Production and characterization of thermostable α-amylase from a newly isolated strain of Bacillus subtilis KIBGE-HAR

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Abstract
Investigation on the fermentation conditions for Alpha-amylase (1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) production was carried out with Bacillus subtilis KIBGE-HAR and a optimal synthetic medium for enzyme production was developed. Alpha-amylase production and cell population reached maximum after 24 hours of cultivation. The optimum temperature and pH for enzyme production were found to be 50°C and 7.0 respectively. Starch (15 g/l), which was used as a carbon source, supported the maximum production of enzyme. Peptone was used as a nitrogen source and the best concentration of peptone for α-amylase formation was found to be 5 g/l. High α-amylase titre was obtained in medium supplemented with 1.0 g/l yeast extract. Calcium chloride was added in the medium as a stabilizer and 0.2 mg/dl CaCl₂ was found to be the most favorable concentration for α-amylase production and stability. Among different reference media tested for α-amylase production, medium 3 gave the maximum yield of enzyme and our own optimized medium 4 was proved to be optimal for α-amylase production in contrast with the reference media. The optimum temperature and pH of the enzyme were found to be 60°C and 7.0 respectively. The most suitable buffer system for pH maintenance was proved to be Tris-HCl buffer (50 mM). Velocity of reaction reached to maximum in the presence of 2 % substrate i.e. starch.

Gene Bank Accession: EU819144 (16S rRNA gene sequencing for Bacillus subtilis KIBGE-HAR)

INTRODUCTION
The α-amylase (E.C. 3.2.1.1) randomly hydrolyzes alpha 1,4 glucosidic linkages in starch, glycogen and related polysaccharides yielding dextrins, oligosaccharides, maltose and D-glucose (Takeshita et al., 1975). Bacterial α-amylases are extensively important in industrial processes such as production of ethanol and high fructose corn syrup, baking, in laundry washing powders and dish washing detergents, textile desizing, and paper recycling (Nigam and Singh, 1995).

Thermostable enzymes are more versatile rather than thermolabile (Fogarth et al., 1974) as they have higher operational stability and a longer shelf life at elevated temperatures (Niehaus et al., 1999). Therefore the thermophilic microorganisms are of special interest for producing thermostable α-amylase, which can be use in a wide array of industrial processes (Chandra et al., 1980; McMohan et al., 1997). Bacteria belonging to the genus Bacillus have been widely used for the commercial production of thermostable α-amylase. These include B. coagulans, B. stearothermophilus, B. caldolyticus, B. brevis, B. acidocaldarius and B. thermoamyloliquefaciens (Campbell, 1954, 1955).

Due to the increasing demand for thermostable α-amylase in various industries, it has been produced and characterized from different sources. The characteristics of α-amylase, such as its thermostability and pH profile should match its application.

In this regard it is essential to work on the conditions that lead to the bulk production of thermostable amylase for industrial applications and to search for α-amylase with improved properties. Therefore, the present study was carried out to optimize the fermentation conditions for the production of α-amylase from Bacillus subtilis KIBGE-HAR and to characterize the enzyme produced in the culture supernatant as well as comparison of different reference media with that of our own optimized medium for the production of α-amylase.

MATERIALS AND METHODS
ORGANISM
The strain was isolated from air and pure culture study was
performed. A pure culture of Bacillus subtilis KIBGE-HAR was used and stock culture was maintained on nutrient agar slant and for alpha amylase production the strain was weekly revived in 5 ml starch broth.

MEDIA COMPOSITION

For β-amylase production, a culture medium was prepared containing (g/l): 15.0 soluble starch, 1.0 Yeast extract, 5.0 Bacto Peptone, 0.5 MgSO$_4$, 0.5 NaCl and 0.002 CaCl$_2$. The pH of the medium was adjusted to 7.0 before sterilization (Aliya et al. 2007).

PRODUCTION OF β-AMYLASE

Starch broth (45 ml) was inoculated with 5 ml of an overnight culture of B. subtilis KIBGE-HAR and then incubated at 50 °C for 24 hours. After 24 hours, fermented broth was centrifuged at 10,000 r.p.m for 10 minutes at 0°C. The cell free filtrate (C.F.F.) was used for enzyme assay.

ENZYME ASSAY AND PROTEIN DETERMINATION

The saccharolytic activity of β-amylase in the culture filtrate was assayed by incubating 0.1 ml CFF with 1 ml soluble starch (2 % w/v, prepared in 50 mM Tris-HCl buffer of pH 7.0) at 60±C for 5 minutes. The β-amylase level was determined by measuring the reducing sugar released from soluble starch (Nelson, 1944; Somogyi, 1945; Somogyi, 1952).

“An enzyme unit is defined as the amount of β-amylase that liberates 1 µmol of reducing sugar from the substrate in one minute at 60±C”.

Total Protein was measured by the Lowry et al. method (1951). Bovine serum albumin (250 µg/ml) was used as a standard.

PARAMETRIC ANALYSIS FOR β-AMYLASE PRODUCTION

The Bacillus subtilis KIBGE-HAR was grown in the medium for 6, 18, 24, 48, 72, and 96 hours for the time course of maximum cellular growth and β-amylase production. Wet cell mass (g/dl) was measured after each interval of time.

The carbon source used was starch and different concentrations of starch (5 – 30 g/l) were tested to get the best one for β-amylase production.

The effects of temperature and pH of the medium were also studied. This was carried out by growing the organism at different temperatures (37 °C – 60 °C) and different pH values (5 – 10).

Various concentrations of nitrogen source i.e. peptone (0 – 20 g/l) were analyzed for the maximum production of β-amylase.

The effects of different levels of Yeast extract (0 – 5 g/l) and CaCl2 (0.001 – 0.01 g/l) were also investigated.

COMPARISON OF DIFFERENT MEDIA COMPOSITIONS FOR β-AMYLASE PRODUCTION FROM BACILLUS SUBTILIS KIBGE-HAR

Four different fermentation media were used for β-amylase production from Bacillus subtilis KIBGE-HAR in order to compare the level of β-amylase produced in them with that of our own optimized medium (Table 1).

Figure 1

Table 1: Media compositions for β-amylase production from KIBGE-HAR

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Media 1</th>
<th>Media 2</th>
<th>Media 3</th>
<th>Media 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>2.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.50</td>
</tr>
<tr>
<td>Peptone</td>
<td>-</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.10</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

(Initial pH values of the media were adjusted to 7.0 before sterilization)

CHARACTERIZATION OF β-AMYLASE

Effect of substrate concentration on enzyme activity was measured at different concentrations of starch in the reaction mixture (0.25% - 2.5 %)

The temperature optimum was evaluated by performing the enzyme assay at different temperatures ranging from 35 °C to 75 °C.

Effect of pH on the activity of β-amylase was determined by measuring the activity at different pH values ranging from pH 5.5 to 9.0.

For the selection of appropriate buffer to get maximum enzyme activity, different buffer systems (50 mM each) including Citrate-Phosphate buffer, Phosphate buffer and
Tris-HCl buffer of pH 7.00 were used.

**RESULTS**

**TIME COURSE OF CELLULAR GROWTH AND α-AMYLASE PRODUCTION**

A linear relationship was found between enzyme synthesis and cell growth i.e. maximum α-amylase production occurred when the cell mass reached to maximum (Fig. 1).

**Figure 2**

Figure 1: Time course for α-amylase production and wet cell mass from KIBGE-HAR

**EFFECT OF STARCH CONCENTRATION ON α-AMYLASE PRODUCTION AND EXTRACELLULAR ACTIVITY**

Figure-2 showed that medium containing 1.5 g% starch supported the maximum production of α-amylase and increasing starch concentration beyond 1.5 g% resulted in the declined enzyme production.

**Figure 3**

Figure 2: Effect of starch concentration on α-amylase production from KIBGE-HAR

**EFFECT OF TEMPERATURE ON α-AMYLASE PRODUCTION AND EXTRACELLULAR ACTIVITY**

It was found that enzyme production in the fermentation medium increased with increase in temperature and maximum enzyme production was obtained at 50°C and after, enzyme production was sharply decreased at 55°C. At 50°C the maximum bacterial cells multiplication occurred and during their multiplication they secreted extracellular enzyme (Fig. 4).

**Figure 5**

Figure 4: Effect of temperature on α-amylase production during fermentation from KIBGE-HAR

The optimum temperature for maximum extracellular α-amylase activity was found to be 60°C. Further increase in temperature resulted in reduce enzyme activity (Fig. 5).
**Figure 6**
Figure 5: Effect of temperature on extracellular \(\beta\)-amylase activity

**EFFECT OF PH ON \(\beta\)-AMYLASE PRODUCTION AND EXTRA CELLULAR ACTIVITY**

Maximum enzyme production was observed when pH of the medium was kept 7.0 before sterilization (Fig. 6), while at pH 5 and pH 9, 68% and 30% production was observed respectively, with reference to the optimum pH.

**Figure 7**
Figure 6: Effect of initial medium pH on the production of \(\beta\)-amylase from KIBGE-HAR

The effect of pH on \(\beta\)-amylase activity is shown in Fig-7. Enzyme showed maximum extracellular \(\beta\)-amylase activity at neutral pH and as the pH increases the enzyme lost its activity.

**Figure 8**
Figure 7: Effect of pH on the extracellular \(\beta\)-amylase activity

**SELECTION OF BUFFER FOR \(\beta\)-AMYLASE ACTIVITY**

Among different buffers tested, Tris-HCl buffer (50 mM) provided the most suitable buffering environment in which \(\beta\)-amylase activity was maximum (Fig. 8).

**Figure 9**
Figure 8: Effect of different buffers (pH 7.00) on extracellular \(\beta\)-amylase activity

**EFFECT OF PEPTONE CONCENTRATION ON \(\beta\)-AMYLASE PRODUCTION**

The effect of peptone on \(\beta\)-amylase production by Bacillus subtilis KIBGE-HAR showed that enzyme production was effected by the change in concentration of peptone in the medium (Fig. 9). Maximum \(\beta\)-amylase production was observed when 0.5 g% peptone was added in the medium.

**Figure 10**
Figure 9: Effect of Peptone concentration in medium on \(\beta\)-amylase production from KIBGE-HAR

**EFFECT OF YEAST CONCENTRATION ON \(\beta\)-AMYLASE PRODUCTION**

It was observed that medium containing 0.1 g% yeast extract supported optimal \(\beta\)-amylase production (Fig. 10) followed by a decline at high concentrations.
Production and characterization of thermostable \( \beta \)-amylase from a newly isolated strain of Bacillus subtilis KIBGE-HAR

Figure 10: Effect of yeast concentration in medium on \( \beta \)-amylase production from KIBGE-HAR

**EFFECT OF CA\(^{+2} \) ON \( \beta \)-AMYLASE PRODUCTION**

Figure 11 showed that when 0.2 mg/dl CaCl\(_2\) was added in the fermentation medium, resulted in the enhancement of \( \beta \)-amylase production.

Figure 11: Effect of CaCl concentration in medium on \( \beta \)-amylase production from KIBGE-HAR

**COMPARISON OF DIFFERENT MEDIA FOR \( \beta \)-AMYLASE PRODUCTION FROM KIBGE-HAR**

It has been observed that the strain of B. subtilis KIBGE-HAR produced 631 U/ml/min in medium-3 while 844 U/ml/min in medium-4, which was our own synthetic medium developed for optimal enzyme production. Medium-2 showed the least \( \beta \)-amylase yield as compare to others.

**DISCUSSION**

Cellular growth of B. subtilis KIBGE-HAR and \( \beta \)-amylase production was affected by the time of incubation. Growth dependent \( \beta \)-amylase production was found by this strain i.e., a linear relationship was found between enzyme synthesis and cell growth. It was observed that maximum \( \beta \)-amylase production occurred when the cell mass reached to its maximum. This suggests that this organism may be sensitive to metabolite repression (Cordeiro et al., 2002). It was also known that optimum temperature for growth and \( \beta \)-amylase production was same. Other investigators also reported the same as above (Bajpai and Bajpai, 1989; Saito and Yamamoto, 1975).

Different carbon sources affect differently on the production of \( \beta \)-amylase (Welker and Campbell, 1963) and the most commonly used carbon source is starch (Bajpai and Bajpai, 1989). Starch at concentration of 15 g/l supported maximum production of enzyme followed by a decline at high concentrations. This may be the effect of catabolic repression i.e. glucose which was formed during the hydrolysis of starch may influenced negatively on \( \beta \)-amylase gene expression. These results are similar to the findings of Haseltine et al. (1996) who observed that glucose repressed the production of amylase by the hyperthermophilic Archaeon Sulfolobus solfataricus.

Starch was also used as a substrate for \( \beta \)-amylase activity. Optimum activity was achieved at 2 % starch, and as the substrate concentration increased, \( \beta \)-amylase activity dropped sharply which may be due to the substrate inhibitory effect.

Fermentation temperature has a profound effect on the level of enzyme produced in the medium. In the present study, \( \beta \)-amylase production by B. subtilis KIBGE-HAR was found to be maximum at 50 °C where after it declined. Amylase production by many Bacillus sp. is known to be affected by the amount of dissolved oxygen in the medium (Wind et al., 1994). At elevated temperature, the solubility of oxygen is decreased in the medium (Campbell and Pace, 1968) leading to limitation of dissolved oxygen for the production of \( \beta \)-amylase, as it has been reported that difference in the level of oxygen in the medium induced changes in the surface protein layer (S-layer) of bacterial cell membrane (Sara et al., 1996) which is involved in the control of extracellular release of enzyme. This may be the reason of decreased amylase production beyond 50 °C.

Temperature is also the most important factor which markedly influences the enzyme activity. In the present study, optimum temperature for \( \beta \)-amylase activity was 60 °C. Further increase resulted in reduced enzyme activity as high temperatures induce conformational changes in three dimensional structure of an enzyme and its accommodation to substrate molecules becomes lower.

Medium pH also affects \( \beta \)-amylase production. Composition of cell wall and plasma membrane of microorganisms is
known to be affected by the medium pH (Ellwood and Tempest, 1972). Due to this change in the nature of cell wall and plasma membrane the growth parameters may vary, especially temperature, and the provided temperature may not remain suitable for the growth of organism (Stutzenberger and Jenkins, 1995). This may be the reason of decreased production of α-amylase at pH 5 and 9 in the present study.

Optimum pH for α-amylase activity was found to be 7.0. The enzyme activity at pH 6.0 and 7.5 were 65 % and 77 % of that at pH 7.0, respectively. At pH 8.0, enzyme activity reduced to 46 %. Thus, we can conclude that α-amylase from Bacillus subtilis KIBGE-HAR is active at or near neutrality.

The nature and concentration of nitrogen source are important for the formation of α-amylase. Lower level of nitrogen is inadequate for the enzyme production and excess nitrogen is equally detrimental causing enzyme inhibition (Dharani Aiyer P. V., 2004).

As a nitrogen source peptone was added for α-amylase production. Similar finding was also reported previously (Davies et al, 1980).

The concentration of yeast extract was found to be crucial for the production of α-amylase and as the concentration increased after reaching maxima in medium a sharp fall of enzyme production was observed. The reason may be that the high concentration of yeast decreased the pH of the medium during fermentation which ultimately destroys the enzyme produced in the medium (Babu and Satyanarayana, 1993). Another reason could be that both yeast extract and peptone affect the surface charge, hydrophobicity and nitrogen-to-carbon ratio of the bacterial cell wall (Schar-Zammaretti Prisca et al., 2005) and the hydrophobicity of the cell wall decreases as the concentration of yeast extract and peptone increase in the medium. This decreased hydrophobicity of bacterial cell wall may result in decreased extracellular release of enzyme.

Calcium ions are known to be the stabilizer and activator of α-amylase and it has been reported that requirement of Ca+2 is different for thermostable α-amylase as compared to thermolabile. In case of thermolabile α-amylase, Mamo and Gassesse (1999) reported that 0.1 g/l CaCl₂ was optimum for amylase production by Bacillus sp. WN 11 whereas Qader et al. (2006) reported that in case of Bacillus sp. AS-1, 0.2 g/l CaCl₂ was optimum for the maximum production of α-amylase. In the present study, thermostable strain required low quantity of 0.002 g/l CaCl₂ for optimum production of α-amylase. Similarly Welker and Campbell (1963) also worked on thermostable α-amylase and used 0.005 g/l CaCl₂ in the fermentation medium. This suggests that thermostable α-amylase has a low requirement for calcium ions as compared to thermolabile or we can say that thermostable α-amylase possesses high affinity for calcium ions (Kindle Karen et al., 1986).

Different media have been compared with that of our own optimized medium i.e. medium 4. Medium 2 reduced markedly the enzyme production which may be due to the greater amount of yeast extract present and the strain did not utilized this extra yeast extract in the fermentation medium and due to this high concentration of yeast extract inhibition started (Babu and Satyanarayana, 1993). Medium 1 contained tryptone as a nitrogen supplement and showed lower enzyme production as compare to medium 3 and 4 which did not contain tryptone but peptone. It has been reported that maximum enzyme production was found with peptone as the nitrogen source (Lin et al., 1998, Dharani Aiyer, 2004) and tryptone resulted in decreased enzyme production. This may be the reason why medium 1 did not produced α-amylase optimally.

CONCLUSION

In the present study, we developed the new medium composition for the maximum production of α-amylase from Bacillus subtilis KIBGE-HAR. Enzyme characterization was performed which indicates that enzyme is thermostable and showed optimum activity at 60°C. This alpha amylase maintains its activity upto high temperature and can easily be used for industrial purpose. Enzyme also showed 20% and 40% loss of activity at 65°C and 70°C respectively.

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References


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