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Isolation of Cyclodextrin Producing Thermotolerant *Paenibacillus* sp. from Waste of Starch Factory and Some Properties of the Cyclodextrin Glycosyltransferase

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Abstract

A Cyclodextrin (CDs) producing bacteria was isolated from waste of starch factory in Thailand and identified as *Bacillus circulans* by biochemical characterization and *Paenibacillus* sp. by 16S rRNA. The *Paenibacillus* grew and produced cyclodextrin glycosyltransferase (CGTase) at temperature range 37–45 °C. The optimum culturing conditions for highest CD-forming activity were pH 10.0 and 40 °C for 72 h in Horikoshi broth containing 0.5% soluble starch. The CGTase was partially purified by starch absorption, with 64% recovery and purification fold of 27. The optimum temperatures for dextrinizing and CD-forming activity were 70 and 50–55 °C. At the optimum temperature, the optimum pH for dextrinizing activity was 6.0, while CD-forming activity was 7.0. When the enzyme was incubated for 1 h at different temperatures, CD-forming activity retained its full activity up to 70 °C while dextrinizing activity dropped to 60%. Cyclodextrin products analyzed by HPLC was α:β = 1:1, temperature of reaction mixture can affect the yield of CDs.

Introduction

Cyclodextrins (CDs) the cyclic oligosaccharides of 6–8 glucose units (α,β,γ forms) and their derivatives, possess unique property to form inclusion complexes with varieties of molecules leading to change in physicochemical properties of the guest molecules. Due to the unique properties of host–guest complexes of CDs, they are increasingly applied for product improvement in many industries. Natural occurring CDs are the products obtained for enzymatic conversion of starch by cyclodextrin glycosyltransferase (CGTase), an enzyme produced by bacteria mainly *Bacilli*. Most CGTase producing bacteria are mesophilic and their enzymes mostly function at moderate temperature. Industrial processes involving CDs production and usage usually operate at high temperatures and require energy consuming process for cooling system Therefore, bacteria which can grow at higher temperature range and produce CGTase which also functions at higher temperature would be beneficial for use in industrial CDs production. This study reports the isolation of a thermotolerant *Paenibacillus* sp. from waste of starch factory, which can grow and produce CDs at high temperature.

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Materials and methods

Screening of bacteria with amylolytic activity

Starch hydrolyzing bacteria were screened from soil contaminated with waste from starch factories in various areas in Thailand, using Medium I agar plates containing soluble starch and stained with iodine solution [1]. Isolates exhibiting amylolytic activity with ratio of clear zone to colony size greater than 3 were selected for further screening.

Screening for CD producing activity

Selected isolates from amylolytic activity screening were grown at 37 °C on Horikoshi medium plate containing 0.03% phenolphthalein and 0.01% methyl orange [2, 3] for 3 days. Isolates producing CDs formed yellow haloed zone on red background. They were further confirmed of CGTase and CD's production with cyclodextrin-trichloroethylene (CD-TCE) assay [4].

Identification of CD producing bacterium

The morphology of the bacterium was examined by light and scanning electron microscopes. Biochemical char-

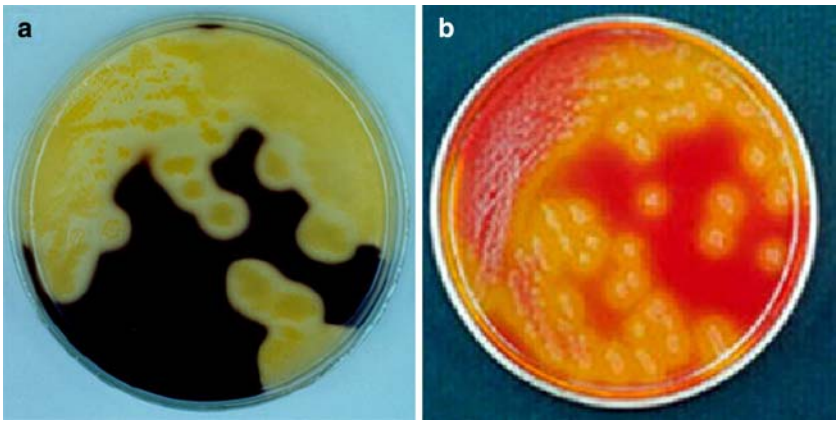

Figure 1. Screening of CD producing bacteria. (a) Dextrinizing activity on solid medium I stained with iodine solution. Light shade = yellowish color of iodine solution. Dark shade = dark brown color of starch-iodine complex. (b) CD-forming activity on solid Horikoshi medium with phenolphthalein (PhN). Light shade = Colorless CD-PhN complex on methyl orange background. Dark shade = methyl orange + PhN background.

acterization of the bacterium was performed based on Bergey's manual of systematic bacteriology using API50CHB system (Bio Mericus). Identification of 16S rRNA was performed by gene fragment amplification according to Edwards *et al*. [5]. 16S rRNA sequence was aligned with reference sequences from GenBank database using Clustal W software (http://www.ncbi.nlm.nih.gov).

Optimization of growth and CD producing conditions

Optimum temperature was determined by culturing the bacterium in Horikoshi broth at temperature range 37–45 °C and determination of growth (A_{420}) and CD forming activity were performed. Optimum pH was then determined by culturing the bacterium in Horikoshi broth at various pHs at the determined optimum temperature for 96 h with monitoring of growth and CGTase production at regular 12-hour intervals. Horikoshi medium contained 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.75% Na_2CO_3 [6].

CGTase assays

CGTase activity was determined by assay of starch degrading (dextrinizing) activity and assay of CD product by formation of CD-trichloroethylene complex (CD-TCE).

Dextrinizing activity assay (Iodine method)

Dextrinizing activity of CGTase was measured by the method of Fuwa [1] with slight modification. Enzyme sample was incubated with 0.3 ml starch substrate (0.2% soluble potato starch in 0.2 M phosphate buffer pH 6.0) at 40 °C for 10 min. The reaction was stopped with 4 ml of 0.2 N HCl and 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and the absorbance at 600 nm was measured. For control tube, HCl was added before the enzyme sample. One unit of enzyme was defined as the amount of enzyme which produced 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition.

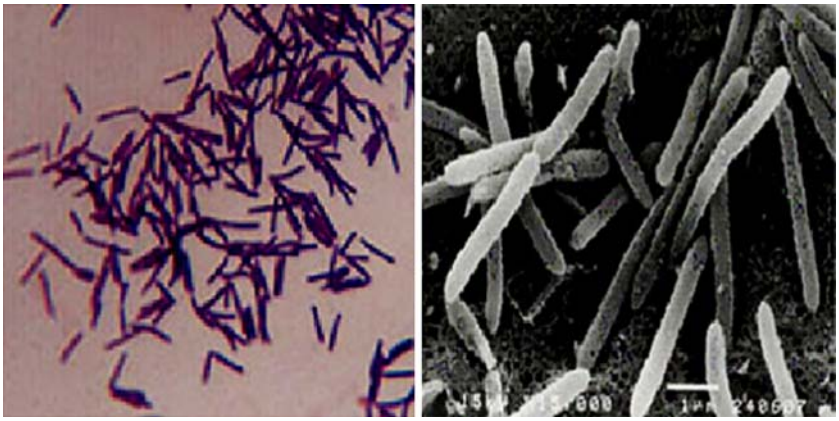

Figure 2. Morphological identification of BT01. (a) Gram stain on light microscope (×100). (b) scanning electron micrograph.

Table 1. Partial purification of CGTase from *Paenibacillus* sp. BT01

Step	Dextrinizing activity (U/ml)	Total activity ($Ux10^3$)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)	CD-TCE ($1:2^n$)
Crude enzyme	18.5	22.2	255.6	86.8	1	100	2^5
Starch adsorption	57.8	14.2	6.0	2366.7	27	64	2^9

Cyclodextrin-Trichloroethylene (CD-TCE) assay

Cyclodextrin-Trichloroethylene (CD-TCE) assay was determined by the method of Nomoto *et al.* [3] with slight modification. Enzyme sample was diluted by serial double dilution with 0.2 M phosphate buffer pH 6.0. The reaction mixture, containing 0.5 ml of enzyme sample and 2.5 ml of starch substrate (0.2% soluble potato starch in 0.2 M phosphate buffer pH 6.0) was incubated at 40 °C for 24 h. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left standing overnight at room temperature in the dark. CD-forming activity was expressed as the dilution limit ($1:2^n$), where n is the highest dilution fold that still produces observable CD-TCE precipitate at the interface between upper starch solution layer and lower TCE layer.

Characterization of CGTase

For biochemical characterization, CGTase produced by the bacterium was partially purified by starch adsorption [7]. Corn starch was oven dried at 100 °C for 30 min and cooled to room temperature. All subsequent steps were conducted at 4 °C. Corn starch was gradually sprinkled onto stirring crude enzyme broth to make 5% concentration for 3 h. The corn starch cake which adsorbed the enzyme was collected by centrifugation at 6,000 rpm for 30 min and washed twice with 200 ml of 10 mM Tris–HCl containing 10 mM $CaCl_2$ pH 8.0 (TB 1). The adsorbed CGTase was eluted from the starch cake by stirring for 30 min with 125 ml of TB1 buffer containing 0.2 M maltose. CGTase was recovered by centrifugation at 6,000 rpm for 30 min. The partial purified enzyme was again checked for enzyme activity and protein content. The enzyme was kept at 4 °C for further study.

Effect of temperature on CGTase activity (in 0.2% soluble starch in 0.2 M phosphate buffer pH 6.0) was performed by incubating the enzyme at various temperatures (0–100 °C) for 10 min and assayed for dextrinizing activity. CD-TCE activity was performed as described in the assay procedure. Optimum pH was

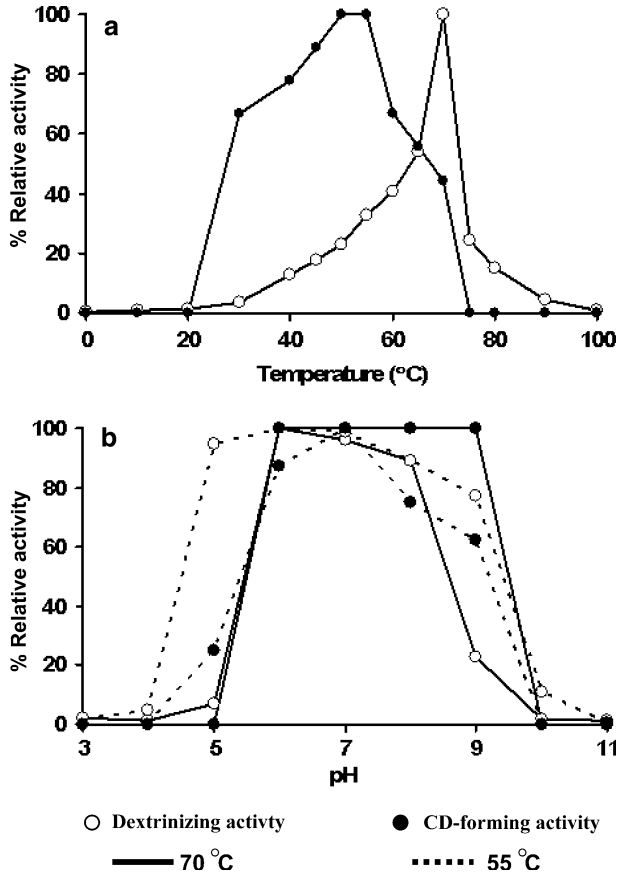

Figure 3. Effect of temperature and pH on CGTase activity. (a) optimum temperature. (b) Optimum pH.

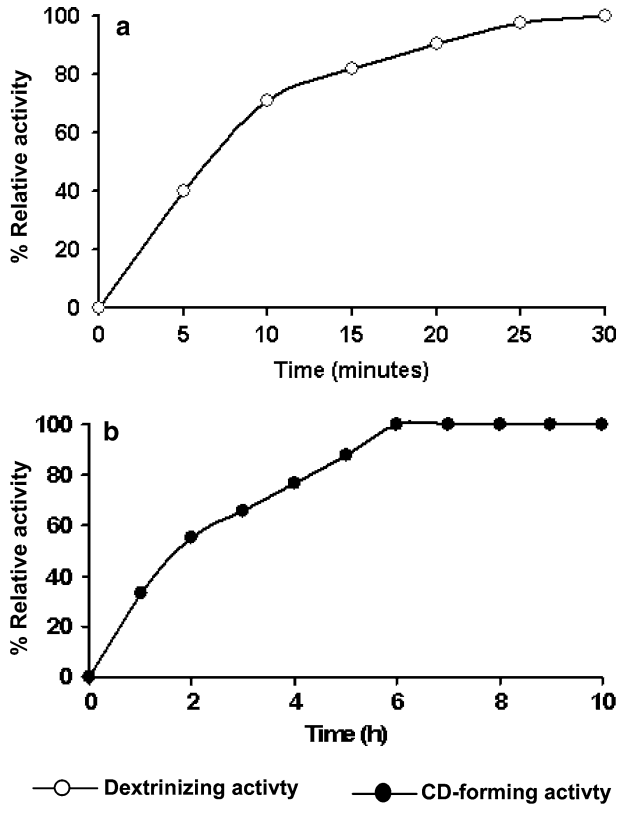

Figure 4. Effect of incubation time on CGTase activity at optimum temperature and pH. (a) Dextrinizing activity. (b) CD-forming activity.

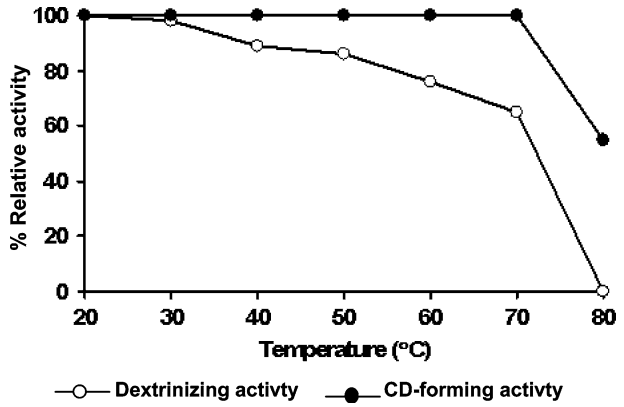

Figure 5. Temperature stability of partially purified CGTase from BT01.

determined by incubating the enzyme at the determined optimum temperature at various pHs (pH 3–11), using universal buffer. Incubating condition was as mentioned in the CGTase assay procedure.

Stability of enzymes

The stability of CGTase was followed by incubating the enzyme at increasing temperatures for 60 min with sample taken at regular intervals. The enzyme activity was assayed at optimal conditions determined from previous experiments.

Identification of cyclodextrins by HPLC

Analysis of cyclodextrins by HPLC was performed with slight modification of the method of Pongsawadi and Yagisama [8]. The HPLC system was a Shimadzu LC-3A equipped with Spherisorb-NH_2 column (0.46 × 25 cm), using Shimadzu RID-3A refractometer as detector. For CD analysis, the reaction was performed by incubating 0.5 ml of enzyme sample with 2.5 ml of starch substrate (0.2% soluble potato starch in 0.2 M phosphate buffer pH 6.0) incubated at 40 °C for 24 h. The reaction was stopped by boiling in water for 5 min. After cooling, the mixture was treated with 20 units of β-amylase at 25 °C for an hour, and the reaction was stopped by heating in boiling water. Prior to injection, the mixture was filtered through a 0.45 μm membrane filter. The mixture was injected and eluted with acetonitrile-water (70:30 v/v) at a flow rate of 1 ml/min. The CD peaks were identified by comparing the retention time with that of standard CDs. For quantitative analysis, peak area corresponded with each cyclodextrin was determined from standard curve.

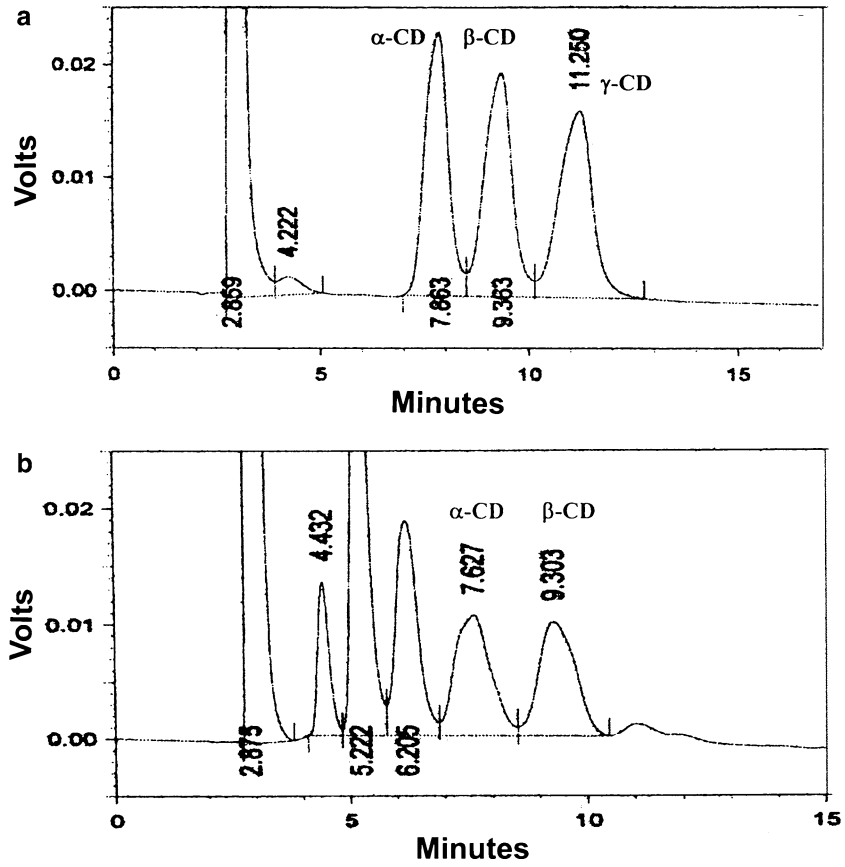

Figure 6. HPLC chromatograms of CDs produced by BT01. (a) standard α,β,γ-CDs. (b) CDs products of BT01.

Table 2. Effect of pH and temperature on CD products of CGTase from BT01

Condition	% Yield			
	α-CD	β-CD	γ-CD	Total CD
(40 °C)				
pH6.0	18.3	17.5	–	35.8
pH7.0	18.2	18.0	0.8	37.5
(pH 7.0)				
Temperature (°C)				
40	18.2	18.0	0.8	37.0
55	20.0	18.2	–	38.2
60	16.8	20.2	0.9	37.9
70	13.8	18.3	1.8	32.9
80	7.4	–	–	7.4

Results and discussions

Isolation and identification of CD producing bacteria

CD producing bacteria were screened from soil samples collected from areas around starch factories by primary screening for amylolytic activity on medium I agar plate containing soluble starch and stained with iodine solution. Positive clones appeared as clear yellowish zone against deep blue background, colonies with ratio of clear zone to colony size greater than 3 (Figure 1a) were further screened for CGTase activity and CD production by inoculation on Horikoshi medium plate containing phenolphthalein. Isolates producing CGTase can convert the starch present in the medium to cyclodextrins. Consequently, β-CD can encapsulate phenolphthalein resulting in yellow zone against red background [2, 8] (Figure 1b). Three clones producing yellow zones were selected and confirmed for cyclodextrin forming activity by CD-TCE assay. Thermotolerant characteristics of the three isolates were studied by monitoring growth and CD forming activity by incubating the bacteria at varying temperatures. One isolate which grew and produced cyclodextrins at 45 °C was named BT01 and further identified. BT01 was found to be rod-shaped gram positive *Bacillus* (Figure 2a and b). Biochemical characterizations based on Bergey's manual of systematic bacteriology identified BT01 as *Bacillus circulans* and 16S rRNA analysis (data not shown) indicated BT 01 belonged to *Paenibacillus* sp., hence named as *Paenibacillus* sp. BT01.

Optimization of BT01 culture conditions

Optimization of culture condition for best CD production of *Paenibacillus* sp. BT01 were performed by growing in Horikoshi broth at 37–45 °C pH 10. BT01 produced highest CD at 40 °C. The bacterium was then grown at 40 °C at varying pHs in the range 6–11. It was found that BT01 grew well at pH 6–10 with highest dextrinizing activity and CD forming activity at pH 10 for 72 h (data not shown). Therefore, BT01 was cultured in Horikoshi medium at 40 °C, pH 10 for 72 h in future study.

Characterization of CGTase and CDs produced by BT01

CGTase from BT01 was partially purified by starch absorption with 64% recovery and 27 folds purification (Table 1). The enzyme showed optimum temperature at 50–55 °C for CD forming activity and dextrinizing activity at 70 °C, respectively (Figure 3). At 55 °C, CD forming activity was highest at pH 7.0. CD-forming activity was maximum at 6 h incubation time at 55 °C and pH 7.0 (Figure 4). When the partially purified CGTase was preincubated at various temperatures for 1 h and assayed for CD-forming activity, its full activity was retained up to 70 °C. On the other hand, dextrinizing activity dropped to 60% at the same temperature (Figure 5).

HPLC analysis of CDs produced by CGTase at the optimum conditions showed only two types of CDs, α and β at 1:1 ratio (Figure 6). Effect of pHs and temperatures on CD production were also analyzed and the results summarized in Table 2. Ratio of α to β- CDs seemed to be unaffected by change in pH but at higher temperatures (60–70 °C) more β-CD was produced with trace amount of γ-CD observed. At 80 °C, only α-CD was detected at lower percentage yield.

Therefore, *Paenibacillus* sp. BT01 was a thermotolerant bacterium producing CGTase which retained activity at temperature up to 70 °C. It should be suitable for use in producing CDs at industrial scale especially α-CD.

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