ISSN: 2278-778X
Research Article
www.ijbio.com

PRODUCTION, PURIFICATION AND ENZYME KINETICS OF AMYLASE FROM BACTERIA ISOLATED FROM A SOIL OF SOLAPUR, MAHARASHTRA STATE, INDIA

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Received for publication: November 17, 2013; **Accepted:** January 05, 2014.

Abstract: Amylase is the enzyme which is used for various purposes. So our aim was to isolate the amylase producing *Bacillus subtilis* spp. from soil. In present work we isolated various *Bacillus* spp. from soil and studied the physical parameters like pH, temperature, and substrate concentration of amylase enzyme. We got four *Bacillus* isolates from soil, which produce amylase. One isolate was selected which shows larger zone of starch hydrolysis. Amylase production was studied by using iodine solution and partial characterization of *Bacillus* was carried out by studying the morphological characterization. As amylase is extra cellular enzyme, it can easily extracted by centrifugation and purification was carried out by salt precipitation and dialysis technique. The purified amylase enzyme activity was determined and also the different parameters of enzyme such as pH, temperature and substrate concentration were studied, the activity of amylase enzyme isolated from "A" is 480 µl/ml of enzyme/min. It's shown maximum activity at pH 6.5 and shown maximum activity at 40°C. Also conclude that the rate of reaction increases with increasing substrate concentration up to 0.19 in which the rate of reaction remains constant. The point of saturation is obtained at 0.19 optical density, hence the value of Vmax and Km value from graph is Vmax = 0.19 O.D, Km = 9.5 O.D (concentration- 0.7mg).

Keywords: Amylase, *Bacillus subtilis* Spp., Soil Source, Physical and Chemical Characteristic

INTRODUCTION

Amylases are enzymes that break down starch or glycogen. The amylases can be derived from several sources such as plants, animals, several fungi, yeasts, bacteria and actinomycetes. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics [2, 7 & 10], however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [11 &12]. Enzymes are biological catalysts, which enhances the rate of reactions at ambient temperature, pH and appropriate substrate concentration. Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries [6 & 8]. The microbial amylases meet industrial demands; a large number of them are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [1 & 9].

The purpose of this work was to study the production of amylase by *Bacillus spp.*, isolated from soil and optimized the cultural conditions for the production of amylase. Since this natural isolate produced low concentration of amylase, attempts were made to increase the productivity by optimizing the cultural conditions.

MATERIALS AND METHOD

Microorganism: *B. subtilis* obtained from soil of solapur, Maharastra and maintained on nutrient agar (Himedia) slants, at pH 7 and 35°C temperature was used in the present investigation.

Inoculum preparation: The spores of *B. subtilis* were transferred aseptically to a 500 mL conical flask containing 100 mL of pre-sterilized inoculum medium containing: glucose- 2g/100mL, yeast extract- 0.3g/100mL, peptone- 0.5g/100mL, NaCl- 1.5g/100mL, $Na_2HPO4._2H2O$- 1.1g/100mL, $NaH_2PO_4.2H_2O$- 0.61g/100mL, KCl- 0.3g/100mL, and $MgSO_4.7H_2O$- 0.01g/100mL in laminar air flow. The flask was then kept on shaker (120 rpm) at 37°C for 24 h. The homogenous spore suspension (10^6-10^7 spores/mL) was used as inoculum.

Microorganism and Growth media: Prepare serial dilution of soil sample of Solapur region, 10^1 to 10^7 from these dilutions 10^3 to 10^6 dilutions are spread on Tendler`s Nonsynthetic Medium (TNS) media with starch- 0.1 gm/ml. Then incubate the plates at 37° C for 48 hrs. After incubation, the colonies surrounded with zone of hydrolysis were observed. From these four isolates, we select one isolate which show maximum zone of hydrolysis. Then we picked and made a pure culture and prepared the suspension in 1% saline solution. Then we studied the morphological characters and gram staining.

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Gram's staining method: Prepared a smear on clean glass slide of selected *B. subtilis* suspension, heats fix the smear and then add crystal violet, wait for one minute. Wash with tap water and then add Grams iodine, wait for one min. Wash the slide with tap water. Then wash the slide with 95% alcohol by drop wise addition, Wash with tap water, air dry it and observe under oil immersion lens (100X).

Motility of test: Take the drop of suspension at center of cover slip and place a wax at four corners of cover slip and inverted the cover slip on cavity slide in such a way that the drop of suspension should touch to the cavity slide and observed the slide at 45X and adjust the edge of that drop and observed motility.

Extraction of amylase enzyme: The TNS broth (For 50ml: Tryptone-0.5gm, Yeast extract- 0.1gm, Sodium citrate- 0.05gm; Ammonium nitrate- 0.05gm, K_2HPO_4-0.015gm, $MgSO_4$-025gm; Starch- 5gm, pH-7.2,) prepared and add the suspension of isolate (1ml) in broth and conical flask is placed on shaker for growth of *Bacillus Spp.* After 48 hrs centrifuge the broth in cooling centrifuge at 6000 rpm for 10min. As amylase is an exoenzyme, it comes in supernatant and this supernatant is used as crude enzyme.

Ammonium salt precipitation: The Supernatant collected after centrifugation was measured using a graduated cylinder. Salting out with 30% salt cut-off was carried out under ice cold conditions with continuous stirring. After that, the enzyme subjected to salt precipitation was incubated at 4°C overnight. After overnight incubation, the precipitated enzyme was centrifuged at 8,000 rpm for 10min. at 4°C. The pellet was collected and dissolved in phosphate buffer, which was then subjected to dialysis.

Dialysis: Dialysis is a common laboratory technique, and operates on the same principle as medical dialysis. Typically a solution of several types of molecules is placed into a semi permeable dialysis bag, such as a cellulose membrane with pores, and the bag is sealed. The sealed dialysis bag is placed in a container of a different solution, or pure water. Molecules small enough to pass through the tubing (often water, salt and other small molecules) tend to move into or out of the dialysis bag, in the direction of decreasing concentration. Larger molecules (often proteins, DNA, or polysaccharides) that have dimensions significantly greater than the pore diameter are retained inside the dialysis bag. One common reason for using this technique would be to remove the salt from a protein solution.

Dialysis bag of about 8cm was warm in 100ml of distilled water for 10min to open the pores of dialysis membrane. Then it was warm in 100ml of sodium bicarbonate solution for 10min for to remove glycerol coated on the dialysis membrane. The bag was again warm in 100ml of distilled water for 10 min for to remove residual sodium bicarbonate. Now the mouth of the dialysis bag was gently rubbed to open it. One end of dialysis membrane was tied with thread and the sample i.e. precipitated protein dissolved in phosphate buffer was placed inside the dialysis bag. After addition of sample, the other end of the membrane was tied with thread tightly. The dialysis bag was then suspended in a beaker containing distilled water (or) phosphate buffer-NaCl solution. This setup was kept in refrigerator overnight.

Study of Amylase Enzyme Kinetics

a) ***Effect of pH:*** Taken well labeled six test tubes with 2.5ml of phosphate buffer then added 1 ml of NaCl and 2.5ml of starch solution in each test tube after that add 1ml of distilled water in each test tube and 0.5ml of enzyme extracted in all test tubes except blank and incubated all tubes at room temperature for 10min. After incubation, added 0.5ml of 1N NaOH in each tube and 0.5ml of DNSA is added in each tube. Kept all the tubes in boiling water bath for 5min. allow all the tubes to cool. After that measure the color by using colorimeter at 540nm wavelength.

b) ***Effect of Temperature:*** we take eleven test tubes, Added 2.5ml of phosphate buffer to each tube. Then add 1ml of NaCl and 2.5ml of starch solution in each tube. Then added 3.5ml of distill water in blank tube and 1ml in other tube i.e. from 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C and 100°C. Incubated all test tubes at respective temperature for 5min. then add 0.5ml of enzyme in each test tube except blank and again incubate all the test tube at respective temperature. After incubation add 0.5ml of 2N NaOH in each tube and 0.5ml of DNSA in each tube. Kept all the test tube in boiling water bath for 5min.Then allow all the tubes to cool at room temperature and measure color obtained by using colorimeter at 540nm wavelength.

c) ***Effect of substrate concentration:*** We take thirteen test tubes and mark them, blank, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, and 2.4. Add 2.5ml of phosphate buffer of pH 6.5 in each tube. Add 1ml of 1 N NaCl in each tube. After that add starch solution in each tube except blank as follows. Add distill water in each test tube to adjust its volume to 10ml. Then add 0.5ml of enzyme in each test tube and mixed well. Incubate all the tubes at room temperature for 10min. Then add 0.5ml of 2 N NaOH in each tube. Add 0.5ml DNSA in each tube. Then keep all the tubes in boiling water bath

for about 5min. Measure color on colorimeter at wavelength of 540 nm.

RESULT AND DISCUSSION

Bacillus spp. Isolate from soil habitat

We got four *Bacillus* isolates from soil, which produce amylase. From them one isolate was selected which shows 40 mm zone of starch hydrolysis. Amylase production was studied by using iodine solution. The partial characterization of *Bacillus* was carried out by studying the gram nature and colony characterization and biochemical tests and found that the isolate was positive rods and motile (Fig.1 and Table.1, 2). Then the amylase production was carried out by inoculating isolated *Bacillus* in TNS broth and incubating at 37°C for 48 hrs on shaker. Amylase in an extra cellular enzyme, it can easily extracted by centrifugation. The purification was carried out by salt precipitation and dialysis technique. In submerged fermentation the production of amylase was reached maximum of 4 U/ml at 10 hours of incubation period. Further increase in incubation period did not show any significant increase in enzyme production rather it was decreased. Thus optimum time of enzyme synthesis was to be 10 hours after inoculation.

Figure 1. Isolated colonies of *bacillus spp.* on TNS medium from soil

Table 1: Colony characterization of isolated bacillus spp.

Colour	Margin	Elevation	Opacity	Shape	Matility	Gram's Nature	Consistency
Creamy White	Regular	Convex	Opaque	Round	Motile	Gram Positive Small rods	Sticky

Effect of Temperature

Results from Fig.3 shows the effect of different incubation temperature on the production of amylase by *Bacillus spp.*, the maximum production of amylase was obtained at 40°C. The optimum temperature was observed for the production of amylase from *B. subtilis* was also 40°C as reported by Krishna and Chandrasekaran, (1996). Increase or decrease in incubation temperature, decreased the production of enzyme. The production of the enzyme was greatly inhibited at 30°C or 50°C. It might be due the effect of temperature, the growth of the bacteria was greatly inhibited and hence, enzyme formation was also prohibited as reported by Lonsane and Ramesh (1990).

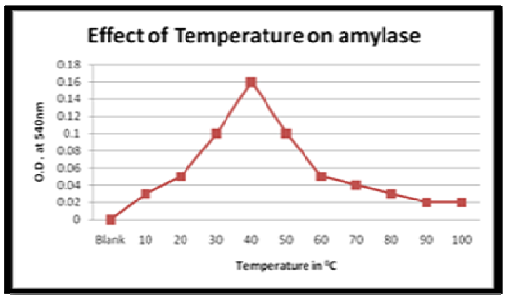

Figure 3. Effect of varying temperature of the medium on amylase production

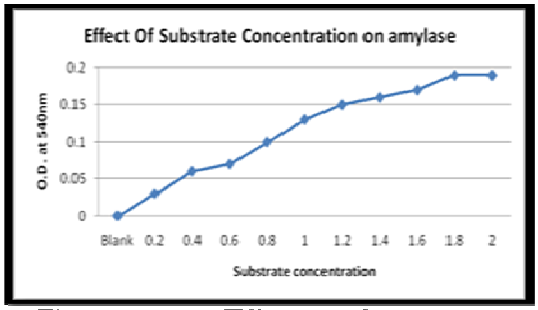

Figure 4. Effect of varying substrate concentration on amylase production

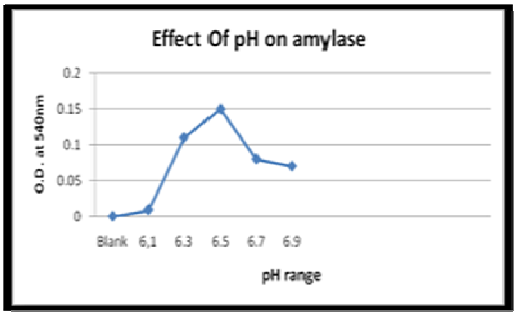

Figure 2. Effect of varying pH of the medium on amylase production

Table 2: Biochemical Test

	Test	Enzyme Control	Substrate Control
Starch	1 ml	1 ml	0 ml
Phosphate Buffer	1 ml	1.5 ml	2.0 ml
Enzyme	0.5 ml	0 ml	0.5 ml
Keep the test tube in boilling water bath for 2min. For inactivation.			
D/W	2.0 ml	2.0 ml	2.0 ml
DNSA	2.5 ml	2.5 ml	2.5 ml
Keep the test tube in boilling water bath for 2min.			
O. D. at 540nm	**0.14**	**0.11**	**0.09**

Effect of pH

In our study the amylase production by *Bacillus spp.,* was found maximum at 6.5 (15U/ml) (Fig.2). Further increase in the pH resulted decrease in the activity of amylase. Terui (1973) reported that 6.8 as optimum pH for the production of amylase by *B. subtilis.*

Effect of substrate concentration

The rate of reaction increases with increasing substrate concentration up to 0.19. Above which the rate of reaction remains constant i.e. increasing concentration does not affect reaction rate. The point of saturation is obtained at 0.19 optical densities. Hence, the value of Vmax and Km value from graph is Vmax = 0.19 O.D and Km = 9.5 O.D (concentration-0.7mg) in (Figure 4).

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Source of support: Nil
Conflict of interest: None Declared