Standardization of optimum conditions for cyclodextrin glycosyltransferase production

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Abstract. The cyclodextrin glucosyltransferase producing bacteria were isolated from soil. The efficient strain was isolated and identified as *Bacillus licheniformis*. Fermentation conditions for CGTase production by *B. licheniformis* were standardized. Maximum CGTase production was obtained at 37°C at pH 8. CGTase production was significantly high when the medium was supplemented with soluble starch. Glucose and cassava supplementation in the media resulted in low level of CGTase production. Yeast extract was found as the best nitrogen source. The enzyme production was inhibited by Mg²⁺, Ca²⁺, Mn²⁺, Fe³⁺ and Zn²⁺.

Key words: CGTase, B. licheniformis, optimum conditions, metal ions

1. Introduction

The enzyme cyclodextrin glycosyl transferase (CGTase E.C 2.4.1.19) is a bacterial enzyme which converts starch into cyclic maltooligosaccharides known as cyclodextrins which are non-reducing, cyclic oligosaccharides composed of D-glucose units linked by α-1,4 glycosidic bonds. CGTase can synthesis all forms of cyclodextrins which are cyclomaltohexaose (α-CD), cyclomaltoheptaose (β-CD) and cyclomaltooctaose (γ-CD) consisting of six, seven and eight glucose molecules respectively (Illias et al., 2003). Among the three types of CDs, β-CD is more suitable for practical use. The main difference among them is the apolar cavity size and solubility in water (Allegre et al., 1994). Due to their apolar cavity they are able to form complexes with molecules and change the physico-chemical properties such as solubility and stability of guest compounds. Cyclodextrins have the ability to form inclusion complexes with organic and inorganic compounds, which have numerous applications in agricultural, food, cosmetics, chemical and pharmaceutical industries (Pszczola, 1998; Szejtli, 2004).

In food industry, cyclodextrins are used for the production of low-cholesterol butter, where the cyclodextrin is used to specifically remove the cholesterol from the milk fat. They are utilized for flavour stabilization and delivery in chewing gum, flavored tea, cinnamon flavored apples, lemon and grapes fruit candies, mints and lemon flavored sugar (Pszczola, 1998). Cyclodextrin is used in fruit juice beverages to mask vitamin odor and to mask bitterness in alone containing beverages. It is used to convert acetic acid to powder. It also increases the solubility of mustard sauce. The main objective of this work was to isolate CGTase producing *Bacillus* sp. from soil and standardization of its production conditions.

2. Materials and methods

2.1 Isolation of CGTase producing organisms

1 g of soil sample was suspended in 50 ml of sterile water and plated on a medium containing soluble starch 2%, peptone 0.5%, yeast extract 0.5%, K₂HPO₄ 0.1%, MgSO₄.7H₂O 0.02%, Na₂CO₃ 1%, phenolphthalein 0.03%,
methyl orange 0.01% and agar 2% and incubated at 37°C. CGTase positive colonies were identified by a clearing zone around them in the phenolphthalein containing media. Colony with a higher zone diameter was selected as an efficient strain and used for further studies.

2.2 CGTase production

The inoculum prepared using the isolated culture was inoculated into production medium containing 1.5% soluble starch, 0.4% yeast extract, 0.15g of KH₂PO₄, MgSO₄.7H₂O, FeSO₄.7H₂O, 0.12g of 20% KCl, and 0.9% NaCl and incubated at 37°C. Samples were withdrawn at specific intervals of time and analyzed for CGTase activity.

2.3 Enzyme assay (Goel and Nene, 1995)

To measure the enzyme activity cell free culture medium was centrifuged at 10000 rpm for 20 minutes at room temperature and supernatant containing cyclodextrin glycosyltransferase was collected. 5ml of the supernatant and 5ml of 1% starch solution (0.1g of soluble starch, 1ml of 0.05mM CaCl₂ and pure water for a total volume of 10 ml) were mixed in a thermostatic reactor at 50°C. Samples were taken periodically from the reactors and inactivated in water at 100°C for 5 minutes. The concentration of cyclodextrin was measured by the addition of 2.5ml of 3mM phenolphthalein solution (5ml of 0.6mM Na₂CO₃ buffer, pH 10.5 and the volume completed with 2.5ml of distilled water in a volumetric flask) to 0.5ml of inactivated samples. The absorbance of the final solution was analyzed in spectrophotometer at 550nm. A unit of enzymatic activity was defined as the quantity of enzyme that produces one µmol of cyclodextrin per minute under standard conditions.

2.4 Standardization of optimum conditions for CGTase production

Factors like carbon, nitrogen sources, microelements, temperature and pH affecting the production of CGTase were optimized by adopting the search technique by varying one factor at a time. The experiments were conducted in 500ml Erlenmeyer flasks. All the experiments were carried out in three sets with a control.

The optimum temperature and pH for the CGTase production were standardized. Different carbon sources like cassava, starch, maltose and glucose were tried to increase the enzyme production. Different nitrogen sources such as ammonium salts, tryptone, urea and yeast extract was supplemented in the production media to analyze their influence on enzyme production. Effect of cation concentration on CGTase production was carried out by the addition of CaSO₄, MgSO₄, MnSO₄, FeSO₄ and ZnSO₄ to the production media.

2.5 Statistical analysis

The mean values and standard deviations were calculated from the data obtained from three different experiments. Analysis of variance was performed by one way ANOVA procedures followed by Tukey HSD Post Hoc tests using SPSS 11.5. Statistical differences at p < 0.05 were considered to be significant.

3. Results and discussion

Ten CGTase positive bacteria (BS 1-BS 10) were isolated from soil sample (Fig. 1). Among them one bacteria showing highest activity (BS 7) was selected and identified as Bacillus licheniformis.

![Figure 1. CGTase activity exhibited by different bacteria isolated from soil sample](image)

At 37°C the CGTase activity was significantly higher than 47 & 27°C during 16 to 36 h of incubation. At 47°C the CGTase activity was significantly less than 37 & 27°C. pH 8 showed maximum CGTase activity at
24h of incubation. But the difference in CGTase activity among the different pH ranges is insignificant (Fig. 2&3). The CGTase activity of Bacillus sp G1 was found to be optimal at pH 6 (Illias et al., 2003). Larsen et al., (1998) observed that the pH optima of the enzyme were 7.5 for the cyclisation activity and 8.0 for the hydrolysis activity. The temperature optima for the cyclisation and hydrolysis activity were 50°C and 60°C respectively. Single optimum pH range for CGtase activity suggests a different degree of ionization of the enzyme catalytic site in order to produce different types of cyclodextrins. A broad range of temperature between 45°C and 70°C was already reported (Salva et al., 1997; Yim et al., 1997; Higuti et al., 2003).

![Figure 2. Effect of different temperature ranges on CGTase activity](image)

Significantly high CGTase activity was observed at 32 h of incubation when soluble starch was supplemented as a carbon source. But with maltose significantly less activity was obtained (Fig. 4). Although cassava supplementation produces a high growth rate for B. licheniformis, it induces low CGTase activity. The supplemented starch in the medium serves as an inducer for CGTase production. Soluble starch was found as a best carbon source for B. firmus and B. macerans (Posci et al., 1998). Glucose supplementation has given only low yields of CGTase production. Varavinit et al., (1998) reported that the production of CGTase by Bacillus sp. MP 523 was repressed significantly by glucose. However studies carried out by Stefanova et al., (1999) showed that 0.5% (w/v) glucose was found to be the most suitable substrate for CGTase production.

![Figure 3. Effect of different pH ranges on CGTase activity](image)
Among the four different nitrogen sources tested yeast extract influenced significantly the productivity of CGTase than urea, tryptone and ammonium salt (Fig. 5). Maximum growth of *B. licheniformis* was also observed in yeast extract containing media. CGTase production was higher when an organic nitrogen source was present in the medium. Enzyme production using inorganic nitrogen sources was found to be low when compared with organic nitrogen source. It was reported that the best nitrogen source for the production of CGTase was peptone (Illias *et al.*, 2002). But Mahat *et al* (2004) observed that sago starch and yeast extract have a significant effect on CGTase production.

The effect of cations on the production of CGTase was studied by substituting different metal ions in the media (Fig. 6). All metal ions inhibited the activity of CGTase. Mg$^{2+}$ showed moderate inhibition but Fe$^{2+}$ and Zn$^{2+}$ were the strong inhibitors of CGTase activity (Table 1). This is in accordance with Higuti *et al.*, (2003). But it was also reported that Mg$^{2+}$ and Ca$^{2+}$ showed little activation on CGTase activity (Freitas *et al*., 2004). The effect of metal ions on enzyme activity seems to depend on the enzyme source (Higuti *et al*., 2003).
Table 1: Effect of metal ions on the activity of CGTase

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Relative activity (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>55.3</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>75.5</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>60.2</td>
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<tr>
<td>Fe$^{2+}$</td>
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<td>Zn$^{2+}$</td>
<td>40.4</td>
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4. References


