

**Full Article**

## **Two-step purification and partial characterization of an extra cellular $\alpha$ -amylase from *Bacillus licheniformis***

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### **ABSTRACT**

The aim of this study was production and partial purification of  $\alpha$ -amylase enzyme by *Bacillus licheniformis*. *B. Licheniformis* was allowed to grow in broth culture for purpose of inducing  $\alpha$ -amylase enzyme. Optimal conditions for amylase production by *B. Licheniformis* are incubation period of 120 h, temperature of 37 °C and pH 7.0. The  $\alpha$ -amylase enzyme was purified by ion exchange chromatography on DEAE-sepharose CL-6B and sephadex G-100 gel filtration with a 19.1-fold increase in specific activity as compared to the concentrated supernatant and with a specific activity of 926.47 U/mg. The  $\alpha$ -amylase had the highest activity at pH 7.0 and 65 °C. According to the data on native polyacrylamide gel electrophoresis, the molecular weight of the purified enzyme was 72 kDa.

**Keywords:**  $\alpha$ -Amylase, *Bacillus licheniformis*, optimal conditions, purification

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### **INTRODUCTION**

One of the most important enzymatic reactions that are carried out at an industrial scale is the enzymatic hydrolysis of starch (Baks *et al* 2006, Kılıc & O'zbek 2005, Yang & Liu 2004). Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca and potato (Haifeng *et al* 2006). Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Asgher *et al* 2007, Kılıc & O'zbek

2005, Najafi & Kembhavi 2005). Amylases constitute a class of industrial enzymes representing approximately 30% of the world enzyme production (Hmidet *et al* 2008, Nguyen *et al* 2002). These enzymes have a great significance with extensive biotechnological applications in bread and baking, food, paper industries, anti-staling agent, production of cyclodextrins, sizing of textile fibers and clarification of haziness in beer and fruit juices (Haifeng *et al* 2006, Asgher *et al* 2007). Amylase which catalyzes the hydrolysis of starch to low molecular weight products, is known to attack both insoluble starch and starch granules held in aqueous suspension (Kılıc & O'zbek 2005). The enzyme is produced by a wide variety of

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microorganisms, but for commercial applications  $\alpha$ -amylase is mainly derived from the genus *Bacillus* (Konsoula & Liakopoulou-Kyriakides 2007, Hmidet *et al* 2008). In this article the production of  $\alpha$ -amylase by a strain *B. licheniformis* isolated in Iran is reported.

## MATERIALS AND METHODS

**Materials.** The *B. licheniformis* strain was obtained from the Boali University of Iran. All the chemicals used were of analytical grade and were purchased from Sigma Chemical Company and Merck.

**Preparation of inoculum.** Two loop of isolated culture was transferred to 5 ml of sterile medium containing: 0.5 g/l MgSO<sub>4</sub>, 3.0 g/l yeast extract, 5 g/l pepton, 0.1 g/l CaCl<sub>2</sub>, 10.0 g/l soluble starch, 0.5 g/l KH<sub>2</sub>PO<sub>4</sub> and 1.5 g/l NaCl in test tubes and incubated for 24 h at 37°C with shaking at 150 rpm (Yazdanparast 1993, Kılıc & O'zbek 2005).

**Biomass determination.** The absorbance of culture broth supernatant at 420 nm was measured for estimation of cell growth and biomass production. Enzyme production medium the enzyme production was carried out in the same medium with inoculum preparation medium. The initial pH of the medium was adjusted to 7.0 unless otherwise mentioned. The medium was sterilized by autoclaving at 121°C for 15 min. Erlenmeyer flasks (500 ml) containing 100 ml of culture medium were inoculated with 5% of prepared inoculum culture and incubated at 37°C in a rotary shaker at 4000 rpm. At regular interval (24 h), the triplicate samples were harvested and the cells were separated by centrifugation (7000 rpm for 30 min) at 4 °C in a refrigerated. The supernatant was used for enzyme assay and characterization studies (Asgher *et al* 2007, Agrawala *et al* 2005, Ramakrishna 1992). Optimization of medium and culture conditions Initially, the organism was grown in the liquid medium at pH 7.0 and 37 °C and then the effect of varying pH values (5 - 10) and temperatures (30 – 70 °C) on  $\alpha$ -amylase production by the bacterium was also investigated.

**Determination of amylase activity.** The reaction mixture containing 450  $\mu$ l of 1.0% (w/v) soluble starch in 0.02 M sodium phosphate buffer (pH 7) and 50  $\mu$ l of the supernatant was incubated at 65°C for 5 min. After appropriate dilution, the reducing sugar in the dilute was determined by using the di nitrosalicylic acid (DNS) method (Yazdanparast, 1993; Yang and Liu, 2004). 1 unit of amylase activity was defined as the amount of enzyme causing release of reducing sugars equivalent to 1  $\mu$ M of glucose from starch per 5 min under the assay conditions. The specific amylase activity was defined as units per mg of protein in the supernatant (Haifeng *et al* 2006). For purification of  $\alpha$ -amylase the supernatant from the bacterial culture was applied on DEAE-sepharose CL-6B (2 cm x 35 cm) column, equilibrated with 20 mM tris-HCl buffer, at pH 8.0. In this step proteins were eluted with a linear gradient of NaCl from 0.0 to 1.0 M. The flow rate was 30 ml/h and 3 ml fractions were collected (Viara *et al* 1993). The fraction showed amylase activity from the ion-exchange chromatography step was applied on a molecular exclusion chromatographic column of sephadex G-100 (2.6 cm x 75 cm), previously equilibrated with the 25 mM tris-HCl buffer and then eluted with the same buffer. Fractions of 3 ml/tube were collected at a flow rate of 15 ml/h.

**Effect of temperature on enzyme activity and stability.** The optimum temperature of the enzyme was evaluated by measuring the  $\alpha$ -amylase activity at different temperatures (30 - 90°C) in 0.01 M sodium phosphate buffer (pH 7). The residual enzyme activity was determined as described earlier (Asgher *et al.*, 2007). Purified amylases in 0.02 M sodium phosphate buffer, pH 7, were incubated at a temperature range of between 40 and 105°C. Aliquots were taken at 30 min interval and assayed for residual activity, taken that of the unincubated control as 100%. The percentage residual activity remaining was then plotted against time for different temperatures.

**Effect of pH on enzyme activity.** The optimum pH of the enzyme was determined by exposure of the enzyme to various pH ranging from 3-10 of the assay

reaction mixture, using the following buffers (0.1 M): citrate sodium phosphate buffer (pH 3.0 - 4.0), sodium acetate buffer (pH 5.0 - 6.0), sodium phosphate (pH 6.0 - 7.5), borate buffer (pH 7.0-9.0), borax/NaOH buffer (pH 9.0-10.5) and phosphate/ NaOH buffer (pH 11- 12) (Asgher et al 2007).

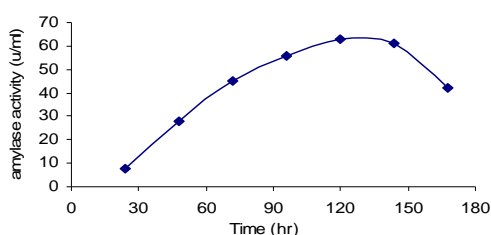
**Protein concentration determination.** The method of Lowry was used to determine the total protein in the samples (Lowry 1950).

**Molecular weight determination.** Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDSPAGE) was performed with 10% acrylamide gel by the method of Laemmili (Adewale et al 2006, Laemmili 1970).

## RESULTS

### Optimization of medium and culture conditions

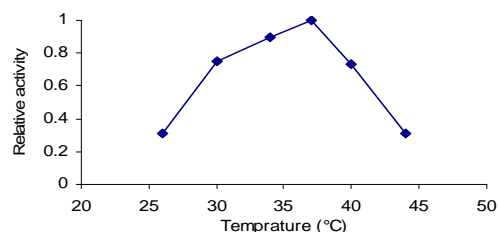
Initially *B. licheniformis* grown on starch containing nutrient agar plates secreted extra cellular amylase which readily digested the starch. Figure 1 shows the ability of *B. licheniformis* to utilizing starch as a carbon source and energy material to produce amylase. Interestingly, the results indicated that *B. licheniformis* exhibited their maximum ability to biosynthesize amylase within 120 h of incubation period.



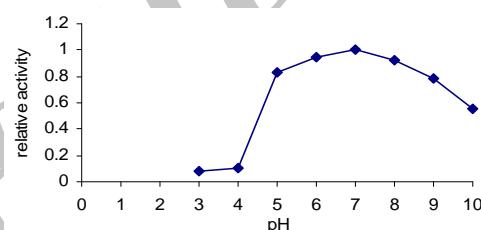
**Figure 1.** Effect of time on production of  $\alpha$ -amylase by *B. Licheniformis*.

Increasing the incubation time to 180 hours caused decreased activity of enzyme. The effects of different incubation temperatures and pH on amylase production were evaluated. As shown in the figure 2, the temperature 37 °C was optimum temperature for amylase production. However, the temperature below or above 37 °C caused decrease in amylase yield as compared to the optimal temperature. When the effect of PH was determined for  $\alpha$ -amylase production by *B.*

*Licheniformis*, the best temperature was found to be pH7 produced maximum levels of amylase at pH 7 and at pH less than 4 almost no enzyme activity was found in the *B. Licheniformis* culture medium (Figure 3).



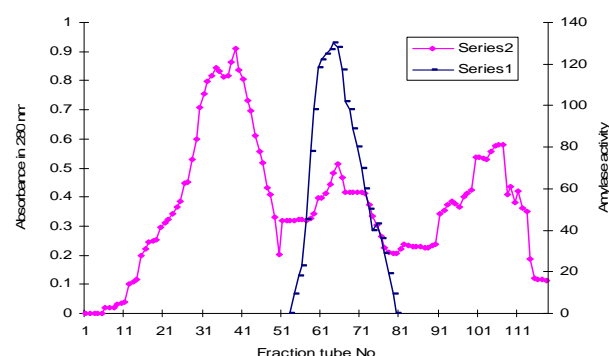
**Figure 2.** Effect of temperature on production of  $\alpha$ -amylase by *B. Licheniformis*.



**Figure 3.** Effect of pH on production of  $\alpha$ -amylase by *B. Licheniformis*.

The effect of time on enzyme yield was determined by determining enzyme activity at various time intervals from 0 to 180 hours of incubation at 37 °C and pH 7. The maximum activity of enzyme found to be at 120 hours incubation.

**Purification of amylase.** In the initial ion exchange chromatography fractionation of the concentrated supernatant three peaks having absorbance at 280 nm were obtained (BL1– BL3) as shown in (Figure 4).



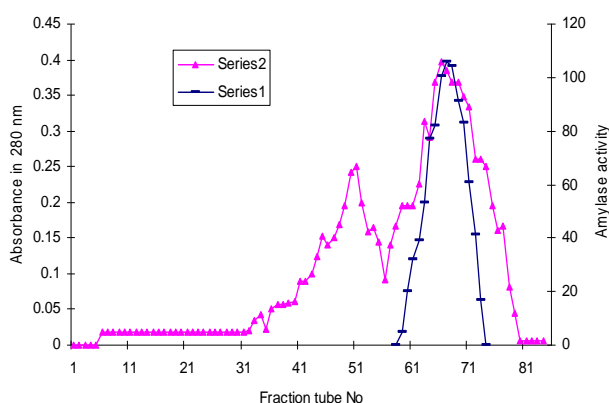
**Figure 4.** DEAE-Sepharose chromatography of *B. Licheniformis* culture supernatant. The pooled culture supernatant was applied to DEAE-Sepharose column (2x35cm) equilibrated with 20 mM Tris±HCl buffer (pH 8.0). Proteins were eluted with a linear concentration gradient of NaCl from 0 to 1 M.

When all the fractions were tested for amylase activity, it was found that fraction BL2 showing positive amylase activity. Determination of amylase activity revealed the fraction BL2 with 260U/min/mg activity. The yield of amylase activity fraction (BL2) was calculated and found to be 55.7 % (Table 1). Further purification was carried out by gel chromatography on Sephadex G-100 (Figure 5).

**Table 1.** Purification of a amylase enzyme (BL21) from *B. Licheniformis*.

Step	Protein (mg)	Total activity (U/min)	Specific activity (U/min/mg)	Purification n fold	Yield (%)
culture	195	9450	48.46	1	100
DEAE-sepharose	20.25	5265	260	5.36	55.7
Sephadex G-100	1.7	1575	926.46	19.1	16.7

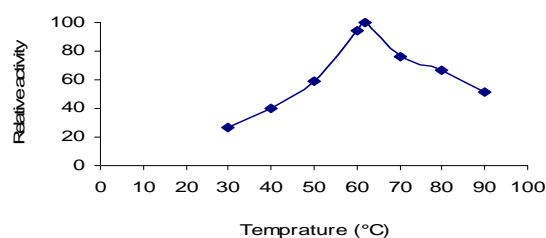
In this step of purification two fractions were obtained (BL21&BL22). Elution profile of this step shows that fraction 2 (BL22) has amylase activity. Therefore, the fractions were collected and concentrated by the ultra filtration. The results in Table 1 show that the enzyme was purified to homogeneity with a 19.1 fold increase in specific activity with a yield of about 16.7% as compared to the concentrated supernatant.



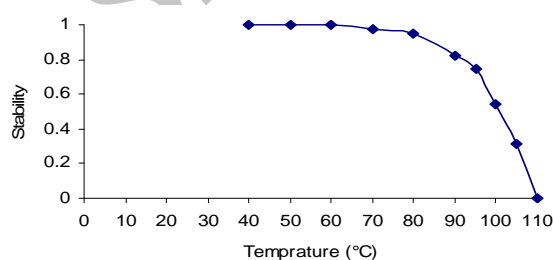
**Figure 5.** Gel chromatography of amylase obtained from DEAE-Sephacrose. The pooled fraction showed amylase activity. (BL2) fraction from Fig 4 were applied to Sephadex G-100 (2.6 x 75cm) equilibrated with 25 mM Tris-HCl buffer and then eluted with the same buffer. Fractions of 3mL/tube were collected at a flow rate of 15 ml/h.

**Optimum temperature and thermal stability for enzyme activity.** The amylase activity measured from

30 to 90 °C. BL22 displayed progressively higher relative hydrolysis rates than 60% from 50 °C up to 80°C. Optimum temperature for its activity was 62 °C. The enzyme retains almost 50% of its maximum activity at 50 °C (Figure 6).



**Figure 6.** Effect of temperature on enzyme activity.



**Figure 7.** Thermal stability of the amylase.

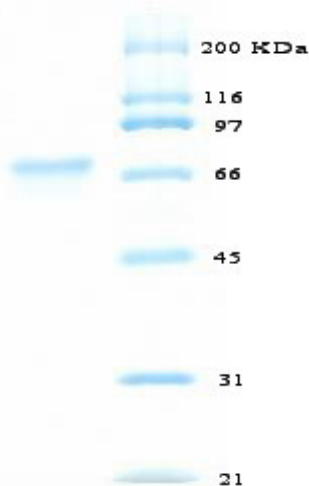
**Optimal pH for enzyme activity.** The amylase activity was measured at various pH in buffers with the same ionic concentrations. The results in Fig. 8 showed that maximum activity was observed at pH 7.0 to 7.5. The activity of BL22 was about 52% at pH 5.0 and about 12% at pH 3.0 and almost 92% of activity was observed at pH 8.0.

**Determination of molecular weight.** The molecular weight of the enzyme under denaturing condition was estimated to be 72 kDa (figure 9).

## DISCUSSION

The *B. licheniformis* strain was gram positive, elliptic and milk white in color. Different culture conditions were used to obtain the maximum levels of amylase productivity by *B. licheniformis*. Interestingly, the results indicated that *B. licheniformis* exhibited their maximum ability to biosynthesize amylase within 120

hours of incubation period. The optimal temperature for amylase production and growth of the *B. licheniformis* were found to be different.



**Figure 9.** SDS-PAGE showing the single band of  $\alpha$ -amylase along with molecular weight maker.

The maximum amylase production was observed after 18-24 h and 36-48 h, when grown at room temperature and 50 °C, respectively (Agrawala *et al* 2005, Soni *et al* 2003). The difference in time to reach maximum yield can be due to the specific strain or the type of culture media used for the growth of *B. licheniformis*. In the present study  $\alpha$ -amylase was purified to homogeneity with a 19.1 fold increase in specific activity with a yield of about 16.7% as compared to the concentrated supernatant. The most important advantage of the present study may be the simple method of purification with high purity and comparatively high activity. The methods used for purification of amylase are different. An  $\alpha$ -amylase enzyme from *B. licheniformis* by ammonium sulphate precipitation, sephadex G-100 gel filtration and sepharose mono Q anion exchange chromatography, with a 3.08 fold increase in specific activity and 15.9% recovery was reported previously (Hmidet *et al* 2008). Yang and Liu purified an amylase enzyme from *Thermobifida fusca* with 7.2 fold and 22% yield from crude culture filtrate by ammonium sulfate fractionation, sepharose CL-6B and DEAE-

sepharose CL-6B column chromatography (Yang and Liu, 2004). The amylase activity measured from 30 to 90 °C. Optimum temperature for its activity was 62 °C. These results are in accordance with the previously reported for amylase from *T. fusca*. The optimum temperature of this enzyme was 60 °C (Yang and Liu, 2004). When the stability of enzyme was carried out, the enzyme retains almost 50% of its maximum activity at 50 °C the residual amylase activity still kept 95% of the control after treatment at 80 °C for 30 min, indicating that the enzyme was stable up to temperature of 80 °C. Also, this figure reveal that the enzyme was inactivated rapidly at temperature higher than 90°C and was inactivated totally at temperature of 110°C within 30 min. From these results, the amylase seemed to have considerable thermostability. The amylase enzyme from marine *Vibrio* showed maximum activity at 55-60 °C and it retains 85% of maximal activity after 30 min pre incubation at 65°C (Najafi & Kembhavi, 2005). However amylase produced by *B. licheniformis* NH1 has a higher optimum temperature, 90°C, and was also less resistant to thermal inactivation (Hmidet *et al* 2008). The amylase activity was measured at various pH in buffers with the same ionic concentrations maximum activity of BL22 was found to be at 7. The activity of BL22 was about 52% of maximum activity at pH 5.0 and about 12% at pH 3.0 while almost 92% of activity was observed at pH 8.0. Optimum pH have been reported for  $\alpha$ -amylases from *B. licheniformis* NH1, *B. licheniformis* 44MB82-A and *B. licheniformis* NRRL B14368, to be 6.5, 6.0 - 6.5 and 5.0 - 7.0 respectively (Hmidet *et al* 2008). The molecular weight of  $\alpha$ -amylase purified in the present study with 72kDa is comparatively higher than other  $\alpha$ -amylase enzymes already studied, for example: 52 kDa (Najafi & Kembhavi 2005), 60 kDa (Yang & Liu 2004), 61 kDa (Nguyen *et al* 2002), 40 kDa (Noman *et al* 2006), and 49 kDa (Thumar *et al* 2007) In conclusion the *B. licheniformis* strain produced a thermo stable  $\alpha$ -amylase with optimum activity at pH 7.0 and temperature of 62 °C, suitable for application in starch processing and other food industries.

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