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

Sunita Bansod*, Deepak Thigale¹, Priyanka Dure² and Harshada Sohoni²
¹Department of Biotechnology, Rajarshi Shahu Mahavidyalaya, Latur, India
²V.G Shivdare College of Arts Commerce and Science, Solapur, India

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 µ/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, Na₂HPO₄·H₂O- 1.1g/100mL, NaH₂PO₄·2H₂O- 0.61g/100mL, KCl- 0.3g/100mL, and MgSO₄·7H₂O-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 (10⁶-10⁷ spores/mL) was used as inoculum.

Microorganism and Growth media: Prepare serial dilution of soil sample of Solapur region, 10¹ to 10⁸ from these dilutions 10³ to 10⁸ 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.

*Corresponding Author: Dr. Sunita Dashrath Bansod, Assistant professor, Biotechnology Department, Rajarshi Shahu Mahavidyalaya, Latur, INDIA.
After overnight incubation, the precipitated enzyme which was then subjected to dialysis. The supernatant was centrifuged at 8,000 rpm for 10min. at 4°C. The to salt precipitation was incubated at 4°C overnight. Graduated cylinder. Salting out with 30% salt cut-off continuous stirring. After that, the enzyme subjected was carried out under ice cold conditions with concentration. Larger molecules (often proteins, DNA, 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 DNA 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 each 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 5 min. 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.

Table 1: Colony characterization of isolated bacillus spp.

<table>
<thead>
<tr>
<th>Colour</th>
<th>Margin</th>
<th>Elevation</th>
<th>Opacity</th>
<th>Shape</th>
<th>Motility</th>
<th>Gram’s Nature</th>
<th>Consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creamy White</td>
<td>Regular</td>
<td>Convex</td>
<td>Opaque</td>
<td>Round</td>
<td>Motile</td>
<td>Gram Positive</td>
<td>Sticky</td>
</tr>
</tbody>
</table>

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).

![Image of isolated colonies of bacillus spp. on TNS medium from soil](image)

![Image of Effect of varying temperature of the medium on amylase production](image)

![Image of Effect of varying substrate concentration on amylase production](image)

Table 2: Biochemical Test

<table>
<thead>
<tr>
<th>Test</th>
<th>Enzyme Control</th>
<th>Substrate Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>1 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>1 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>D/W</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>DNSA</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

Keep the test tube in boiling water bath for 2 min. For inactivation.

O. D. at 540nm

<table>
<thead>
<tr>
<th>Test</th>
<th>O. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Control</td>
<td>0.14</td>
</tr>
<tr>
<td>Substrate Control</td>
<td>0.11</td>
</tr>
<tr>
<td>D/W</td>
<td>0.09</td>
</tr>
</tbody>
</table>
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).

REFERENCE


Source of support: Nil
Conflict of interest: None Declared