Synthesis of β- Cyclodextrin by Cyclodextrin glycosyl transferase produced by
Bacillus licheniformis MCM –B 1010

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ABSTRACT

Cyclodextrins (CD’s) are closed-ring structures having six or more glucose units joined by means of α-1, 4 glucosidic bonds. They are synthesized enzymatically by Cyclodextrin glycosyl transferases (CGTase; EC 2.4.1.19) that convert starch into cyclodextrins. CD’s have a variety of applications in food, pharmaceutical and cosmetic industry. In this study we have reported enzymatic synthesis of β- CD from CGTase produced by alkaliphilic Bacillus licheniformis isolated from Lonar lake, India.

Keywords: β -cyclodextrin, CGTase, Bacillus licheniformis, alkaliphilic, Lonar lake

INTRODUCTION

Cyclodextrin glycosyl transferase (CGTase; EC 2.4.1.19) is an enzyme that converts starch into cyclodextrins (CD’s) which are closed-ring structures having six or more glucose units joined by means of α-1, 4 glucosidic bonds. CGTase is classified in the α-amylase family and is known to catalyze four different transferase reactions: cyclization, coupling, disproportionation, and hydrolysis (1).Three major types of cyclodextrins are produced by CGTase depending on number of glucose units, α – CD, β- CD and γ- CD (2). Cyclodextrins have a variety of applications in food, pharmaceutical and cosmetic industry.

Cyclodextrins are used as drug carriers and tabletting vehicles. They are used to reduce the bitter or aggravating taste and bad odour of drugs. Cyclodextrins can improve the stability of active pharmaceutical ingredients and increase the shelf life of drugs (3).

They can improve the cord strength of polyester fibres used for reinforcement of rubbers (3). It is observed that there is a distinct increase in the rate of formation of penicillin-G from phenyl acetic acid and 6-aminopenicillanic acid if both substrates used are in a cyclodextrin complexed form. In environmental biotechnology, cyclodextrin is used to remove organic pollutants and heavy metals from soil, water, and atmosphere. Fava et al., (1998) found that cyclodextrin has the potential of being successfully used in the bioremediation of chronically polychlorinated biphenyl-contaminated soils (2). However, its application is drastically limited because of its low yield and excessive price. Cyclodextrins are declared to be “Generally Recognized As Safe” (GRAS) and have no adverse effects on the absorption of certain nutrients (4). It has been proposed that they can be used in all kinds of food and nutraceutical applications as a food ingredient and additive. Cyclodextrin can also stabilize emulsions of fats and oils. This property is useful for the preparation of bread spreads, dairy ice creams and breads (4).
It is evident from the above mentioned applications that CGTase enzyme is industrially a very important enzyme because of its application in production of CD’s. However there are two problems associated with CGTase production. The yield of cyclodextrin is low, thus cost is high and solvents are used to precipitate CD’s are highly toxic (5).

CGTase from alkalophilic Bacillus sp. was the first bacteria that lead to mass production of α – CD, β- CD and γ- CD by overcoming these problems (5). Since then it is known that alkaliphiles are candidate organisms for CGTase production. In this study we have reported the production of cyclodextrin by CGTase produced by alkalophilic Bacillus licheniformis MCM – B 1010 isolated from soda lake of Lonar, India (6).

MATERIALS AND METHODS

Microorganism:
Bacillus licheniformis MCM – B 1010 isolated from Lonar lake, India was employed for production of CD (6). The culture was maintained on nutrient agar pH 10.

Production of CGTase:
B. licheniformis was inoculated in Nutrient medium containing (g/L) soluble potato starch 10, peptone 10, yeast extract 5 and sodium chloride 5 for production of CGTase. The pH was maintained as 10 by addition of sterile 10 % sodium carbonate after autoclaving. The organism was cultivated in 250 ml flasks containing 50 ml medium incubated in an orbital shaker at 150 rpm at 30° C for 24 hours. After 24 h, the broth was centrifuged at 10,000 g at 4°C for 20 min. The cell free supernatant was used as crude enzyme.

Enzyme Assay:
The assay for CGTase was performed using phenolphthalein reagent as described by Goel and Nene (1995) (7). 100 µl of crude enzyme extract was added to one ml of 1 % soluble potato starch in 0.005 M Tris- HCl buffer pH 8.5 and incubated at 60° C for 20 min. After incubation, this reaction mixture was cooled in ice. Four ml of one mM phenolphthalein reagent was added to the tubes and the absorbance measured immediately at 550 nm. The amount of β-CD produced is estimated from standard graph of 50 – 200 µg /ml of standard β-CD (Sigma-Aldrich) against decrease in absorbance. One unit of CGTase activity is defined as amount of enzyme required to produce 1 µg of β-CD /ml/min.

Tilden and Hudson test for detection of CGTase:
Starch substrate was prepared by adding three percent soluble potato starch in Tris-HCl buffer 0.05 M, pH 7.2. 500 µl of appropriately diluted enzyme was incubated with 1 ml of soluble starch and incubated at 60 °C. Three drops of reaction mixture (approx 300 µl) are withdrawn every five minutes and mixed with one drop of iodine reagent and observed under a Light microscope under high power (8).

Detection of Cyclodextrin by HPLC:
The amount of β- Cyclodextrin produced by the CGTase enzyme was estimated by HPLC using Perkin Elmer Amino column (5 µm, 250 X 4.6) was used. Mobile phase was acetonitrile: water (65:35) and detection was by online Perkin Elmer series-200 Refractive Index detector. The reaction mixture consisted of 100 micro liters of enzyme and one ml of one percent starch. The mixture was incubated at 60 °C for 20 min. Five hundred micro liters of this reaction mixture was mixed with equal volumes of HPLC grade Acetonitrile (Merck) and centrifuged at 20,000 g for 15 min at room temperature and then filtered through a 0.45 μ filter (Millipore, USA). Ten µl was injected in the column with a syringe and the pressure was maintained between 2800 to 3070 psi. The standard cyclodextrins were prepared in HPLC grade water. The output was read using a TotalChrom Navigator Software.

Detection of Cyclodextrin by SEM:
For microscopic observation, the cyclodextrin produced was precipitated by addition of complexing agent (5 (w/v) trichloroethylene and toluene) and incubation at 4°C for 2 h. After incubation, the reaction mixture was centrifuged at 5, 000 g for 30 min and the precipitate was dried in a hot air oven at 80°C. The cyclodextrin powder was observed using a JSM-7600F Field Emission Gun Scanning Electron Microscope (FEG-SEM) with a resolution of 1nm and accelerating voltage of 15 kV under high vacuum of ~ 10^-5 Pa.
RESULTS AND DISCUSSION

Production of CGTase:
CGTase was produced in 250 ml shake flask conditions. After 24 h, the broth was centrifuged and assessed for production of CGTase. *Bacillus licheniformis* CGTase had specific activity of 0.5 U/mg protein and enzyme activity of 2.2 U/ml. *Bacillus* sp. have been previously reported for CD and CGTase production by Kaneko *et al.* (1988) (9), Kimura *et al.* (1989) (10), Nakamura *et al.* (1992) (11) and Haga *et al.* (1994) (12). *Bacillus licheniformis* MCM B-1010 was found to produce 140 µg/ml β-CD from soluble starch (Fig. 1).

**Fig. 1:** Production of β-cyclodextrin by *Bacillus* sp. isolated from Lonar lake

The production of CD by *B. licheniformis* was compared with other bacilli viz. *B. flexus* and *B. fusiformis* isolated from Lonar lake (6). It was observed that highest production of β-CD was obtained from *B. licheniformis* in comparison with the other two isolates. *B. licheniformis* isolated from cassava culture soil has been reported by Bonilha *et al.* (2006) (13). The production of CGTase can be enhanced by optimization of medium components and fermentation parameters (14).

Tilden and Hudson test for detection of CGTase:
The enzyme was used for production of CD and it was qualitatively detected by Tilden and Hudson microscopic test. Typical hexagonal and dichoric crystals due to formation of cyclodextrins were observed in reaction mixture (Fig. 2).

**Fig. 2:** Detection of CD’s using microscopic assay a) Starch granules (Control), b) Colorless starch granules with CGTase after 10 min incubation, c) Colorless starch granules with CGTase after 10 min incubation

Starch granules with CGTase after 30 min showing typical dichoric crystals

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<th>Amount of β-CD produced (µg/ml)</th>
<th>Specific activity X 10^-3 (U/mg protein)</th>
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<tr>
<td>Bacillus firmus</td>
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<td>Bacillus fusiformis</td>
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<td>Bacillus licheniformis</td>
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Detection of Cyclodextrin by HPLC:
CGTase activity can be confirmed by isolation and identification of the endproducts, i.e. cyclodextrin. The cyclodextrins synthesized were estimated by HPLC. Standard β cyclodextrin showed a peak at 3.1 min. The mixture of standard cyclodextrins showed distinct peak of β and γ CD at 3.1 and 5 min respectively. The cyclodextrins produced could be detected by HPLC and visualised by scanning electron microscope.

Detection of Cyclodextrin by SEM:
The cyclodextrins produced were visualized using scanning electron microscope (Fig.3).

CONCLUSION
Alkaliphilic Bacillus licheniformis originally isolated from alkaline soda lake of Lonar, India was assessed production of CD and CGTase. The enzyme activity and yield was comparable with other reported organisms. B.licheniformis could produce β- CD from soluble starch using the enzyme cyclodextrin glycosyl transferase. This one step enzymatic synthesis can be optimised further and used for industrial production of cyclodextrins. To the best of our knowledge this is the first report of production of β- CD by alkaliphilic Bacillus licheniformis MCM- B 1010 from Lonar lake India.

REFERENCES

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