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Production, purification and characterization of thermostable α-amylase from soil isolate *Bacillus sp*. strain B-10

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ABSTRACT

A bacterial strain B-10 that produces α-amylase was isolated from compost and kitchen waste receiving agricultural soil. Based on microbiological and biochemical tests the isolate B-10 was identified as *Bacillus sp*. Alpha-amylase produced by this isolate was purified by $(NH_4)_2SO_4$ precipitation and DEAE cellulose ion-exchange chromatography showing 15.91 and 48.21 fold purification, respectively. SDS-PAGE of the purified enzyme confirmed the purification and monomeric nature of the enzyme. The purified α-amylase showed maximum activity at pH 7 and temperature 50°C. The enzyme was significantly active in the temperature range of 30-60°C for the studied period of 2 h. During the incubation of purified enzyme at pH ranging from 5 to 10 for 24 h the maximum stability was observed at pH 7 followed by pH 8, whereas at extreme pH, the stability was very poor. K_m and V_{max} were found to be 1.4 mg/mL and 6.2 U/mL, respectively.

Key words: *Bacillus*, α-amylase, SDS-PAGE, ion exchange chromatography, Michaelis constant, K_m

Introduction

Alpha (α)-amylases (E.C. 3.2.1.1) catalyzes hydrolysis of α-1, 4-glycosidic bond in starch and converts it into low molecular weight products, such as glucose, maltose and maltotriose units (Gupta et al., 2003; Rajagopalan & Krishnan, 2008). These are one of the most important classes of industrial enzymes and account around 25% of the world enzyme market (Reddy et al., 2003; Rajagopalan & Krishnan, 2008). Amylases can be obtained from many different sources such as plants, animals and microbes, but the industry relies generally on microbial amylases. Easy manipulation of microbes to get the enzyme of desired characteristic and less capital input for bulk commercial production enable microbes to be used for the production of amylase (Aiyer, 2005). Various microorganisms produce α-amylases, but *Bacillus sp*. are the most commonly used microbes by the industry for production of the enzyme. Amylases have numerous useful applications in food, brewing, textile, detergent and pharmaceutical industries. They are principally used for starch liquefaction to minimize viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetrose syrup. They are used to improve cleaning effect in detergents and starch de-sizing in textile industry (Haq et al., 1997).

Thermal stable amylases are the basic requirement of current starch processing industries (Bolton et al., 1997; Uguru et al., 1997). Thermophilic and moderate thermophilic organisms are of special interest as a source of novel thermostable enzymes. Minimized risk of contamination and

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decreased cost of external cooling are the main advantages of using thermostable α-amylases in industrial processes (Lin et al., 1998). With the diverse use of α-amylases, it has become essential to isolate new potent amylase producing microbial strains.

The present study deals with production of α-amylase from an isolated microorganism, purification and characterization of the enzyme and its suitability for industrial application.

Materials and Methods

Isolation of bacterial cultures

Soil samples were collected from different agricultural farms, receiving kitchen waste and compost from Bijnor (U.P.), India and mixed properly. The mixed soil sample was suspended in normal saline (0.85% w/v) with vigorous stirring for 10 min and then allowed to settle at room temperature for one hour. The supernatant was collected and serially diluted up to 10^{-7}. 100 µL inoculum of 10^{-7} diluted supernatant was spread on nutrient agar plate and incubated for 48 h at 37°C. The appeared bacterial colonies were aseptically removed and reselected on nutrient agar plate to obtain their pure culture.

Screening of microorganisms

Isolated bacterial colonies were screened for amylase production by spot plate technique. Soil isolates were spotted aseptically by tooth pick on starch agar plate (1% starch, 0.5% peptone, 0.1 % NaCl, 0.1% $MgSO_4$, 2% agar, pH 7) and incubated for 48 h at 37°C. After incubation zone of clearance were checked around the spotted colonies by flooding iodine solution (1%) on the plate. The cultured microorganism that produced maximum zone of clearance was selected as best amylase producer (isolate B-10).

Identification of bacterial strain B-10

Best amylase producer strain (B-10) was identified by performing various microbiological and biochemical tests (Claus & Berkeley, 1986).

Time dependent growth and amylase production by strain B-10

A single colony of B-10 was inoculated in 10 mL nutrient broth and incubated at 37°C with constant stirring for 24 h. After incubation, cell density was adjusted to 10^7 cells/mL (A_{600} = 0.9). 1.0 mL culture (10^7 cells) were inoculated in 250 mL starch fermenting medium (2% starch, 0.5% yeast extract, 0.02% $CaCl_2$, 0.1% NaCl, 0.1% $MgSO_4$, 2% agar, pH 7) and grown for different time points (1, 2, 4, 6, 12, 24, 36, 48, 60 and 72 h) at 37°C under constant shaking (130 rpm) condition. At each time point, 5 mL culture was taken out from fermentation broth. Growth was monitored by measuring the absorbance at 600 nm, followed by centrifugation of the withdrawn culture at 8000 rpm for 5 min. Supernatant was used for checking production of α-amylase by standard amylase assay.

Production and purification of alpha amylase

Production of α-amylase was started by inoculating 10^5 cells of strain B-10 in starch fermenting medium and incubated at 37°C for 48 h under shaking condition. After incubation whole broth was centrifuged at 8000 rpm for 5 min to get cell free filtrate (CFF) and used as a source of crude enzyme. Purification process was started by adding ammonium sulfate (65% saturation level) in the CFF at 4°C for 2 h followed by centrifugation at 10000 rpm for 15 min. Pellet was dissolved in minimum volume of 0.1 M phosphate buffer and dialyzed against phosphate buffer at 4°C till the complete removal of ammonium sulfate. Partially purified enzyme was further purified by DEAE cellulose column (1×15 cm) pre equilibrated with 0.1 M phosphate buffer (pH 7.2). The column was gradient eluted with 0.1 to 0.5 M sodium chloride (NaCl) in the 0.1 M phosphate buffer (pH 7.2) at a flow rate of 20 mL/h. Fraction of 2 mL size was collected. The amylase activity and protein concentration were checked in each fraction. Enzymatically active fractions were pooled and concentrated to 3 mL using finely powdered sucrose at 4°C.

Polyacrylamide gel electrophoresis (PAGE)

Purification was monitored on silver stained SDS-PAGE (8%) by the method described by Laemmli (1970). Alpha amylase activity was also monitored on 8% native polyacrylamide gel (clear zone against blue background). For activity staining, resolved native gel was immersed in 1% soluble starch for 1 h at room temperature and transferred to 0.1 M phosphate buffer, incubated for 10 min and stained with 1% iodine solution.

Enzyme assay

Amylase assay was done as described by Bernfeld (1956) with some modifications. 0.5 mL of 1% starch in 0.1 M phosphate buffer (pH-7.2) was added to 0.5 mL of enzyme and incubated for 30 min at 37°C. The reaction was stopped by adding 1 mL of dinitrosalicysilic acid (DNS) reagent and

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kept on boiling water bath for 5 min. Absorbance was measured at 540 nm against blank, which was devoid of enzyme. Enzyme unit: one unit of enzyme activity is defined as amount of enzyme that liberated one micromole of reducing sugar (glucose) per min under assay conditions.

Determination of the specific activity of α-amylase

The specific activity (SA) of the α-amylase was calculated according the following equation:
SA=enzyme activity (U/mL)/protein concentration (mg/mL).

Protein estimation was done by the method of Lowery et al. (1951) using bovine serum albumin (BSA) as standard.

Effect of temperature on the activity and stability of α-amylase

Effect of temperature on purified amylase activity was examined by performing the enzymatic reaction at different temperatures (30°C - 80°C). Thermal stability was investigated by incubating purified enzyme at varied temperatures (30°C - 80°C) for 2 h prior to adding it in standard amylase assay. At every 10 min intervals enzyme was withdrawn from each tube incubated at specific temperature and examined for enzymatic activity. Thermal stability was expressed as percent residual activity, considering the initial enzyme activity at each temperature as 100%.

Effect of pH on the activity and stability of α-amylase

Effect of pH on purified amylase activity was investigated by performing the enzyme assay at different pH ranging from 5 to 10 by using the following buffer systems: 0.1 M sodium acetate (pH 4-5.5); 0.1 M sodium phosphate (pH 6-7.5); 0.1 M Tris-HCl (pH 8-9); 0.1 M glycine NaOH (pH 9.5-12). Enzyme assay was performed at optimum temperature. pH stability was checked by incubating enzyme at different pH for 24 h. Amylase activity was measured by standard enzyme assay at every 4 h interval. The pH stability was expressed as percent residual activity, considering the initial enzyme activity at each pH as 100%.

Effect of substrate concentration on amylase activity

The effect of substrate concentration on enzyme activity was measured by performing enzyme assay at different substrate (starch) concentrations ranging from 0.5-6.5 mg/mL and keeping other parameters constant. The values of K_m and V_{max} were determined by Lineweaver-Burk plot.

Statistical analysis

All the experiments were carried out in triplicates. The results were expressed as mean of three independent experiments. MS Excel was used for statistical analysis to calculate standard deviation, regression equation and the correlation coefficient. The significance of test was evaluated by analysis of variance (ANOVA) using SPSS17 software.

Results and Discussion

Isolation screening and identification of bacteria

Total 27 bacterial colonies were isolated from soil sample in nutrient agar plate. These isolates were further checked for the production of α-amylase on starch agar plate. Only 11 isolates were able to produce α-amylase as indicated by zone of clearance around them. Among these 11 positive isolates, the bacterial isolate B-10 showed maximum zone of clearance, thus selected as best amylase producer for further studies. B-10 was identified on the basis of its microscopic examination and various biochemical tests. These studies revealed B-10 as Gram positive, motile, rod shaped, spore former and non-acid fast bacterium. Biochemical activities showed that B-10 was positive to glucose fermentation, catalase, oxidase, nitrate reduction, gelatin liquefaction, starch hydrolysis and Voges Proskauer, while negative for others (Table 1). On the basis of various staining reactions and biochemical tests the isolate B-10 was identified as *Bacillus sp.*

Time dependent growth and amylase production by strain B-10

Time dependent growth and amylase production was carried out by growing B-10 for 72 h in starch containing medium. As shown in Figure 1 after 4 h lag phase the growth of B-10 increased exponentially with increase in incubation time up to 36 h (A_{600}, 0.923). Afterward, its growth became almost stationary during further growth up to 72 h. On the other hand detectable amylase activity (0.0952±0.02 U/mL) was recorded after 2 h incubation, which increased exponentially after 6 h and reached maximum (0.81±0.017 U/mL) at 48 h incubation. Further increase in incubation time up to 72 h did not show any significant increase in enzyme production rather it was decreased (Figure 1). The time dependent production of α-amylase is in accordance with previous findings showing enzyme production abilities of many *Bacillus sp*. (Ashger et al., 2007; Mishra & Behera, 2008).

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Table 1. *Physical and biochemical characteristic of isolate B-10.*

Test	Results
Morphological characterization	
Gram staining	positive
Spore staining	positive
Acid fast staining	non acid-fast
Motility	motile
Biochemical tests	
Catalase	positive
Oxidase	positive
Nitrate reduction	positive
Urease	negative
H_2S production	negative
Methyl red	negative
Vogues proskaeur	positive
Citrate utilization	negative
Indole production	negative
Glucose fermentation	positive
Starch hydrolysis	positive
Gelatin hydrolysis	positive
Lipid hydrolysis	negative

Nurullah (2011) studied time dependent amylase production from Bacillus licheniformis ATCC 12759, which reached maximum at 72 h further incubation did not showed any enhancement in amylase production.

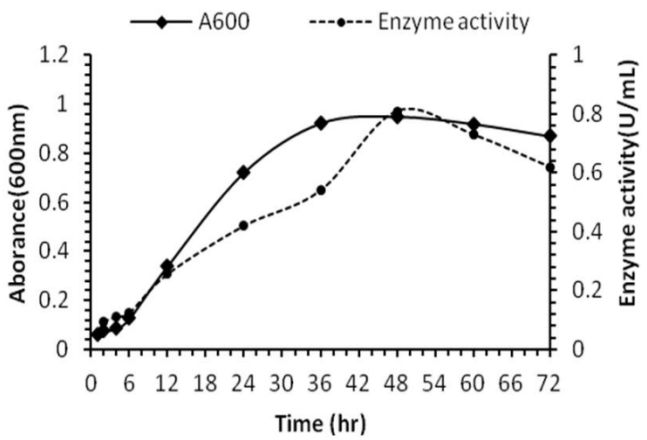

Figure 1. *Time dependent growth and amylase production of isolate B-10.*

Production and purification of alpha amylase

B-10 was grown for 48 h in starch fermenting medium for the production of enzyme. The purification of enzyme was done by ammonium sulfate precipitation (65%) followed by DEAE cellulose ion-exchange chromatography. Protein concentration of crude extract (0.41±0.02 mg/mL), partially purified enzyme by ammonium sulfate precipitation (0.28±0.044 mg/mL) and DEAE cellulose purified enzyme (0.10±0.026 mg/mL) were quantified by Lowery et al method (1951) using BSA standard (y=0.0012x+0.05, R^2=0.994). Specific activity of partially purified enzyme (20.62±1.16 U/mg of protein) and DEAE cellulose purified enzyme (62.44±2.49 U/mg of protein) exhibited 15.91 and 48.21 fold purification, respectively, as compared to crude enzyme. Many researchers used the similar strategies for purification of microbial amylase (Eratt et al., 1984; Khoo et al., 1994). A study carried out by Sajedi et.al (2005) showed 3.4 fold purification with ammonium sulfate precipitation and 45.2 fold purification after DEAE cellulose ion-exchange chromatography in comparison to crude enzyme. In similar kind of study on amylase isolated from pine seed showed 1286.1 fold purification after $(NH_4)_2SO_4$ fractionation, DEAE ion-exchange and starch affinity chromatography (Azad et al., 2009).

Polyacrylamide gel electrophoresis (PAGE)

Purified enzyme showed a single sharp band in comparison to three prominent bands of crude enzyme in reducing SDS-PAGE (Figure 2A).

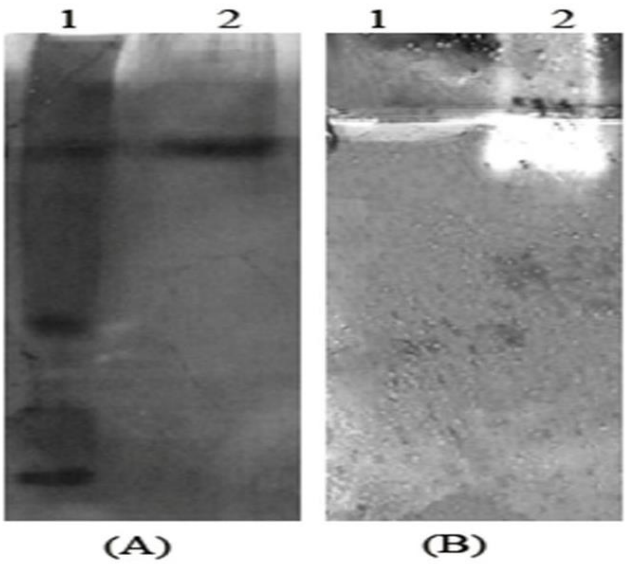

Figure 2. *SDS PAGE (silver stained)* ***(A)****, Native PAGE (activity staining)* ***(B)****. Lane-1 crude enzyme, Lane-2 purified enzyme.*

Presence of single band in lane-1 confirmed the monomeric nature of the enzyme and success of purification process. Results from the native gel electrophoresis followed

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by activity staining showed a sharp clear band against blue background that confirmed the activity of amylase in the gel (Figure 2B). PAGE is the most frequently used method to monitor success of the enzyme purification (Sajedi et al., 2005; Dutta et al., 2006; Kumar et al. 2010; Bano et al., 2011). The result of activity staining was in agreement with the findings in literature showing activity of α-amylase in the gel (Dutta et al., 2006; Bano et al., 2011).

Effect of temperature on the activity and stability of α-amylase

The amylase activity at different temperature points were significantly different ($p<0.05$). As shown in Figure 3 activity of α-amylase increased with increase in temperature and reached to maximum at 50°C (6.37±0.67) followed by sharp decline in activity with increasing of the temperature.

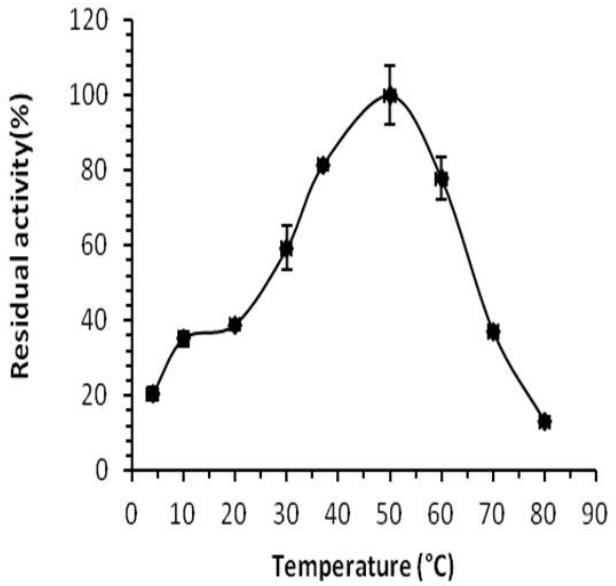

Figure 3. *Effect of the temperature on the amylase activity.*

Figure 4 shows the stability of amylase at various temperatures when incubated for 2 h prior to use in enzyme assay. At temperature 30, 40, 50 and 60°C the enzyme showed 95±3.97, 74±2.11, 59±1.88 and 23±1.01% residual activity, respectively. The activity of enzyme declined gradually with increase in incubation time and finally became zero after 2 h incubation at temperature beyond 60°C. Results indicated the thermostable nature of the enzyme, which is an important desired characteristic that suits its applicability in various enzyme based industries. The results are in commensurate to findings of many researchers who have reported 50°C as an optimum temperature for α-amylase isolated from *Bacillus sp*. (Soni et al., 2003; Ashger et al., 2007; Mukherjee et al., 2009).

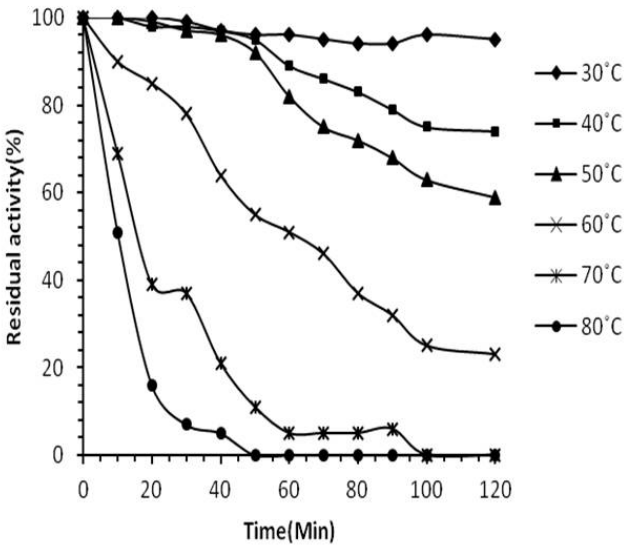

Figure 4. *Thermal stability of the amylase produced by Bacillus sp. strain B-10.*

Effect of pH on the activity and stability of α-amylase

Significant difference ($p<0.05$) were observed in amylase activity at different pH values. A bell shaped curve was obtained when the enzymatic activity was plotted against different pH values. There was an increase in α-amylase activity with increasing pH from 5 to 7. Maximum amylase activity was recorded at pH 7 and it declined with further increase in pH up to 10 (Figure 5).

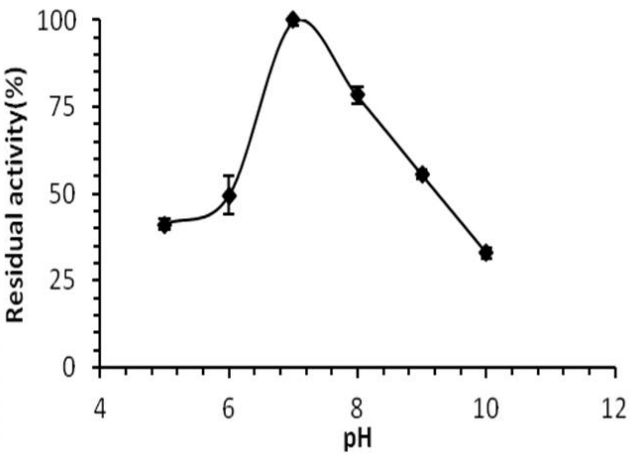

Figure 5. *Effect of pH on the amylase activity.*

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The activity reported at pH 5 and 10 were 41.19±1.52% and 32.93±1.44% as compared to that of pH 7, respectively. pH stability study revealed that enzyme activity decreased by 5±1.35% at pH 7 when incubated for 24 h. In the same study, enzyme activity decreased by 71±0.34% and 80±0.89% at pH 5 and 10, respectively (Figure 6). For most *Bacillus sp*., the pH optimum and stability of α-amylase has been reported in the range of pH 5 to 9. The results are similar to the previous findings that showed pH optimum and stability of α-amylase in the range of pH 5 to 9 for most *Bacillus sp*. (Goyal et al., 2005; Ashger et al., 2007; Konsoula & Liakopoulou-Kyriakides, 2007; Tanyilidizi, 2007).

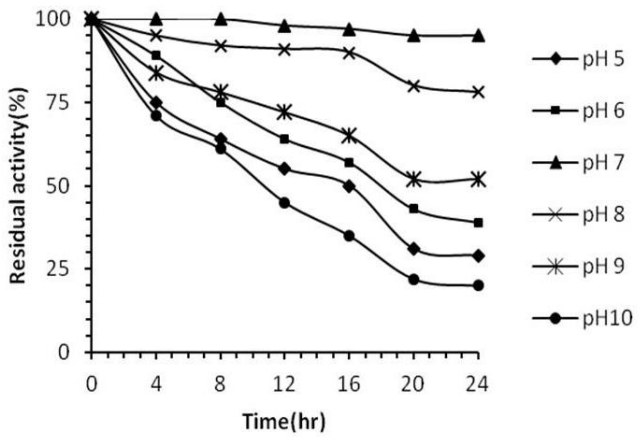

Figure 6. *pH stability of the amylase produced by Bacillus sp., strain B-10.*

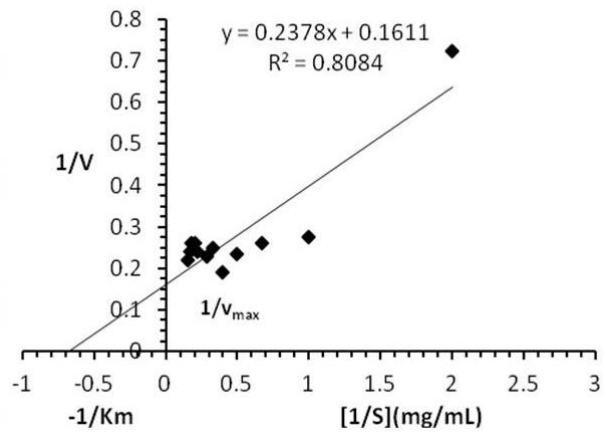

Figure 7. *Line-Weaver Burk plot of purified α-amylase.*

Effect of substrate concentration on amylase activity

Enzyme activity was evaluated at various substrate concentrations (0.5-6.5 mg/mL). Initially, activity of enzyme increased with increasing substrate concentration up to 3.5 mg/mL starch. Further increase in substrate concentration had no effect on enzyme activity. K_m and V_{max} were found to be 1.4 mg/mL and 6.2 U/mL, respectively, using Lineweaver-Burk plot (Figure 7). The observed K_m value was found much lower in comparison to the K_m values of amylase isolated from different *Bacillus* sp. (1, 28-30). Lower K_m showed the high affinity of isolated amylase towards its substrate that favors its application for industrial uses.

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