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Characterization and optimal production of alkaline α-amylase from *Bacillus* sp. DLB 9

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The present study is concerned with the characterization and production of alkaline α-amylase from newly isolated *Bacillus* sp. DLB 9. Maximum enzyme synthesis occurred at 2nd day of incubation. Maximum amylase activity was observed at pH 9.0 and 60°C. Urea was found to be most inhibitory for the activity of amylase. Wheat bran was found to be a suitable natural source for maximum production of amylase. Media supplementation with nitrogen and amino acids increased amylase yields. More amylase (36.0 U/ml/min) was produced when organic nitrogen sources were used as compared to inorganic nitrogen sources. Supplementation of medium with valine resulted in the highest production of α-amylase 50.4 U/ml/min.

Key words: Amylase, alkaliphilic, *Bacillus* sp., submerged fermentation.

INTRODUCTION

α- Amylases (1, 4- α- D- glucan glucanohydrolases, E.C. 3.2.1.1) are one of the most important and oldest industrial enzymes. They cleave the α-1, 4-glycosidic linkage in starch with an endo mechanism, that is, in a random fashion within the polysaccharide molecule. Due to retaining catalytic mechanism the released cleavage products have an α-anomeric configuration. α- Amylases stand out as a class of industrial enzymes having approximately 30% of the enzyme market (Van Der Maarel et al., 2002). They can be derived from several sources such as plants, animals and microbes. The major advantage of using microorganisms for production of amylase is economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). A large number of α-amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch (Aiyer, 2005). Although many microorganisms produce this enzyme but the ones most commonly used for the industrial application are *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger*. α- Amylases have potential applications in number of industries such as starch, baking, analytical chemistry, automatic dishwashing detergents, textile desizing, medicine, pulp and paper industry (Pandey et al., 2001).

Irrespective of the wide range of applications one major limitation of the amylases is their ineffectiveness in the detergent industry (Khan and Briscoe, 2011). It has been shown in earlier research that alkaliphilic amylases can be good source for detergent industry. Alkaliphiles are the extremophiles that occupy extreme pH environments (Horikoshi, 1999). The first alkaline amylase of an alkaliphilic *Bacillus* strain was reported by Horikoshi (1971). The main reasons for selecting enzymes from alkaliphiles are their long term stability in detergent products, energy cost saving by lowering the washing temperature, quicker and more reliable product, reduced effluent problem during the process and stability in the presence of detergent additives such as bleach activators, softeners and perfumes. Keeping in mind the recent activities on alkaline α-amylases, the present study was designed to isolate alkaliphilic bacterial species in the soil sample enriched with starchy material under alkaline conditions, to decipher some cheaper substrate for the production of alkaline α-amylases by submerged fermentation and to characterize the extra cellular

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physiological and biochemical tests. The pH was maintained in the mixture by preparing substrate in sap water followed by distilled water. Various soil samples were collected from the industrial areas in and around Ludhiana and Patiala district of Punjab. The protocol for isolation of alkaliphilic microbes was essentially based on established procedures reported in literature by Horikoshi and Akiba (1982) particularly through addition of sterile Na₂CO₃ to autoclaved media to obtain a pH in excess of 9.5 to 10. Media (g/l)

Yeast extract  -  5.0
Peptone  -  5.0
Potato starch  -  10.0
(soluble/raw)
K₂HPO₄  -  1.0
MgSO₄  -  0.20
Agar  -  20.0
pH  -  10.0
Distilled water  -  1000 ml

The test culture was streaked directly on to the starch agar plates and incubated for 48 h at 37°C and flooded with Gram’s iodine solution. A clear area around the line of growth indicated the starch hydrolysis. A negative control was maintained by adding heat denatured crude enzyme sample. Denaturation was done by boiling enzyme extract at 110°C for 20 min and then cooled suddenly in an ice bath for 5 min.

Enzyme production

Amylase production was carried out in submerged fermentation (SmF). One ml of 24 hrs old bacterial culture was transferred in to 50 ml of sterile Horikoshi broth medium and was incubated at 37°C for 48 hrs on a rotary shaker at 150 rpm.

Enzyme recovery

The bacterial culture was centrifuged at 10,000 rpm for 15 minutes at 4°C in a refrigerated centrifuge. The resulting supernant was used as crude enzyme for estimation of α-amylase.

Amylase enzyme assay

The amylase enzyme assay was performed according to the method described by Miller (1959).

Characterization of enzyme

Effect of pH on enzyme activity

The amylase enzyme was incubated at different pH 8.0 to 13.0 for determination of optimum pH. The pH was maintained in the reaction mixture by preparing substrate in 100 mM Na-phosphate buffer (pH 6 to 8), 100 mM Glycine - NaOH buffer (pH 9 to 10) and 100 mM Borax- NaOH buffer (pH 11 to 13) and activity was measured using standard assay conditions.

Effect of temperature on enzyme activity

The enzymatic reaction was carried out at different temperatures ranging from 37 to 90°C. Reaction mixtures were incubated at respective temperatures and enzyme activity was determined to find out the optimum temperature of amylase.

Effect of chemical agents on enzyme activity

The chemical agents used were sodium dodecyl sulfate (SDS), ethylene diamine tetraacetic acid (EDTA), urea and acrylamide. All the chemical agents were added in the incubation mixture at 1mM concentration and enzyme assay was carried out.

Production of amylase on pre-treated starch waste

Amylase production using natural starch was studied under submerged fermentation (SmF). Various natural starch products like wheat bran and rice flakes were used as substrate for effective amylase production. These materials were obtained from local market, were washed first with tap water followed by distilled water to remove the adhered surface dust particles. Then bleaching operation was carried out by immersing them in hot water (75 to 80°C) for 20 min followed by oven drying at 45°C. The dried material was ground in a mixer grinder and sterilized at 121°C, 15 psi for 15 min and stored at 4°C before further use. The amount of enzyme produced from different substrates was estimated using standard assay.

Amylase enzyme optimization

Effect of substrate concentrations on amylase production

Out of the two substrates mentioned above, the potent producer of amylase was taken at concentration 1, 2, 3, 4 and 5% (w/v) to evaluate the optimum amylase production by bacterial cultures. Starch substrate of desired concentration was added in culture medium and after incubation period amylase assay was performed.

Effect of nitrogen sources on amylase production

Different organic (yeast extract, peptone and tryptone) and inorganic (ammonium sulphate, potassium nitrate and sodium nitrate) nitrogen sources at a concentration of 0.5% (w/v) were used. The optimum nitrogen source was found by analyzing the results of amylase production.

Effect of amino acids on amylase production

To observe the effect of different amino acids on enzyme production, amino acids viz. valine, glycine, tryptophan, asparagine and arginine were taken. 0.2% (w/v) each of amino acid was added to the growth medium. After incubation period the enzyme assay was carried out.

RESULTS AND DISCUSSION

The morphological, physiological and biochemical tests performed on the isolate DLB9 resulted into identification on the basis of Bergey’s Manual of Determinative Bacteriology. The characteristics of the isolate confirm
Table 1. Amylase enzyme assay of Bacillus sp. DLB 9.

<table>
<thead>
<tr>
<th>Bacterial sp</th>
<th>No of days</th>
<th>*Enzyme production (U/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp. DLB-9</td>
<td>2</td>
<td>10.0 ± 0.283</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.2 ± 0.141</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8.6 ± 0.141</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.0 ± 0.283</td>
</tr>
</tbody>
</table>

*Values are mean ± standard deviation (SD). Culture conditions: pH-10.0; Temperature - 37°C.

Figure 1. Amylase enzyme assay of Bacillus sp. DLB 9.

the Bacillus sp. (Table 1). The DLB9 strain produced appreciable amount of amylase enzyme on 2nd day of incubation and thereby slightly decreased on 4, 6 and 8th day of incubation (Table 2 and Figure 1).

The Table 3 and Figure 2 illustrates that the enzyme was active at alkaline pH of 8.0 to 13.0, with optimum pH 9.0 (12.2 U/ml/min) indicating broad pH range for enzyme activity. Maximum amylase activity from Bacillus sp. GM8901 with optimum pH 11.0 to 12.0 was reported by Kim et al. (1995). Similarly, Yang et al. (2011) reported that the recombinant alkaline α-amylase from Bacillus alcalophilus in Bacillus subtilis was stable at pH from 7.0 to 11.0 with an optimum pH of 9.5.

Temperature is a vital biochemical factor which controls the enzymatic activities. Optimum temperature was found to be 60°C showing enzymatic activity of 16.2 U/ml/min thereby indicating its thermophilic nature (Table 4 and Figure 3). Aygan et al. (2008) reported that enzyme obtained from Bacillus sp. AB68 was active in a broad temperature range, between 20 and 90°C, with an optimum of 50°C.

The chemical agents viz. SDS, EDTA, acrylamide and urea were used to study their effect on α-amylase activity. The enzyme activity showed a dramatic decrease to 4.2 U/ml/min with urea (Table 5 and Figure 4). Inhibition of the amylase enzyme by EDTA suggests that it is a metalloenzyme as reasoned by Aygan et al. (2008).

Natural sources could serve as economical and readily available raw material for the production of valuable enzymes. In this study wheat bran and rice flakes were used. As presented in (Table 6 and Figure 5) significant enhancement in enzymatic activity was observed when Bacillus sp. DLB-9 was grown on natural starch. The amylase production was 17.2 U/ml/min on wheat bran and 16.8 U/ml/min on rice flakes as compared to 16.2 U/ml/min on purified starch (control). Higher production using wheat bran can be correlated with its starch content (75.6%) as compared to rice flakes (55.6%). In the previous studies also, wheat bran was found to be best substrate for the production of amylase. Amylase production and physico-chemical parameter optimization using wheat bran has been studied under SSF and SmF by Kocher et al. (2003).

The substrate (wheat bran) concentration in the medium was varied from 1 to 5% keeping all other conditions of the medium constant. Table 7 and Figure 6 shows that maximum amylase production (38.4 U/ml/min) occurred at 4% substrate concentration. The data further shows that the enzyme does not behave typically according to Michaelis-Mentens kinetics but more like a substrate-inhibited enzyme as the enzyme production decreased beyond 4%. The kinetics study suggests that this inhibition is due to hydrolase’s behaviour. The water may be considered one of the substrate, increasing
Table 2. Effect of pH on bacterial amylase activity.

<table>
<thead>
<tr>
<th>Bacterial sp.</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
<th>pH 11</th>
<th>pH 12</th>
<th>pH 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Enzyme Activity (U/ml/min)</td>
<td>9.6 ± 0.707</td>
<td>12.2 ± 0.283</td>
<td>10.0 ± 0.848</td>
<td>9.8 ± 0.141</td>
<td>9.4 ± 0.141</td>
<td>9.2 ± 0.283</td>
</tr>
</tbody>
</table>

*Bacillus sp. DLB-9*

*Values are mean ± SD. Culture conditions: pH range 8.0 to 13.0; Temperature 37°C; Incubation period 2nd day.*

![Figure 2. Effect of pH on amylase activity.](image)

Table 3. Effect of temperature on bacterial amylase activity.

<table>
<thead>
<tr>
<th>Bacterial sp.</th>
<th>Temperature (°C)</th>
<th>37</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Enzyme activity (U/ml/min)</td>
<td>12.2 ± 0.424</td>
<td>16.0 ± 0.283</td>
<td>16.2 ± 0.141</td>
<td>9.4 ± 0.566</td>
<td>1.5 ± 0.141</td>
<td>1.1 ± 0.566</td>
<td></td>
</tr>
</tbody>
</table>

*Bacillus sp. DLB-9*

*Values are mean ± SD. Culture conditions: pH 9; Temperature 60°C. Incubation period 2nd day.*

![Figure 3. Effect of temperature on amylase activity.](image)
Table 4. Effect of chemical agents on bacterial amylase activity.

<table>
<thead>
<tr>
<th>Chemical agent (0.2%v/v)</th>
<th>Bacillus sp. DLB-9 *Enzyme activity (U/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.0 ± 0.283</td>
</tr>
<tr>
<td>Urea</td>
<td>4.0 ± 0.565</td>
</tr>
<tr>
<td>EDTA</td>
<td>4.4 ± 0.424</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>4.6 ± 0.563</td>
</tr>
<tr>
<td>SDS</td>
<td>4.2 ± 0.707</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. Culture conditions: pH-9; Temp. 60°C, incubation period- 2nd day.

Figure 4. Effect of chemical agents on amylase activity.

Table 5. Production of bacterial amylase on natural starch substrate.

<table>
<thead>
<tr>
<th>Bacterial sp.</th>
<th>Natural starch substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat bran</td>
</tr>
<tr>
<td>Bacillus sp. DLB-9</td>
<td>17.2 ± 0.424</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. Culture condition: pH-9; Temp. 60°C, incubation period- 2nd day. Substrate – 1% natural starch

Figure 5. Production of amylase on natural starch substrate.
Table 6. Effect of substrate at different concentrations on bacterial amylase production.

<table>
<thead>
<tr>
<th>Substrate (wheat bran) concentration (%)</th>
<th>*Enzyme production (U/ml/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.2 ± 0.141</td>
</tr>
<tr>
<td>2</td>
<td>30.0 ± 0.566</td>
</tr>
<tr>
<td>3</td>
<td>31.6 ± 1.414</td>
</tr>
<tr>
<td>4</td>
<td>38.4 ± 0.141</td>
</tr>
<tr>
<td>5</td>
<td>36.8 ± 0.566</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. Culture conditions: pH-9; Temp. 60°C, incubation period- 2nd day. Substrate: 1 to 5.

Table 7. Effect of different nitrogen sources on bacterial amylase production.

<table>
<thead>
<tr>
<th>Nitrogen sources (0.5% w/v)</th>
<th>*Enzyme production (U/ml/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.4 ± 0.141</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>32.8 ± 0.424</td>
</tr>
<tr>
<td>Peptone</td>
<td>36.0 ± 1.131</td>
</tr>
<tr>
<td>Tryptone</td>
<td>34.4 ± 0.282</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>26.4 ± 1.131</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>24.8 ± 0.282</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>29.6 ± 0.282</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. Culture conditions: pH-9; Temp. 60°C, incubation period- 2nd day.

substrate concentration leads to diminished water concentration due to the encroachment of the acceptor site by polysaccharide at the expense of water as reasoned by Vasant (2010). Various organic (yeast extract, peptone and tryptone) and inorganic (ammonium sulphate, potassium nitrate and sodium nitrate) nitrogen sources were evaluated using an optimum substrate concentration of 4%. It was observed that organic nitrogen sources gave comparatively higher yields than inorganic nitrogen sources (Table 8 and Figure 7). Maximum production was obtained in control flasks where a combination of yeast extract and peptone was used. As reported by Tiwari et al. (2007) addition of peptone shortens the lag period and improves the α-amylase synthesis.

Effect of different amino acids viz. glycine, valine, tryptophan, asparagines and arginine at concentration of 0.2% (w/v) was studied on the production of amylase. It
was observed (Table 9 and Figure 8) that supplementation of medium with valine resulted in highest production of α-amylase with (50.4 U/ml/min) followed by glycine, tryptophan, arginine and asparagine. As reported by Ikura and Horikoshi (1999) β-Alanine, DL-nor valine and D-methionine were effective for the production of alkaline amylase by Bacillus sp. A-40-2. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion. α-Amylase production by Bacillus amyloliquifaciens ATCC23350 increased by supplementation of medium with valine resulted in
highest production of α-amylase with (50.4 U/ml/min) followed by glycine, tryptophan, arginine and asparagine. As reported by Ikura and Horikoshi (1999) β-Alanine, DL-nor valine and D-methionine were effective for the production of alkaline amylase by Bacillus sp. A-40-2. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion. α-Amylase production by Bacillus amyloliquefaciens ATCC23350 increased by factor of 300 in the presence of glycine (Zhang et al., 1983).

Conclusion

The Bacillus sp. DLB 9 produced appreciable amount of amylase enzyme on 2nd day of incubation. The α-amylase was active at alkaline pH of 8.0 to 13.0, with optimum pH 9.0. Optimum temperature was 60°C. In the presence of urea alkaline α-amylase showed a dramatic decrease in its activity.

Under the optimal conditions (natural starch 4%, combination of yeast extract and peptone 0.5% (w/v), Valine 0.2% (w/v), the yield of alkaline α-amylase reached 50.4 U/ml/min.

REFERENCES


