.05$ , Duncan’s multiple range test).

\* Basal Medium (BM):  $\alpha$ -amylase production and bacterial growth in basal medium at pH 7.0, temperature 40°C and 1% (v/v) inoculum after 120 h.

### Effect of inoculum size

The inoculum size plays an important role in the fermentation of enzymes (29). The different inocula, 1 to 7 % (v/v) of basal medium at optimal pH and temperature were tested for the production of  $\alpha$ -amylase and bacterial growth (Figure 4). The production of  $\alpha$ -amylase was found to be optimal at 2% (v/v) of the basal medium. As

the inoculum size was further increased, we did not observe any significant increase in the production of  $\alpha$ -amylase. Decline in enzyme production at 7% (v/v) of inoculum is likely due to the fact that at higher inocula, the bacteria grow rapidly, exhausting essential nutrients for growth at the initial stages that results in accumulation of other byproducts in the fermentation medium (4).



**Figure 5.** Effect of incubation time on  $\alpha$ -amylase production and bacterial growth in basal medium at optimal pH 9.0, temperature 40°C and 2% (v/v) inoculum.

Mean  $\pm$  S.E.M = Mean values  $\pm$  Standard error of means of three replications.

Our results are in good agreement with Dash *et al.* (30), for production of extracellular  $\alpha$ -amylase from *B. subtilis*-BI19.

### Effect of incubation time

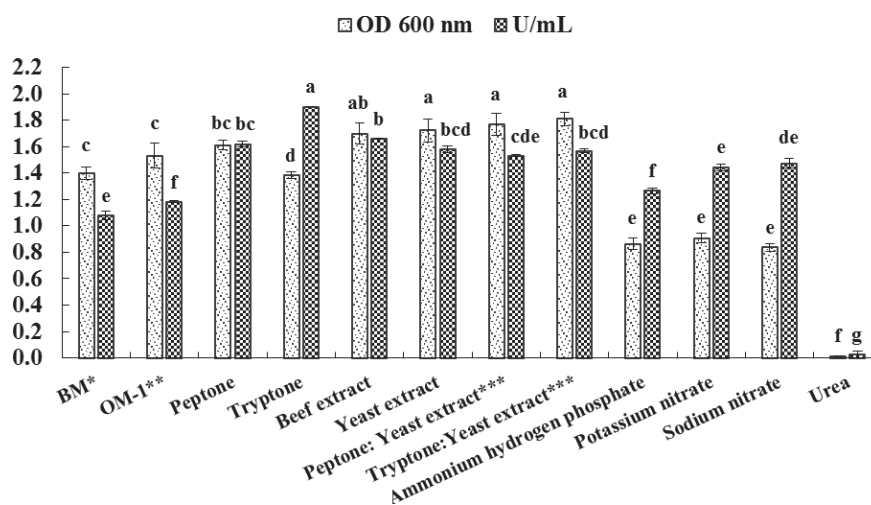
The production of  $\alpha$ -amylase and bacterial growth in basal medium with optimal cultural conditions i.e. pH 9.0, 40°C and 2% (v/v) inoculum at different time courses are shown in Figure 5. Maximal  $\alpha$ -amylase production and bacterial growth were obtained after 84 h of incubation. After that bacterial growth and enzyme production gradually decreased. Optimal incubation time may vary in other *Bacillus* species. In the case of *B. megaterium* Aq-2007 (5), *Bacillus* sp. Strain KCPSS-12ss (2), *B. licheniformis* SPT27 (17) and *B. licheniformis* Shahed-07 (23) maximal  $\alpha$ -amylase productions have been reported at 12, 24, 24 and 26 h of incubation, respectively. While  $\alpha$ -amylase production of *B. subtilis* JS-2004 was reported to be the highest at 48 h declining gradually up to 96 h (31). In some *Bacillus* strains the maximal  $\alpha$ -amylase production is reached in the exponential phase, whereas some other species achieve at the middle stationary phase (32). *B. licheniformis*-AZ2 produces the highest levels of  $\alpha$ -amylase when cell mass in shaking flasks enters the stationary phase of growth (Figure 5). It means that enzyme secretion in stationary phase is not growth associated and effective induction of  $\alpha$ -amylase may not occur until the stationary phase of growth has been reached and the readily available carbon sources were depleted from the fermentation medium (33).

### Effect of nitrogen source

$\alpha$ -amylase synthesis has been widely studied in the presence or absence of different nitrogen sources in several microorganisms (34). It has been correlated to the various amino acids and organic nitrogen sources like yeast extract, peptone and tryptone in the fermentation medium (23).  $\alpha$ -amylase production was investigated by replacing peptone and yeast extract in the basal medium with different organic and inorganic nitrogen sources at 1% (w/v) concentration under optimal cultural conditions i.e. pH 9.0, 40°C and 2% (v/v) inoculum after 84 h of incubation (Figure 6). Among all organic nitrogen sources, tryptone showed the maximal enzyme production. Our result was similar to Iraj *et al.* (23). Maximal bacterial growth was also observed in the presence of organic nitrogen sources, particularly yeast extract. It has been found previously (35) that yeast extract can significantly reduce the lag phase promoting fast bacterial growth. Among inorganic nitrogen sources,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  ions stimulated  $\alpha$ -amylase production, but urea showed inhibitory effect on both enzyme production and bacterial growth (Figure 6). Either positive (5, 17) or negative (2, 3, 23) effect of inorganic nitrogen sources on  $\alpha$ -amylase production have been reported previously.

### Effect of carbon source

The addition of carbon sources, either in the form of monosaccharides or polysaccharides could affect the en-



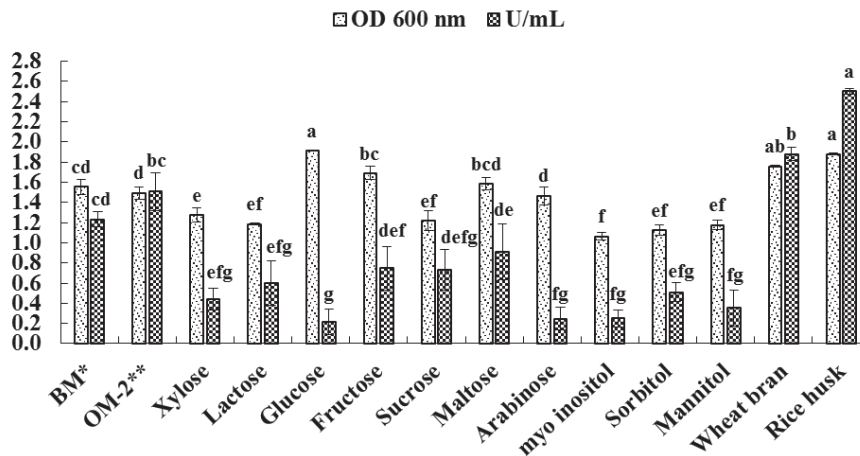
**Figure 6.** Effect of different nitrogen sources at 1% (w/v) on  $\alpha$ -amylase production and bacterial growth by replacement of peptone and yeast extract in the 1<sup>st</sup> optimized medium.

Each letter denotes the mean of three replications and bar represents standard error. Means with different letters indicate significant differences between treatments ( $P < 0.05$ , Duncan's multiple range test).

\* Basal Medium (BM):  $\alpha$ -amylase production and bacterial growth in basal medium at pH 7.0, temperature 40°C and 1% (v/v) inoculum after 120 h.

\*\* The 1<sup>st</sup> Optimized Medium (OM-1):  $\alpha$ -amylase production and bacterial growth in basal medium at optimal pH 9.0, temperature 40°C and 2% (v/v) inoculum after 84 h.

\*\*\* Used at a ratio of (1:1) at a final concentration of 1% (w/v).



**Figure 7.** Effect of various carbon sources at 1% (w/v) on  $\alpha$ -amylase production and bacterial growth by replacing starch in the 2<sup>nd</sup> optimized medium.

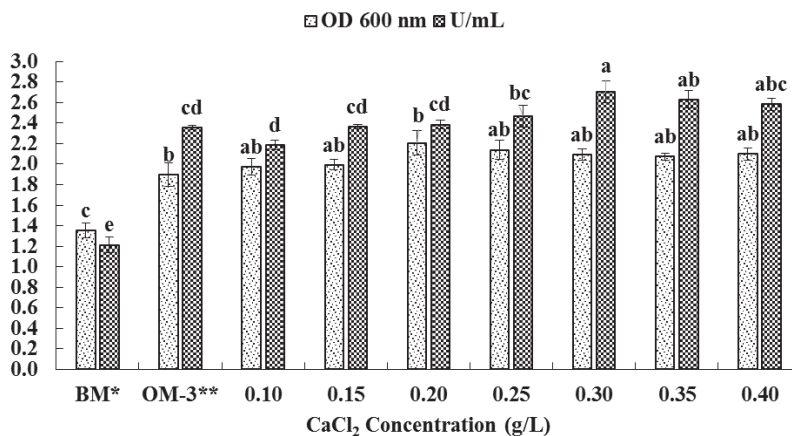
Each letter denotes the mean of three replications and bar represents standard error. Means with different letters indicate significant differences between treatments ( $P < 0.05$ , Duncan's multiple range test).

\* Basal Medium (BM):  $\alpha$ -amylase production and bacterial growth in basal medium at pH 7.0, temperature 40°C and 1% (v/v) inoculum after 120 h.

\*\* The 2<sup>nd</sup> Optimized Medium (OM-2):  $\alpha$ -amylase production and bacterial growth in the presence of 1% (w/v) tryptone, 1% (w/v) starch, NaCl 3.0 (g/L) and MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 (g/L) at optimal pH 9.0, temperature 40°C and 2% (v/v) inoculum after 84 h.

zyme production (36). Agro-wastes also used as substrates for production of several enzymes either by solid state or submerged mode of fermentation to reduce the cost of fermentation media (37). The use of agro-wastes could be more effective, economic and help to solve environmental problems (38). However, effect of carbon source differ widely, depending on bacterial strains (39). Therefore,  $\alpha$ -amylase production was investigated by replacing starch

in the basal medium with different sugars and agro-wastes at 1% (w/v) concentration under optimal cultural conditions and nitrogen source [i.e. pH 9.0, 40°C, 2% (v/v) inoculum and 1% (w/v) tryptone] after 84 h of incubation. Starch is a commonly accepted nutritional component for induction of amyolytic enzymes. This material was considered as a reference (2). Figure 7 shows that highest  $\alpha$ -amylase production was obtained in basal me-



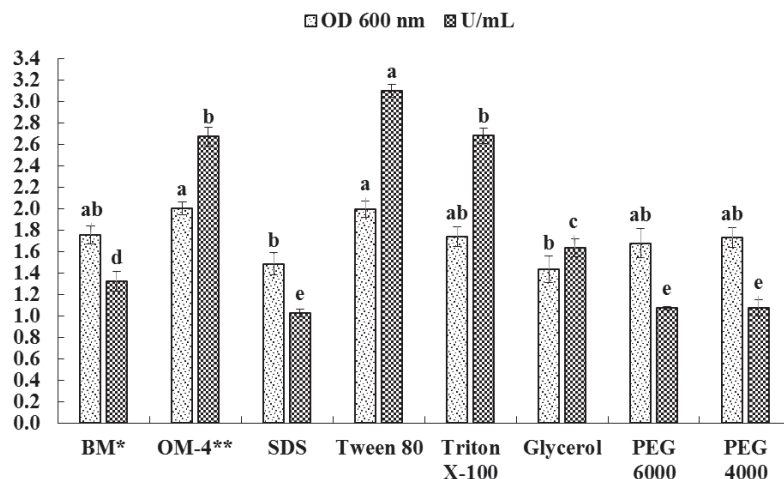
**Figure 8.** Effect of CaCl<sub>2</sub> concentrations on  $\alpha$ -amylase production and bacterial growth by replacement of NaCl and MgSO<sub>4</sub>·7H<sub>2</sub>O in the 3<sup>rd</sup> optimized medium.

Each letter denotes the mean of three replications and bar represents standard error. Means with different letters indicate significant differences between treatments ( $P < 0.05$ , Duncan's multiple range test).

\* Basal Medium (BM):  $\alpha$ -amylase production and bacterial growth in basal medium at pH 7.0, temperature 40°C and 1% (v/v) inoculum after 120 h.

\*\* The 3<sup>rd</sup> Optimized Medium (OM-3):  $\alpha$ -amylase production and bacterial growth in the presence of 1% (w/v) tryptone, 1% (w/v) rice husk, NaCl 3.0 (g/L) and MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 (g/L) at optimal pH 9.0, temperature 40°C and 2% (v/v) inoculum after 84 h.





**Figure 9.** Effect of 2% (w/v) surfactants on  $\alpha$ -amylase production and bacterial growth in the 4<sup>th</sup> optimized medium.

Each letter denotes the mean of three replications and bar represents standard error. Means with different letters indicate significant differences between treatments ( $P < 0.05$ , Duncan's multiple range test).

\* Basal Medium (BM):  $\alpha$ -amylase production and bacterial growth in basal medium at pH 7.0, temperature 40°C and 1% (v/v) inoculum after 120 h.

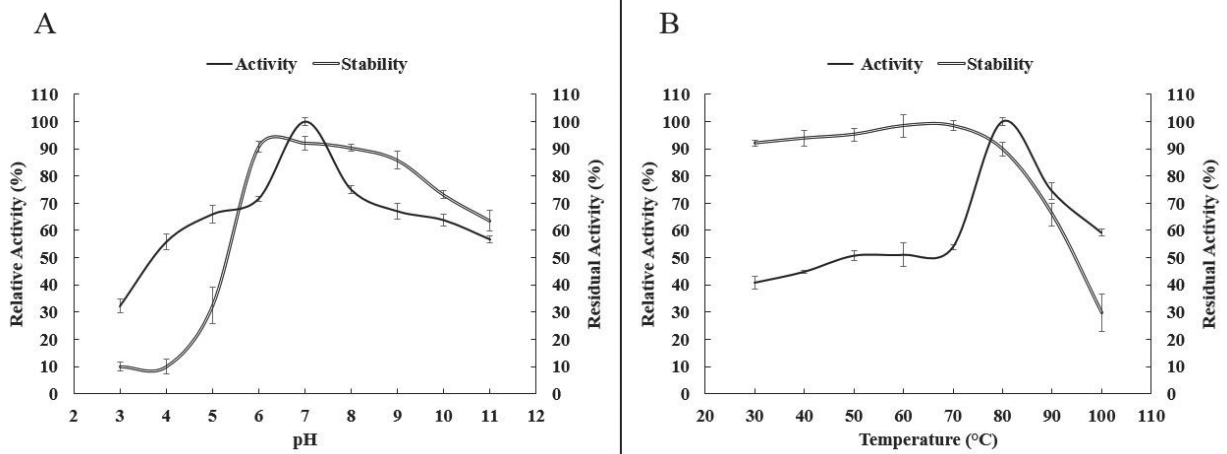
\*\* The 4<sup>th</sup> Optimized Medium (OM-4):  $\alpha$ -amylase production and bacterial growth in the presence of 1% (w/v) tryptone, 1% (w/v) rice husk and 0.3 (g/L)  $\text{CaCl}_2$  at optimal pH 9.0, temperature 40°C and 2% (v/v) inoculum after 84 h.

dium supplemented with rice husk followed by wheat bran. Other carbon sources reduced  $\alpha$ -amylase production compared to starch. Starch was used as a substrate for the production of  $\alpha$ -amylase from *Bacillus* sp., *Bacillus* spp. NCIB 11203 and IMD 370, *B. subtilis* (18). Although in some *Bacillus* spp. simple sugars such as lactose (2, 40), fructose (5, 17), glucose (41), dextrose (5) and maltose (18, 36) induced the highest  $\alpha$ -amylase production. However, it has also been demonstrated that in most species of genus *Bacillus*, the synthesis of carbohydrate degrading enzymes is subjected to catabolic repression by readily metabolisable substrates such as glucose and fructose (29). Results of our study showed that despite the higher cell density and bacterial growth rate in the presence of glucose in basal medium, highest enzyme production was obtained in the presence of rice husk (Figure 7). Our results are in agreement with reports of Lin *et al.* (29) and Thippeswamy *et al.* (3) who demonstrated that  $\alpha$ -amylase synthesis is under catabolic repression by readily metabolisable substrates. Among different agro-wastes, wheat bran and rice husk have been used to maximal extent due to the presence of considerable amount of carbohydrates and nitrogen sources which stimulate the cells to express many hydrolytic enzymes (37, 38). Rice husk and wheat bran were also used as a low cost carbon substrates for  $\alpha$ -amylase production by *B. subtilis* (42).

### Effect of inorganic salts and surfactants

Effect of inorganic salts at different concentrations was separately examined on  $\alpha$ -amylase production and bacterial growth by replacing NaCl and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in

basal medium at optimal conditions obtained before optimization. Among different inorganic salts i.e. NaCl,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{K}_2\text{HPO}_4$  and  $\text{CaCl}_2$ , only calcium chloride at 0.3 g/L significantly increased the  $\alpha$ -amylase production (Figure 8), while other inorganic salts had no positive effect on  $\alpha$ -amylase production, moreover at higher concentrations (data not shown) a significant inhibition of bacterial growth and  $\alpha$ -amylase production was observed. The inhibitory effects of some inorganic salts may be related to the pH changes associated with their use in fermentation medium (43). Most of the  $\alpha$ -amylases are considered as metalloenzymes which require calcium ions ( $\text{Ca}^{2+}$ ) for their activity, structural integrity and thermal stability (44). It has been predicted from the crystal structure of  $\alpha$ -amylase that calcium ion was involved in ionic interaction with charged residues like Asn 100, His 201 of domain A, and Asp 159 and Asp 167 of domain B of  $\alpha$ -amylase. Active site of  $\alpha$ -amylase was located between domains A and B and calcium ion formed an ionic bridge between A and B domains of  $\alpha$ -amylase promoting its stability and catalytic activity (45). In the case of *B. licheniformis* previous studies suggested that  $\alpha$ -amylase production was enhanced due to the increasing availability of the calcium ion in the fermentation medium (8). Among all surfactants, addition of a non-ionic detergent, Tween 80 causes the maximal  $\alpha$ -amylase production (Figure 9). It has been demonstrated that addition of surfactants into fermentation medium can stimulate the release of extracellular enzymes by increasing cell membrane permeability (4). Surfactants contain fatty acids and silicates, and therefore can affect the enzyme secretion by decreasing surface tension and increasing the



**Figure 10.** Effect of pH (A) and temperature (B) on activity and stability of partially purified  $\alpha$ -amylase.

Relative activity was defined as the percentage of maximal activity detected in the standard assay conditions, while residual activity is expressed as a percentage of the original activity.

Mean  $\pm$  S.E.M = Mean values  $\pm$  Standard error of means of three replications.

air supply of liquid medium (4).  $\alpha$ -amylase production seems to be greatly inhibited by the addition of sodium dodecyl sulfate (figure 9).

### Partial purification by ammonium sulfate precipitation

Among different ammonium sulfate fractions, maximal  $\alpha$ -amylase activity was detected in the 60-90 % fraction with a 6.6 fold purification and 54% yield after dialysis (data not shown). This partially purified fraction was used for the characterization studies on  $\alpha$ -amylase. Since the activity and thermal stability of  $\alpha$ -amylase at high temperatures were significantly enhanced in the presence of 5 mM  $\text{CaCl}_2$ , all thermal and pH stability assays were performed in the presence of  $\text{Ca}^{2+}$  ions (data not shown).

### Effect of pH on amylase activity and stability

Influence of pH on activity and stability of partially purified  $\alpha$ -amylase from *B. licheniformis*-AZ2 is presented in Figure 10. The optimal pH for  $\alpha$ -amylase activity was found to be at 7.0. The activity increased from pH 3.0 and peaked at pH 7.0, beyond which it gradually decreased until pH 11.0. The result of this study is in accordance with Iraj *et al.* who has reported that *B. licheniformis* Shahed-07 had an optimal activity at pH 7.0 (23). The enzyme was also found relatively stable over the pH range 6.0 and 10.0 after 24 h of incubation at 30°C and retained more than 75% of its original activity, while the maximal residual activity (about 92%) was observed at pH 6.0-7.0. Stability of the enzyme at alkaline pH range indicates its alkali-tolerance. Comparable values for optimal pH and stability at high pH of *Bacillus* spp. have been reported by various researchers (31, 46, 47).

### Effect of temperature on amylase activity and stability

Thermostability is an important factor for the use of amylolytic enzymes in starch processing industries (23). The  $\alpha$ -amylase activity was assayed at different temperatures ranging from 30°C to 100°C at optimal pH 7.0 under standard assay conditions (Figure 10). The optimal temperature for  $\alpha$ -amylase activity was found to be around 80°C, the habitat temperature of the organism. The enzyme showed stability over a temperature range of 30°C to 80°C (more than 80% of the residual activity after 1 h of incubation). The stability of the enzyme could be due to the genetic adaptability to carry out its biological activities at higher temperatures (48). Original activity of the enzyme was lost at 90°C and 100°C, 34% and 70%, respectively. This might be due to loss of integrity of the enzyme structure as well as of its original activity (49). Some other  $\alpha$ -amylases from thermophilic strains of *B. licheniformis* are reported to exhibit somewhat higher optimal temperature (~90°C), but similar temperature stability to that obtained in our study (25, 50). Thermophilic  $\alpha$ -amylase from *B. licheniformis*-AZ2 seems to possess higher temperature optimum than the enzyme from moderately thermophilic *B. subtilis* (31) and the thermostable enzyme from *Bacillus* sp. TULH, isolated from a hot water spring (51).

### CONCLUSIONS

Findings of this study show a 2.4-fold increase in  $\alpha$ -amylase production by *B. licheniformis*-AZ2 strain in optimized medium containing 1% (w/v) of tryptone, 1% (w/v) of rice husk, 0.3 g/L of  $\text{CaCl}_2$  and 2% (w/v) of Tween 80 with 2% (v/v) inoculum after 84 h at 40°C and pH 9.0 compared to the basal medium. Stability of the

partially purified enzyme over a broad range of pH (6.0 to 10.0) and temperature (30°C to 80°C) in the presence of CaCl<sub>2</sub> implies that the produced calcium dependent thermostable  $\alpha$ -amylase has suitable characteristics for application in starch processing and other industries such as detergents. To be commercialized, further investigations are required for enhancement of the enzyme production.

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