JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. **4**(1), p. 303-308

Characterization and Quantification of Amylase from *Bacillus stearothermophilus*

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(Received: 07 August 2009; accepted: 11 October 2009)

The present study is concerned with the production of α-amylase by *Bacillus stearothermophilus*. The fermentation was carried out by continuous shaking in 250ml Erlenmeyer flask containing 100ml of the medium. The maximum production of the enzyme was optimized at the pH 7.5, while the incubation temperature investigated was 37ºC. The production of enzyme was obtained maximum at 120hours after incubation (14.496 IU/ml/min).

Key words: α-amylase, *Bacillus stearothermophilus*.

Alpha(α)-amylase , an extracellular enzyme degrades α-1,4-glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin (Calik & Ozdamar, 2001). Amylases are produced in a variety of living organisms like bacteria, fungi, plants and animals. Microbial amylases are used for industrial purposes (Alva *et al.,* 2007). Using current technology, microbial amylases are commercially produced and these synthetic types have completely replaced the chemical hydrolysis of starch in industries. With the discovery of new strains of microorganisms and development of more efficient production strategies, microorganisms and development of more efficient production strategies, microorganisms have substantial potential to contribute to a number of industrial applications. Such industrially important microorganisms are found with in the Bacillus species because of their rapid growth rates that lead to short fermentation cycles, their capacity to secret proteins into extracellular medium and general handling safety (Pandey *et.al.,* 2001). However, catabolic repression of the enzyme synthesis has been reported in submerged fermentation of bacteria and fungi(Tomanga

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1966; Heinkes & O'Connor 1972). Amylases have potential application in several industrial processes e.g. food, fermentation, detergent, textile, pharmaceutical, chemical, brewing and paper industries(Moreira *et.al.,* 1999; 2001; Kathiresan & Manivannan, 2006).

The objective of the present study was to isolate bacteria from water source and screen them for the production of α-amylase, optimize the culture conditions for enzyme production and characterize the enzyme for industrial applications.

MATERIAL AND METHODS

Bacillus stearothermophilus was isolated from water sample obtained from Hot Spring Atri of Orissa and was maintained as pure culture after identifying by standard identification protocols.

Screening of bacterial isolates

Primary screening of bacterial isolate for production of α-amylase was done by the starch agar plate method (Aneja, 2002).

Inoculum preparation

The bacterial isolate was transferred from stock culture to 100ml Nutrient broth. The inoculated flasks were incubated overnight at 35±2ºC .The broths were then centrifuged at 10,000rpm for 10 min. After centrifuging the pellet was resuspended in 10ml of sterile distilled water and the absorbance (A) read at 660nm.Cell density of the inoculum suspension was adjusted to an OD of 0.5 which contains 4.5×105cells/ml.

Fermentation technique

The fermentation was carried out in 250ml Erlenmeyer flask taking 100ml of the fermentation medium containing peptone(2%), soluble starch(0.5%), 0.3% dipotassium hydrogen phosphate and 0.1% hydrated magnesium sulphate .The flasks were sterilized in the autoclave at 121ºC and 15 lbs pressure and then cooled at room temperature. 1ml of bacterial cell suspension was transferred to each flask. The flasks were then placed at 37ºC for 96 hours in the rotary incubator shaker. After 96h of fermentation, the broths were centrifuged at 8,000rpm for 10min.In both cases the cell free supernatant obtained after centrifugation was used as the source of enzyme. All the experiments were carried out in triplicates.

Enzymatic assay

Estimation of amylase activity was carried out according to the 3,5-dinitro salicylic acid (DNSA) method of Miller (1959). One hundred micro liter of 1% starch was incubated with 1mL of enzyme extract and 1mL of phosphate buffer (pH - 6.5). The reaction mixture was incubated for 20min. before stopping the reaction by addition of 0.5mL DNSA reagent followed by heating for 5 minutes, then cooling in a water bath for 10min. A volume of 2.5ml of distilled water was added and the absorbance read at 540nm using UV-Visible Spectrophotometer against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole 0f reducing sugar as glucose per minute, under the assay condition (IU/ml /min). The experiments were carried out in triplicates and standard error was calculated.

Protein estimation

Protein content of the enzyme extract was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Enzyme activity is expressed as specific activity, which is equivalent to U/mg protein. All the experiments were carried out in triplicates and the standard error was calculated.

Evaluation of factors affecting enzyme activity

pH

The effect of pH on amylase activity was assayed by incubating the reaction mixture at different pH values such as 4.5, 6.0, 7.5 and 9.0.

Different carbon and nitrogen sources

The effect of amylase production was carried out taking different carbon sources viz. glucose, maltose, sucrose and soluble starch and at different nitrogen sources like beef extract, yeast extract, peptone, casein and urea.

Thermal stability of amylase

The thermal stability of enzyme was assayed by incubating the fermentation medium at various temperatures between 30ºC-70ºC for 96hours.

Effect of different metal chloride ions

Enzyme activity was assayed in the presence of 10Mm concentrations of various metal ions (Na^{2+}, Ca^{2+}, K^{+}, Co^{2+} and Fe^{3+}). The chloride salts of these metal ions were used (NaCl, $CaCl_2$, KC_l, $CoCl_2$, $FeCl_3$).

Different concentrations of starch

The effect of amylase production was carried out taking different concentrations (0.5%, 1.0%, 1.5%, 2.0%) of starch in the fermentation medium.

RESULTS AND DISCUSSION

Bacillus stearothermophilus was tested for the production of amylase by starch hydrolysis tests. Amylase production in case of *B.stearothermophilus* was found to be 14.496 IU/ml/min in the standard fermentation medium. Protein value of this enzyme was found to be 3.166U/ mg protein.

Effect of incubation temperature

The data of Fig.1 shows the effect of different incubation temperatures on the production of α-amylase by *B.stearothermophilus*. The fermentation was carried out at 30ºC, 37ºC, 40ºC, 50ºC, 60ºC and 70ºC in the rotary incubator shaker. The maximum production of α-amylase was obtained at 37ºC (14.496 IU/ml/min). As the incubation temperature was increased, the production of the enzyme was greatly inhibited at 70ºC (1.491 IU/ml/min). Thus, the incubation temperature, 37ºC was selected for maximum production of enzyme.

Effect of pH on the activity of enzyme

Fig.2 shows the effect of initial pH of the reaction mixture (enzyme substrate complex) for the activity of α-amylase. The enzyme activity was extremely low at pH-9 (0.707 IU/ml/min). The activity of the enzyme was gradually increased from 4.5-7.5 and found maximum at pH-7.5 (14.496 IU/ml/min). Further increase in the initial pH resulted decrease in the activity of α-amylase. However, the pH of reaction mixture for the hydrolysis of starch was found to optimum at 7.5.

Effect of different carbon sources on amylase production

Fig.3 shows the effect of different carbon sources on amylase production. From the figure it was observed that amylase production was more on glucose (55.695 IU/ml/min) than any other carbon sources. Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides showed substantial difference in effect to α-amylase

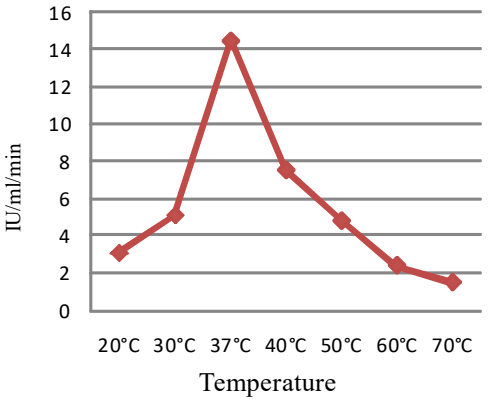

Fig. 1. Effect of temperature on amylase production

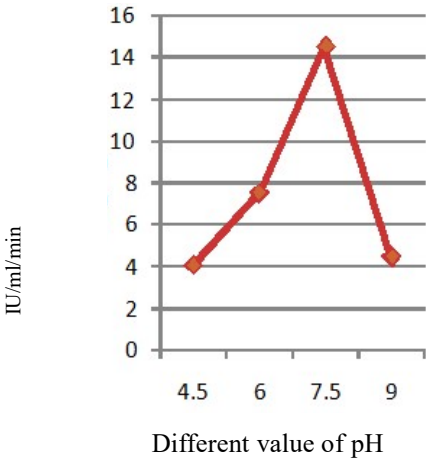

Fig. 2. Effect of different pH on amylase production

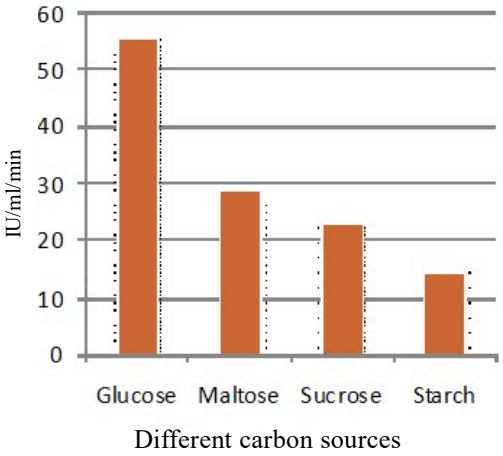

Fig. 3. Effect of different carbon sources on amylase production

production.

Effect of different nitrogen sources on amylase production

Fig.4 shows the amylase production from *B. stearothermophilus* is more on Beef extract (16.507 IU/ml/min) than any other nitrogen sources. Addition of organic nitrogen sources such as casein, yeast extract, urea and peptone to the medium resulted in a considerable increase in the production of α-amylase. Media supplemented with peptone showed (14.496 IU/ml/min) followed by casein (1.841 IU/ml/min). Amylase production is least incase of yeast extract (3.974 IU/ml/min).

Effect of different period of fermentation for amylase production

Time dependence of the rate of hydrolysis of starch by amylase was calculated by recording the period of fermentation up to 8 days. Fig.5 provides a detailed view of the results. The results of the period of fermentation study showed that there is a gradual increase in the amylase production up to 5 days and subsequently the rate of production decrease after 5 days. *B. stearothermophilus* showed amylase activity on fifth day (18.940 IU/ml/min) and least amylase activity on 8th day (1.963 IU/ml/min).

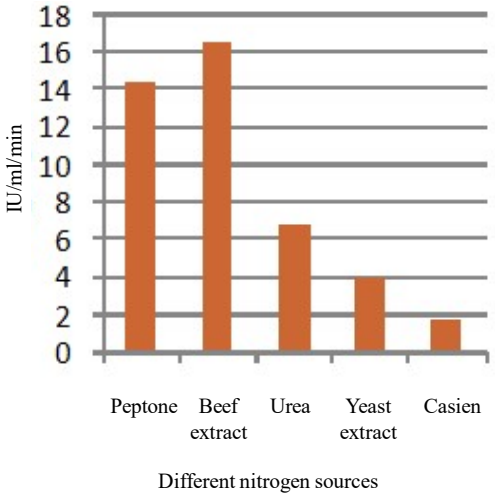

Fig. 4. Effect of different nitrogen sources upon amylase production

Effect of different metal chloride ions for amylase production

From the metal chloride optimization study, it was clearly observed that *B. stearothermophilus* was able to produce maximum amylase enzyme (7.014 IU/ml/min) in the presence of $FeCl_3$ than any other metal chloride in the fermentation medium. The detailed results are shown in fig. 6.

Effect of different concentrations of starch

Starch was also used as a substrate for α-amylase activity. Optimum activity achieved at 1% starch which was clearly indicated in Fig.7. As the substrate concentration increased, α-amylase activity dropped sharply which may be due to substrate inhibitory effect. *B.stearothermophilus* showed higher amylase activity on 1% starch (18.939 IU/ml/min) and least amylase activity on 2% starch(10.205 IU/ml/min).

The production and stability of α-amylase depends upon temperature. In the present study, the fermentation was carried out at different incubation temperature. The maximum production of enzyme was observed at 37ºC. Biosynthesis of α-amylase was significantly decreased with the increase in the incubation temperature beyond 37ºC. It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and enzyme fermentation was also prohibited (Haq *et.al.,* 1997; Chengyi *et.al*., 1999). Composition of cell wall and plasma

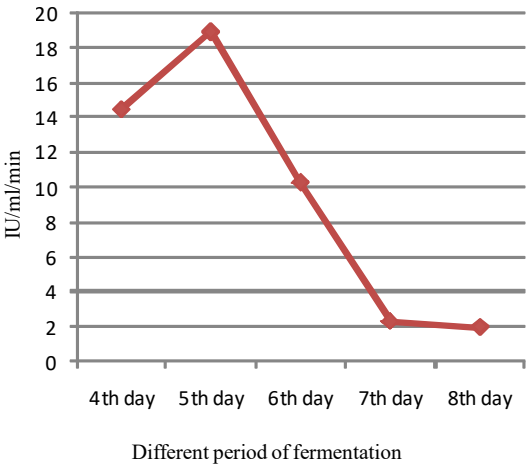

Fig. 5. Effect of different fermentation period on amylase production

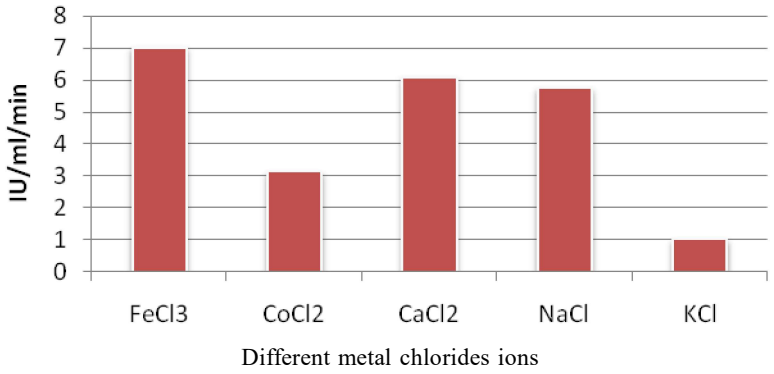

Fig. 6. Effect of different metal chlorides ions on amylase production

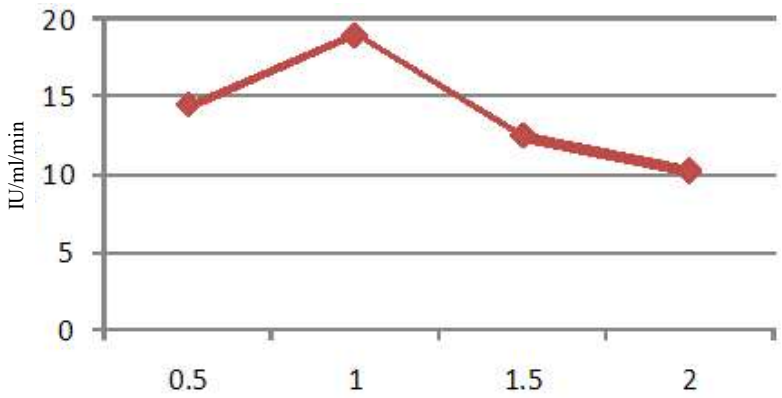

Fig. 7. Effect of different concentrations of starch on amylase production

membrane of microorganisms are known to be affected by the medium pH which was reported by Ellwood and Tempest (1972). Due to this change in the nature of cell wall and plasma membrane the growth parameters may vary. This may be responsible for decreased production of amylase at pH 4.5 and 9.0 in the present study. Optimum pH for amylase activity was found to be 7.5. The findings are similar to the previous findings of Fisher and Stein (1961).

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