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Optimization of Culture Conditions for Production and Activity of Thermostable Amylase by using *Bacillus* Strain A_4

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The present study deals with the production and activity of thermostable amylase by using *Bacillus* strain A_4. The conditions for production of amylase was optimized. Maximum production of amylase was obtained in presence of wheat bran (270 U/mL) and cassava flour (110 U/mL) after 48 h of incubation. The optimum temperature and pH were found to be 50 ºC and 10, respectively. Maximum activity was observed at pH 9.0 at 90 ºC in crude extract, after 20 min of incubation. The presence of EDTA and SDS inhibits enzyme activity whereas Mn^{2+} and Fe^{2+} ions increased the amylase activity. Due to maximum production of amylase in presence of cheap, low concentration of substrates, stability at alkaline pH and high temperature, all the above characteristics makes the *Bacillus* strain A_4 and its enzyme useful in different industries.

Key Words: α-Amylase, Thermostable enzymes, *Bacillus* sps.

INTRODUCTION

α-Amylase, an extracellular enzyme degrades α, 1-4-glycosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and α limit dextrin[1]. This enzyme was extensively used in many industries including starch liquefaction, brewing, paper, food, textile and distilling industry[2]. Microorganisms like fungi and bacteria have been extensively screened for α-amylase production[3]. The production of amylase was depend on the strain, composition of media, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermostability. The effect of temperature on relative activity of α-amylase from *B. subtilis* was detected and temperature optimized between 60-70 ºC for maximum activity[4,5]. The most important characteristics of thermophillic organisms are the ability to produce thermostable enzymes with a higher operational stability and a longer shelf-life[6]. Recent research with thermostable α-amylases has concentrated on the enzymes of thermophiles and extreme thermophiles[7-11]. Hence there is a considerable interest in the production of thermostable amylase from bacterial source. Therefore the present study was aimed to optimize cultural conditions for production and activity of thermostable amylase from *Bacillus* strain A_4.

EXPERIMENTAL

Screening of microorganism: For isolation of amylase producing organism, soil samples were collected from different sites in Vizianagaram district of Andhra Pradesh, India where generally the flour mill wastes are disposed off. These soil samples are made different dilutions (10^{-1}-10^{-7}) with sterile distilled water and plated on starch agar plates (nutrient agar with 1 % starch), incubated at 37 ºC for 24 h. After incubation, the plates were flooded with 4 % lugol solution. Colonies showing clear zone of hydrolysis were picked and purified. Isolate $11A_4$ showed maximum activity and it was selected and maintained on nutrient agar at 4 ºC. The cultures were examined for various morphological and biochemical characteristics as per Bergey's manual of determinative bacteriology[12].

Amylase assay: Amylase assay was done by using phosphate buffer (0.1 M, pH 7.0), starch solution (1 %), dinitro salicylic acid (DNS) reagent and standard maltose[13]. The reaction mixture contained 0.5 mL of starch solution, 0.3 mL of phosphate buffer and 0.2 mL of enzyme. The tubes were immersed in boiling water bath for 15 min and after cooling the absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that liberated one μ mol of reducing sugar (maltose equivalent) under assay condition.

Optimization of culture conditions for enzyme production

Effect of incubation time on production of amylase: Amylase production was carried out in a production medium containing (g/L) soluble starch-15; yeast extract-10; peptone-10; NaCl-10 at pH 7.0. The production medium was inoculated with 50 μL of the *Bacillus* strain A_4 overnight culture (1×10^8

cells/mL) and incubated at 37 ºC in shaking incubator at 150 rpm (Remi-Ris-24, Mumbai, India) for 96 h[14]. Samples were collected after 24, 48, 72 and 96 h, centrifuged at 5,000 rpm for 10 min at 4 ºC (Plastocraft Super spin-RV/FM high speed, Mumbai, India). The clear supernatant was used as enzyme source.

Effect of culture conditions on production of amylase: Different culture conditions like different substrates (1 % wheat bran, 1 % cassava flour, 1 % corn flour, 1 % soya flour and 1 % rice flour) were used for the production of α amylase by adding 50 µL of the *Bacillus* strain A_4 overnight culture (1×10^8 cells/mL) in a production medium and incubated at 37 ºC in a shaking incubator at 150 rpm for 48 h (Remi-Ris-24, Mumbai, India). In order to optimize the effect of pH on extracellular amylase production, pH of the production medium was adjusted to 4.0-10. Amylase production was followed at different temperature (30-100 ºC) for maximum production. Samples were collected after 48 h and centrifuged at 10,000 rpm for 10 min at 4 ºC (Plastocraft Super spin-RV/FM high speed, Mumbai, India). Supernatant was used as crude extract and amylolytic activity was measured under standard assay condition.

Stability of enzyme in crude extract: Amylase was produced from *Bacillus* strain A_4 under optimized conditions. After 48 h of incubation, cell free supernatant was taken as crude extract and used for the characterization of enzyme. The enzyme was incubated at different pH (4.0-10) to determine the stability of α amylase and enzyme was pre-incubated in different buffers (4.0-10) at 45 ºC for 20 min. The residual enzyme activity was determined. The effect of temperature on amylase stability was also determined by measuring the residual activity after 20 min of pre-incubation in 0.1 M sodium phosphate buffer (pH 9.0), at temperature ranging from 30-100 ºC. The effect of metal ions (NaCl, $MnCl_2$, $CaCl_2$, $CoCl_2$ and $FeCl_2$) on the activity of amylase was determined by adding different metal ions in the reaction mixture at 1 mM concentration and incubated for 20 min. All the chelating agents (SDS and EDTA) are added in the incubation mixture at 1 mM concentration and enzyme assay was carried out as discussed above.

RESULTS AND DISCUSSION

Using morphological and biochemical characteristics[12] the selected isolate (strain A_4) was identified as *Bacillus*. Maximum production of amylase by *Bacillus* strain A_4 was 300 U/mL after 48 h of incubation. The production was 150, 125 and100 U/mL after 24, 72 and 96 h and it was shown in the Fig. 1.

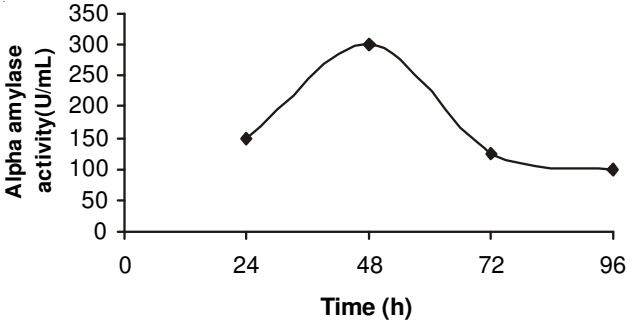

Fig. 1. Effect of incubation time on amylase production

Effect of substrates on amylase production: The use of cheap sources like wheat bran, cassava flour, corn flour, soya flour and rice flour are very important as these can reduce the cost of production of amylase. Maximum production of α-amylase (275 U/mL) was observed in case of wheat bran after 48 h of incubation. While in case of cassava flour and corn flour, the production of amylase was found to be 110 and 100 U/mL, respectively (Fig. 2). Most of the studies reported that wheat bran acts as the good substrates for α-amylase production by *Bacillus* licheniformis using different agricultural byproducts[15]. In our subsequent optimization studies, wheat bran was used as the best substrates for the production of α-amylase.

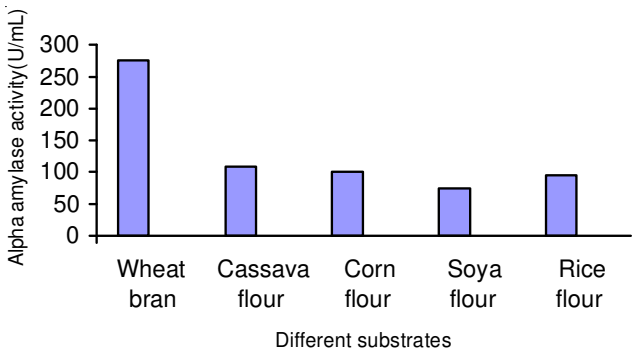

Fig. 2. Effect of substrates on amylase production

Effect of pH on amylase production: Production of α amylase was observed at various pH values ranging from 4-10. The maximum production of α amylase was 250 U/mL at pH 10 after 48 h of incubation (Fig. 3). Most of the *Bacillus* strain used commercially for the production of α amylases have an optimum pH between 6-10 for growth and enzyme production[16].

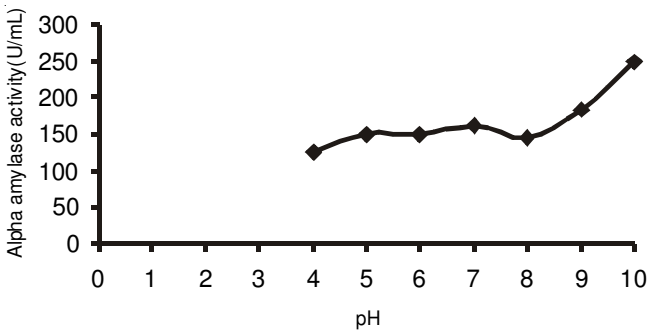

Fig. 3. Effect of pH on amylase production

Effect of temperature on amylase production: Production of α amylase was observed at different temperature ranging from 30-100 ºC. The maximum α amylase production was 300 U/mL at 50 ºC (Fig. 4).

Stability of enzyme in crude extract

Effect of temperature on amylase activity: Effect of temperature on amylase activity was measured by incubating the reaction mixture from 30-100 ºC. Maximum activity was showed at 90 ºC (95 %), respectively and it was shown in the Fig. 5. Most of the *Bacillus* species had showed the stable activity after 2 h of incubation at 90 ºC[17].

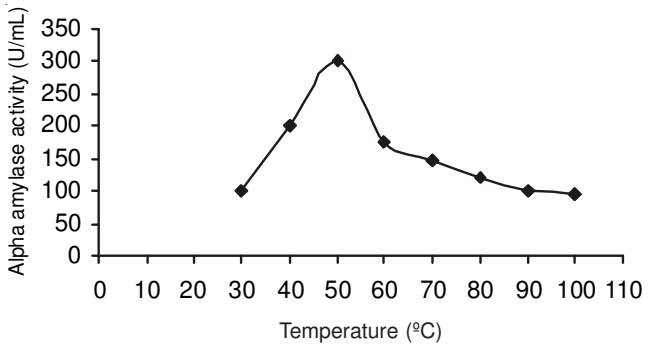

Fig. 4. Effect of temperature on amylase production

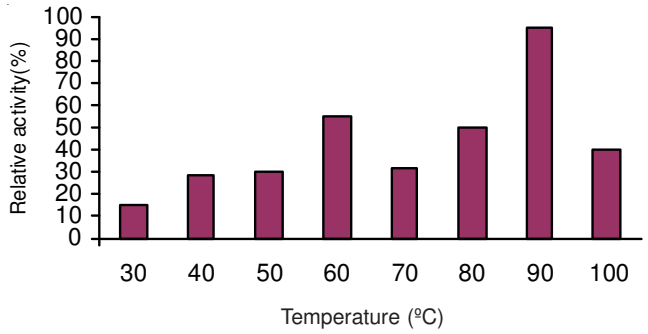

Fig. 5. Effect of temperature on enzyme activity

Effect of pH on enzyme activity: The enzyme activity of the amylase produced by *Bacillus* strain A_4 was determined after incubating the crude extract for 20 min at 90 ºC at different pH values (4.0-10). Maximum activity was observed at pH 9.0 (95 %). It was showed that the enzyme was 90 % stable at pH 7.0 and 80 % stable at pH 8.0 and 10 (Fig. 6). Amylase activity from novel strain of *Bacillus licheniformis* at alkaline pH (8.0-10) were studied[18].

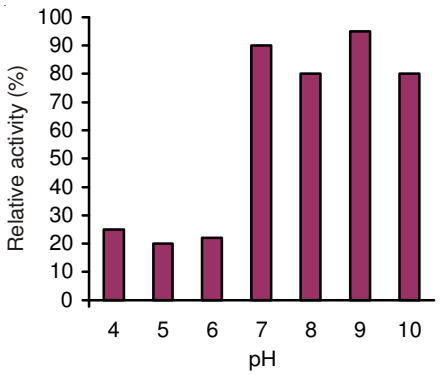

Fig. 6. Effect of pH on enzyme activity

Effect of metal ions on enzyme activity: Different metal ions were used to study their effect on amylase activity. Na^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} and Fe^{2+} were used in 20 mmol concentration. Enzyme reaction mixture was incubated with each metal ion and it was found that Mn^{2+} (120 %) and Fe^{2+} (100 %) showed highest amylase activity while others metal show the activity of Na (80 %), Ca (60 %) and Co (40 %).

Effect of inhibitors on enzyme activity: Different chelating agents are used to analyze the effect of inhibitors on enzyme activity. EDTA and SDS were used to find out best inhibitor of amylase. EDTA (120 %) and SDS (100 %) were found to inhibit amylase activity.

Conclusion

The results obtained in the present study indicated that *Bacillus* strain A_4 as a potential strain for thermostable α-amylase production by using wheat bran as best substrate. Interesting observation was that it showed and retained 95 % enzyme activity at 90 ºC compared to the optimum temperature at 50 ºC. Furthermore, the enzyme was found to show optimum activity under alkaline condition (pH 9.0). This makes that α-amylase of the organism (*Bacillus* strain A_4) useful for industrial applications like starch liquefaction at increased temperature.

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