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ISOLATION AND CHARACTERIZATION OF A HIGHLY THERMOSTABLE ALPHA-AMYLASE ENZYME PRODUCED BY *BACILLUS LICHENIFORMIS*

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Bacterial amylases play an important role in industrial production and contribute significantly to meet our commercial needs in a variety of ways. The present study was designed to report the isolation, identification and characterization of a thermostable α-amylase enzyme produced from a bacterium isolated from indigenous soil samples. The isolated bacterium was identified as *Bacillus licheniformis* and confirmed through a variety of morphological, biochemical and hydrolysis based tests. The isolated bacterium was cultured in amylase producing media and found to produce α-amylase at the rate of 92 U/ml after 24 h cultivation at pH 7.0 and at 100°C. Enzyme induction was found to be the highest utilizing potato starch followed by corn and wheat. The characterization of α-amylase was done to establish its suitability for industrial applications in starch processing and food manufacturing industries among others.
Keywords: *Bacillus licheniformis,* α-amylase, potato starch, characterization, thermostable

INTRODUCTION

Enzymes are catalysts of biological processes. They possess extraordinary catalytic properties far exceeding those of synthetic agents (Nelson and Cox, 2000). Alpha (α) amylase is an enzyme that breaks down α-1, 4 linkages between large polysaccharides such as starch and glycogen, producing glucose and maltose (Lamabam *et al.,* 2010). Amylases are produced from numerous sources such as animals, plants and microorganisms etc. Microbial amylases have successfully replaced the chemical hydrolysis methods in starch-processing industries. The majorities of amylases are produced from microbial sources for the main reason that microbes can be cultured under strictly controlled environmental conditions in greater quantities and are relatively easy to purify and process. Microorganisms also provide different varieties of α-amylases in terms of their amylolytic action and protein structures. Such amylases are used in many industries e.g. the pharmaceutical and chemical industries. With the advent of biotechnological production procedures, mostly in the form of fermentation tools and technologies, many new areas have opened up for the processing of food wastes and manufacture of value added products (Pandey and Nigam, 2000). Among microbes, species of genus Bacillus have been widely used for the production of thermostble α-amylases due to their wide distribution in nature and easy adaptability to varying environmental conditions (Ajayi and Fagade, 2006; Kubrak *et al.,* 2010). *Bacillus (B) subtilis, B. stearothermophilus, B. licheniformis,* and *B. amyloliquefaciens* are known to be good producers of thermostable α-amylases (Prakash and Jaiswal, 2009; Sumrin *et al.,* 2011).

Thermostable α-amylase is one of the most important and widely used enzymes whose spectrum of application has widened in food, paper and detergent industries (Nigam and Singh, 1995). Due to its importance in a number of industrial applications, this novel study was designed to the isolation, identification and characterization of bacteria which produce highly thermostable α-amylase was initiated to screen for new sources of production of this enzyme.

MATERIALS AND METHODS

***Isolation, identification and characterization of bacterial strains*:** Potato baiting technique was used for the isolation of a highly thermophilic bacterium in the Institute of Microbiology, University of Agriculture Faisalabad. Ten grams of the treated potato were mixed in 90 ml sterile distilled water. Serial dilution (10-fold) was made, out of which 0.1 ml diluted sample was taken and spread on nutrient agar plates containing 1% (w/v) soluble starch and then incubated at 37°C for 24 hrs. Starch hydrolysis was assessed as clearing zones around the colonies. Bacterial strains were identified on the basis of various morphological {shape, size, aerobic, motile, spore forming} bio-chemical {Citrate, catalase, Voges-Proskauer, Indole, oxiadase, nitrate reduction and sugar fermentation test} cultural characteristics {white to cream color colony on nutrient agar medium, grow at various concentrations of NaCl (3, 6, 9 and12%), temperature above 50^{o}C} and hydrolysis test {Starch, dextrin, arginine and rhamnose hydrolysis test} following the criteria laid down by Claus and Berkeley, 1986. Culture purity was further checked by Gram's staining (Giffel *et al.,* 1995).

***Temperature tolerance*:** Temperature tolerance of various isolates was examined on nutrient agar medium containing 1% starch (w/v) by incubating the petriplates for 24hrs in the temperature range of 30-70°C.

***Bacterial amylase production medium (APM)*:** 10% starter culture of *B. licheniformis* was made in nutrient broth containing 1% (w/v) soluble starch and was incubated for 24 hours at 37°C. The starter culture mix was transformed into amylase production medium and incubated for 37°C for 24 hours. The composition of amylase production medium was in gram/liter starch 10, KCl 0.5, $MgSO_4.7H_2O$ 0.5 and peptone 6, respectively (Vasantha and Hemashenpagam, 2012).

***Extraction of crude enzymes*:** The amylase production medium was poured into centrifuge tubes and centrifugation was carried out for 20 minutes at 5000 rpm. The supernatant containing cell free extract was removed and served as crude enzyme extract. This crude enzyme extract was used in amylase enzyme activity measurement and characterization studies (Diomi *et al*., 2008).

***Partial purification of α-amylase*:** Partial purification of crude enzyme sample was achieved by fractionation using ammonium sulphate (60%). Briefly, the crude sample was fractionated into two, 0-30 and 30-60% based on saturation of ammonium sulphate. The two fractions were separately dissolved in minimum amount of 0.1 M acetate buffer (pH 5) and dialyzed overnight against the same buffer. The purified fractions were assayed individually for protein and α-amylase activity (Patel *et al*., 2005).

***Determination of protein in crude extracts*:** Soluble protein concentrations were determined in the aqueous extract of fermented matter by Bradford method (1976). Bovine serum albumin was used as a standard.

***Determination of α-amylase activity*:**

a. Starch-iodine method: α-amylase production was detected after flooding the plates with 10 ml iodine solution. This method determines the dextrinising activity of α-amylase in term of decrease in the iodine color reaction (Saxena *et al.,* 2007).

b. *DNS method*: The activity of α-amylase was examined and measured by using DNS reagent (Haq *et al.,* 2010). For this purpose, 1 ml of culture extract was added into test tube. One ml of 1% soluble starch in citrate-Phosphate buffer (pH 6.5) was added and the mixture was incubated in a water bath at 40°C for 30 min. After incubation the reaction was stopped by adding 2 ml of DNS reagent. The mixture was boiled for 5 minutes and the color intensity was determined at 540 nm by using lBM S-2600 spectrophotometer. One unit of activity is defined as that amount of enzyme which releases 1 µ mol of reducing end groups per minute in 0.1 M sodium phosphate buffer.

c. *Benedict method*: This method is used to determine the reducing sugars i.e. glucose which is liberated by the action of α-amylase on starch. 1 ml of curd enzyme extract was taken and mixed with 3 ml of Benedict reagent and boiled for 5-10 min. The reducing sugar is checked for the change in color of solution after boiling (Robert *et al.,* 2002).

***Comparison of different substrates concentrations on the production of α-amylase*:** The optimum enzyme production was examined utilizing different substrates (potato, corn and wheat) concentrations (1, 2, 3, 4, 5 and 6%). The isolate was grown on nutrient agar medium which contained 1% w/v potato starch, wheat starch and corn starch and incubated for 24 hours at 37°C. After incubation, the hydrolysis zone of various substrates was checked and measurement of the zone of hydrolysis was made (Vasantha and Hemashenpagam, 2012).

***Effect of pH on enzyme activity and stability*:** Optimally produced α-amylase was assayed at different levels of pH viz; 4, 5, 6, 7, 8 and 9 in Na-phosphate buffer (pH 6.5-8.0), glycine-NaOH buffer (8.5-10.5), respectively in order to check out the optimum conditions for enzyme activity (McTigue *et al.,* 1995).

***Effect of temperature on the activity and stability of enzyme*:** Enzyme produced under optimum conditions was assayed at different temperatures (50-110^oC) in order to find out the optimum temperature for the activity of enzyme (Asghar *et al.,* 2007).

RESULTS AND DISCUSSION

In the present study, *B. licheniformis* was isolated and characterized by performing different morphological, biochemical, hydrolysis and cultural tests. The isolated bacterium was Gram positive (Plate 1), rod shaped, aerobic, motile and spore forming. Acid was produced from the hydrolysis of sucrose, melibiose, cellobiose, galactose, xylose and mannose but gas was produced only from glucose. Citrate, catalase and Voges-Proskauer tests were positive. Indole, oxiadase and nitrate reduction test were negative. Similar results have been reported by other investigators using other *Bacillus* species (Ljungdahl, 1979; Hussein and Janabi, 2006; Reda, 2010). The isolated Bacillus strain has the ability to grow at various concentrations of NaCl (3, 6, 9 and12%). It was able to grow on nutrient agar plates with 1% soluble starch at pH 7, and may be characterized as a mesophile to thermophile with growth temperature ranges from 30-70°C (Barrow and Feltham, 1993; Fisher, 1975; Rasooli *et al.,* 2007). Starch, dextrin, arginine and rhamnose were hydrolyzed. Utilization of arginine as an energy source for growth is well documented in a variety of micro-organisms, e.g. *B. licheniformis* (Asghar *et al.,* 2007). Our isolate were primarily identified through various methods as *B. licheniformis* or *B. subtilis* followed by their phenotypic differentiation based on their abilities to hydrolyze arginine

(Plate 2). Since our isolated strain was able to grow above 50°C, it fulfilled the criteria of growth for thermophilic microorganisms (Perry and Staley, 1997). This strain was subsequently used for the production of α-amylase in submerged cultures as well as through solid state fermentation. It has been reported that the species of genus Bacillus are good producers of α-amylase (Khajeh *et al.,* 2011). Ammonium sulfate saturation fraction (30%-60%) exhibited maximum enzyme specific activity of 92 units/ml protein. To determine the starch hydrolysis zone 2% starch iodine solution was utilized. The results obtained from Gram iodine test showed color change to dark blue which represented positive indication for starch whereas, the zone of hydrolysis was observed as red color as shown in Plate 3.

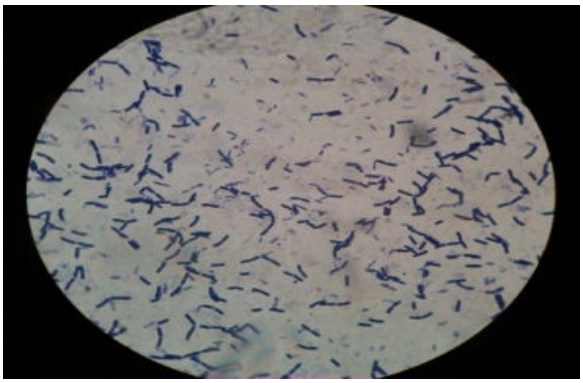

Plate 1. Rod shaped *Bacillus licheniformis*

Plate 2. Arginine hydrolysis test

Plate 3. Clear zone of hydrolysis

Similar results have been obtained by many scientists (Asad *et al.,* 2011). Our isolated *B. liceniformis* strain had the ability to produce thermostable α-amylase which was subsequently used to hydrolyze different starches such as corn and wheat starch. Soluble potato starch was used as a standard. Hydrolysis zone was observed around the bacterial colonies which were measured as 24 mm for potato starch, 20 mm for corn starch and 18 mm for wheat starch. These results indicated that the hydrolyzing ability and effectiveness of our α-amylase is the highest for potato starch followed by corn starch and wheat starch as shown in Figure 3. The main hydrolysis products of starch are glucose, maltose and malt-triose. *Bacillus spp.* (1-3) has the ability to digest potato starch. Maximum 1% concentration of potato starch hydrolysis occurred at 70°C after 5 hours (Goyal *et al.,* 2005). Similar findings have been reported (Niazi *et al.,* 2010) by comparing wheat bran and maize bran as a substrates for the production of α-amylase.

It was observed that the production of α-amylase increases with increase in the concentration of starch in the medium and reaches an optimum activity level at 1% starch level. Further increases in the level of starch results in significant decrease in α-amylase production, it may be due to the end product repression which is supported by other reports on the α-amylase production in *B. licheniformis* strains (Omidiji *et al.,* 1997). Such a repression may also be strain dependent. However, in present finding 1% starch was found to be sufficient as a carbon source to promote the production of maximum α-amylase (Rasooli *et al.,* 2007). The α-amylase obtained in this research may have a great potential to digest the raw potato starch in food and fermentation industries. After boiling with Benedict reagents, different colors (from light to dark green) were observed, indicating the presence of glucose in the solution. The standard method of reference for estimation of α-amylase is that of Somogyi method for glucose determination in which the amount of reducing substances formed by enzymatic action on a known amount of starch was determined quantitatively (copper reduction) and the results expressed as color change through Benedict reagent (Nelson, 1944). We observed different color changes from green to yellow, which indicated that reducing sugar e.g. glucose was present in the solution.

In this study the effect of different temperatures at various levels (50, 60, 70, 80, 90, 100 and 110°C for 30 minutes) were examined to test the extent of thermostability and result was depicted in Figure 2. It was observed that the α-amylase enzyme was stable up to 100°C for 30 minutes duration. The activity then declined precipitously above 110°C and at 120°C the enzyme became largely inactive. Correlation between enzyme thermostability versus time of enzyme exposed to heat was found maximum at 60°C for up to 10 min. It has been previously demonstrated (Goyal *et al.,* 2005) that α-amylase from a hyper producing strain of Bacillus sp. E2 was also stable at 70°C for 30 min but

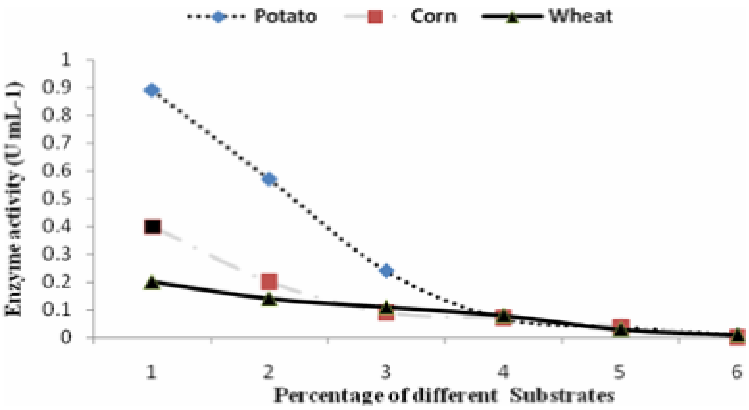

Figure 1. Effect of substrate concentration on alpha amylase productivity

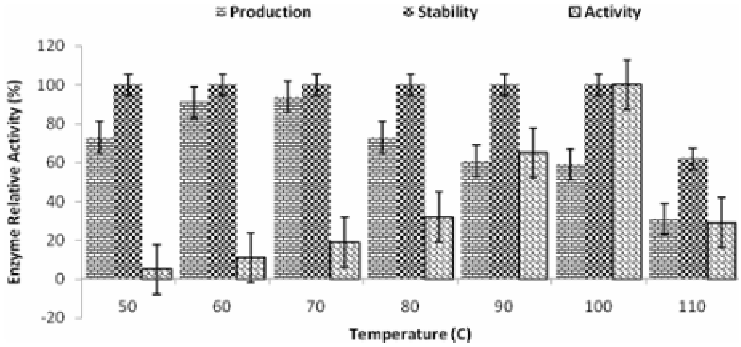

Figure 2. Effect of temperature on stability, productivity and activity of alpha amylase

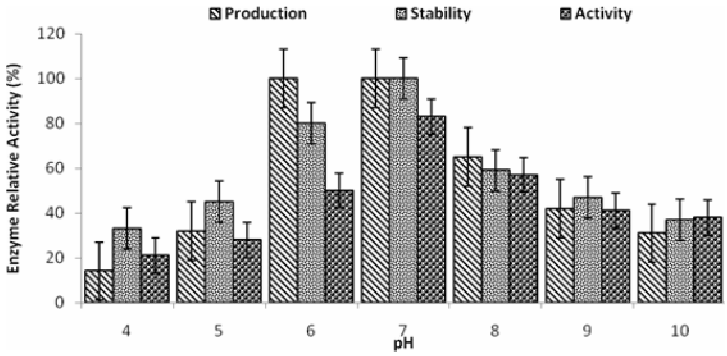

Figure 3. Effect of pH on stability, productivity and activity of alpha amylase

quickly inactivated at temperature above 70°C. Similar results have also been reported by others where the enzyme was found to be stable at 70°C to 80°C for 20-30 minutes (Asgher *et al.,* 2007; Rasooli *et al.,* 2007). Again activity and stability of α-amylase is very sensitive to temperature depending on specific strains utilized (Robert *et al.,* 2002). In starch-processing industries, *B. stearotermophilus* and *B. licheniformis* are utilized as source of α-amylases (Xiangli *et al.,* 1984). Since, thermostability is a significant feature for the use of hydrolytic enzymes; thermostable enzymes produced by the thermophilic bacteria are of utmost importance and are considered as the best for the production of industrial by products (Saxena *et al.,* 2007; Leveque *et al.,* 2000). The pH of the fermentation medium was profound effect on the growth of the organism and overall enzyme synthesis (Horikoshi, 1971). Microbial growth and productivity of enzymes are very sensitive to any changes in pH (Krishnan and Chandra, 1983). To examine the effect of initial pH on the biosynthesis of α-amylase we also carried out carefully crafted experiments. We observed that the optimum occurred when the initial pH of the fermentation medium was maintained at 7.0 as shown in Figure 1 while

other scientists have been reported optimum production of α-amylase in pH ranges from 8.0-9.5. These differences might be due to the fact that our organism required neutral pH for its growth. Therefore, a neutral pH of 7.0 was selected in our fermentation medium for maximum expression and production of α-amylase in our bacterial cultures. It should be noted here that most strains of Bacillus used commercially for the production of α-amylase utilize an optimum pH in ranges somewhere between 6.0 to 9.0 for growth as well as enzyme production (Asghar *et al.,* 2007; Niazi *et al.,* 2010; Asad *et al.,* 2011). In summary various Bacillus sp. produced α-amylase at rather wide pH ranges between 3 to 12 (Horikoshi, 1971; Hayashi *et al.,* 1988; Kim *et al.,* 1995).

***Conclusions*:** Our study showed, we have isolated and characterized strain of *B. licheniformis* that is α-amylase producing and this enzyme showed maximum activity at a pH 7.0 and a temperature of 100°C.

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