Production and purification of CGTase of alkalophilic Bacillus isolated from Marneri pond in Tirunelveli District, Tamil Nadu

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Abstract

Cyclodextrin glucosyltransferase (CGTase) producing alkalophilic bacteria were isolated from the water samples collected from Marneri pond of Tirunelveli, Tamil Nadu by serial dilution and plating method. Totally 22 bacteria were isolated from the collected water samples and screened for CGTase activity by Horikoshis medium II agar plate method. Among 22 bacterial isolates, 15 isolates showed CGTase activity and better zone producing strain was selected for further studies. The potential strain was identified as Bacillus circulans by 16S rDNA gene sequencing experiment. The best enzyme activity was observed at pH 10.5, 32°C, supplemented with cassava as carbon source and beef extract as nitrogen source. CGTase was purified to 20.21 fold with a yield of 55.14% recording a single protein band in SDS-PAGE.

Keywords: CGTase, alkalophilic bacteria, Horikoshis medium, Bacillus circulans, cassava, beef extract.

Introduction

Biotechnology and bioengineering is the phenomenon of molecular complexation which is useful in selecting, separation and solubilization of various bio-molecules. Cyclodextrins (CDs) are advantageous molecular complexation agents (Singh et al., 2002). CDs are cyclic oligosaccharides consisting of six (a-CD), seven (b-CD), eight (g-CD) or more gluco-pyranosyl units linked by α-(1,4) bonds. They are also known as cycloamylose, cyclomaltoase and Scharnderg dextrins (Del Valle, 2004). They are produced by reacting liquefied starch with the enzyme cyclodextrin glycosyltransferase (CGTase).

Usually, a mixture of CDs is formed and their concentration ratios depend on the enzyme source. The CGTase is usually a monomeric enzyme with a molecular weight of the order of 74,500 Da that presents a sequence of amino acids which reveals a structural similarity to the enzyme a-amylase.

The enzyme CGTase (EC 2.4.1.19), is responsible for the conversion of starch and related sugars into non-reducing cyclic oligosaccharides, cyclodextrins (CDs) comprising D-glucose residues linked by α-1,4 glucosidic bonds (Schmid, 1989). The most common are α-, β- and γ-CD containing 6, 7 and 8 glucosyl residues respectively. CDs are doughnut shaped molecules with a hydrophilic outer surface and a relatively hydrophobic cavity. With such structural features these molecules and their derivatives can accommodate various organic molecules to form inclusion complexes.

This property has been used for stabilizing and solubilizing of various substances of interest to pharmaceutical, cosmetic and food industry as well as in bioconversions and separation processes (Starnes, 1990). Another industrial CGTase application in addition to the production of CDs by means of the cyclization reaction is the steviolide glycosylation to reduce bitterness, improve sweetness and enhance water solubility (Jung, 2007). CGTase can also be utilized in the preparation of baked products, in which the incorporation of the enzyme into the dough increases the volume and delays the process of bread staling during storage (Jemli et al., 2007). Yet, because of the inability of CGTase to bypass α-(1,6) bonds in gelatinized starches, degradation of these substrates leads to a reduction in viscosity without a corresponding decrease of the high-molecular character of starch. Thus, for surface sizing or coating of paper, the action of CGTase improves the writing quality of the paper, resulting in a glossy surface that is good for printing (Van der Veen et al., 2000).

However, over 100 years after their discovery, CDs are still regarded as having a number of possible applications. The aim of the present study was to produce, purify, characterize and investigate the environmental parameters which affect CGTase production in the synthetic medium.
Materials and methods

Sample collection and isolation of alkaline CGTase producing bacteria

Pond water samples were collected in a sterile container from Manneri pond near Tirunelveli, Tamil Nadu. The samples were serially diluted and pure cultures were maintained in nutrient agar slants. The pure cultures were screened for CGTase production using Horikoshi medium II plates containing soluble starch; 2% peptone; 0.5%, yeast extract; 0.5%, K2HPO4; 0.2%, MgSO4·7H2O; 0.02%, Na2CO3; 1%, phenolphthalein; 0.03%, methyl orange; 0.01% and agar; 2% (37°C, pH 10.5). Alkaline CGTase positive colonies were identified by a clearing zone around them in the phenolphthalein containing alkaline media. Colony with a higher zone diameter was selected as an efficient strain and used for further studies (Park et al., 1989).

16S rDNA sequencing

For the sequence analysis, bacterial genomic DNA was extracted and purified using CTAB method. Two primers annealing at the 5’ and 3’ end of the 16S rDNA were 5’-GAGTTTGATCCTGGCTCAG-3’ and 5’-AGAAAGGAGG TGATCCAGCC-3’. PCR amplification was performed in a final reaction volume of 100 µL. The PCR reaction was run for 35 cycles in a DNA thermal cycler. The amplified PCR products were then analyzed in a 1% (w/v) agarose gel, excised from the gel and purified. The amplified DNA sequence was then sequenced in Chromous biotech, Hyderabad using automated sequencer. The 16S rDNA gene sequence of the isolates was aligned with reference 16S rDNA sequences of the GenBank using the BLAST algorithm (Altuschul et al., 1997; Neethu Shah et al., 2010) available in NCBI (National Centre for Biotechnology information).

CGTase production medium

Selected strain was cultivated in 100 ml of modified culture medium consisting of 1% starch, 0.5% peptone, 0.5% yeast extract, 0.1% K2HPO4, 0.02% MgSO4·7H2O and 1% Na2CO3 and incubated at 37°C for 24 to 48 h with an agitation at 100 rpm. Samples were drawn during various time intervals and the cell-free supernatant was used as the source for the enzyme analysis.

CGTase activity

The CGTase activity was measured according to the method of Kaneko et al. (1998) with some modifications. The reaction mixture containing 1 ml of 0.04 g starch in 0.1 M phosphate buffer (pH 6) and the mixture was incubated at 60°C for 10 min in a water bath. The reaction was stopped by adding 3.5 ml of 0.03 M NaOH solution. 0.5 ml of 0.02% (w/v) phenolphthalein in 0.005 M Na2CO3 was added to the reaction mixture. After 15 min, the decrease in colour intensity was measured at 550 nm. The percentage of reduction in the original colour intensity was interpreted with a standard curve (%OD reduction versus β-CD in mg produced) for the calculation of CGTase activity.

One unit of enzyme activity was defined as the amount of enzyme that forms 1 umole of β-CD from soluble starch in 1 min. Protein content was determined by the method of Bradford method (1976) with bovine serum albumin as the standard.

Optimization of CGTase production

To optimize the culture conditions for maximum production of CGTase, different parameters such as incubation periods (0-72 h), medium pH (9, 9.5, 10, 10.5, 11 and 11.5) and temperature (28°C, 32°C, 37°C, 42°C, 47°C and 52°C) were used. The influence of various carbon and nitrogen sources for the maximum production of CGTase production was studied. The carbon sources (Corn starch, Wheat starch, Rice starch, Potato starch and Cassava) in the production medium were replaced with starch and the nitrogen sources (Yeast extract, Beef extract, Urea and Ammonium chloride) were substituted with peptone in the production medium.

Purification of CGTase

The cells were separated from supernatant by centrifugation at 8000 rpm for 10 min at 4°C. CGTase was precipitated by the addition of solid ammonium sulfate (NH4)2SO4 to give a 70% saturation. The mixture was stirred slowly and gently in order to obtain a better dissolution rate of ammonium sulfate and promoting the salting out effect. The mixture was set to stand overnight at 4°C to enhance the precipitation and stabilization of the enzyme. The resulting precipitate was separated from the supernatant by centrifugation at 3400×g for 20 min at 4°C. The precipitate was re-suspended in 800 ml of 0.01 M acetic acid buffer (pH 5.5). The mixture was subjected to subsequent purification procedures. Then, the mixture was spun at 8000 rpm for 10 min at 4°C to remove any remaining insoluble material. CGTase was purified using Sepharose 6B column biospecific-affinity chromatography (Laszlo et al., 1981). Three milliliters of fractions were collected and each one was assayed for CGTase activity and protein content.

Molecular weight determination

The molecular weight of the purified enzyme was determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) on a vertical slab gel using 150 V for 3 h at 25°C. The gel was stained with 1% Coomassie Brilliant Blue R-250.

Results and discussion

Naturally occurring environments like alkaline soils, ponds and lakes were characterized by their extremely alkaline pH between 8 and 11. The alkaline microorganisms present in these alkaline environments were paying much attention for their ability for producing extracellular enzymes that are extremely stable at high pH values (Van den Burg, 2003; Antranikian, 2005). The alkaline cyclodextrin glucosyltransferase production is a thirst field in the industries because of its versatile industrial uses. In this study, the bacterial strains were
isolated from Marneri pond, Tirunelveli, Tamil Nadu. Totally 22 bacteria were isolated from the collected water samples and alkaline CGTase producing bacteria was isolated using Horikoshis medium II containing 0.03% (w/v) phenolphthealin at the pH of 10.5. Fifteen isolates showing halo zones around their margins were obtained. Among the 15, the better zone producing strain was selected and used for further studies (Fig. 1).

Fig. 1. Bacillus circulans in CGTase screening plate.

The isolated bacterium was identified as Bacillus circulans by using 16S rDNA sequencing. The better CGTase producing strain was designed as MKPTK8. The DNA from the strain MKPTK8 was isolated and the 16S rDNA was amplified and sequenced. The BLAST analysis of the strain using its 16S rDNA sequence data showed that strain MKPTK8 had highest homology (100%) with Bacillus circulans. The nucleotide sequence of the isolate was deposited in the GenBank database with accession number GQ468586. 16S rDNA analysis is more advanced and accurate since the difference in properties between the bacterial strains such as B. anthracis, B. cereus and B. thuringiensis, are <1%. Such small differences cannot be analyzed using conventional methods. The study made by Claudio et al. (2002) clearly demonstrates that such small differences also might be important for species identification. DNA hybridization studies have shown that these three Bacillus species are closely related and probably represent a single species (Somerville and Jones, 1972; Seki et al., 1978; Ash et al., 1991). If the three were classified as a single species, 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level. Production of CGTase activity Bacillus circulans was studied under different culture conditions, by varying the medium composition and growth temperature. Incubation time plays a substantial role in the maximum enzyme production. It has been reported that Bacillus circulans showed maximum CGTase production in 42 h.

Results of the present study indicated that the production of CGTase was dependent on the bacterial cell growth (Fig. 2). It is clear from figure 3 that the maximum enzyme production was obtained during continuous growth of the culture at the late exponential phase and early stationary phase of the growth and thereafter number of viable organism decreased due to the depletion of readily available carbon source and other nutrients.

Fig. 2. Effect of incubation time on CGTase production and total protein content.

The growth and enzyme production was studied at different pHs 8–11.5 and maximum CGTase activity was recorded at pH 10.5 by the test isolate. Increased alkalinity beyond 10.5 was not favorable and enzyme production was reduced. Further, the reduction in enzymatic level was more pronounced than that in growth pattern at alkaline pH at 10.5 (Fig. 3).

Fig. 3. Effect of pHs on CGTase production and total protein content.

Temperature is one of the most important factors affecting any enzyme production. The results referred to a positive relationship between CGTase production and incubation temperature up to 32°C, while the activity decreased rapidly above 52°C (Fig. 4). Yeast extract and trace elements were found to be essential components of the culture medium. Cell growth in the absence of either of these components was diminished significantly while enzyme production was nil.
The highest cell growth and enzyme production was obtained during cultivation at 32°C. The effect of carbon sources on CGTase production showed that enzyme production was greatest when cassava was used as the carbon source, followed by rice starch (Fig. 5). Ibrahim et al. (2005) suggested that the production of CGTase is a specific reaction process between the microorganism and the carbon source and *Bacillus circulans* appears to favor starch more than simple sugars. Investigations on the influence of nitrogen sources on CGTase production indicated that CGTase production was higher when beef extract was present in the medium (Fig. 6). In a study on the influence of organic and inorganic sources of nitrogen, the highest production of CGTase was found using organic sources, such as peptone and yeast extract (Ibrahim et al., 2005). According to Mahat et al. (2004) the concentration of yeast extract in the medium is the most important variable for the production of CGTase, presumably because of the presence of some essential nutrients or inductors that stimulate the production of CGTase.

The results for each purification step are presented in Table 1. To purify the crude CGTase to homogeneity, the enzyme purification procedure included two steps: ammonium sulfate precipitation, and Sepharose 6B column biospecific-affinity chromatography. These purification protocols resulted in CGTase purification around 20.21 fold with a yield of 55.14%. The purified enzyme gave a single protein band by SDS-PAGE. The molecular weight of the purified denaturated CGTase by

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>107</td>
<td>47</td>
<td>2.28</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>78</td>
<td>12</td>
<td>6.5</td>
<td>72.89</td>
<td>2.85</td>
</tr>
<tr>
<td>Sepharose 6B</td>
<td>59</td>
<td>1.28</td>
<td>46.09</td>
<td>55.14</td>
<td>20.21</td>
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The effect of temperature on CGTase production and total protein content is shown in Fig. 4. The effect of carbon sources on CGTase production and total protein content is shown in Fig. 5. The effect of nitrogen sources on CGTase production and total protein content is shown in Fig. 6.
SDS-PAGE was estimated to be 83 kDa and this value was quite close to the established molecular weight of 89 kDa of the purified native enzyme by gel filtration on a superdex-200 (Fig. 7). Most of the reported CGTases from bacteria are monomeric with molecular weight between 69 and 80 kDa (Gao et al., 2005; Sian et al., 2005; Atanasova et al., 2011). CGTases with a lower molecular weight between 36 and 59 kDa such as those from *Bacillus lentus* (Sabioni and Park, 1992) and *Bacillus sphaericus* strain 41 (Moriwaki et al., 2009) was also reported.

**Conclusion**

Twenty two bacterial strains were isolated from the collected water samples and alkaline CGTase producing bacteria was isolated using Horikoshis medium. Fifteen isolates showed halo zones around their margins and the better zone producing strain was evaluated for CGTase production. CGTase was purified around 20.21 fold with a yield of 55.14%. Molecular weight of the purified denaturated CGTase by SDS-PAGE was estimated to be 83 kDa.

**References**


