Optimization for Cyclodextrin-Glucanotransferase (CGTase) Production by an Alkalophilic Bacterium, *Bacillus* sp.

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บทคัดย่อ

จากการศึกษาสภาวะที่เหมาะสมสำหรับเอ็นไซม์ไซโคลเดกซ์ทริน กลูคาโนทรานสเฟอเรส เพื่อผลิตไซโคลเดกซ์ทริน โดยแบคทีเรียไอโซเลต #10 ที่แยกจากดินมหาวิทยาลัยขอนแก่น พบว่า อุณหภูมิและระยะเวลาที่เหมาะสมในการเพาะเลี้ยงคือ 37 องศาเซลเซียส ภายใน 72 ชั่วโมง โดยแป้งข้าวเหนียวที่ความเข้มข้น 1% เป็นสารต้นตอคาร์บอนที่เหมาะสมต่อการผลิตเอ็นไซม์ และที่ความเข้มข้น 4% เป็นสับสเตรทที่เหมาะสมต่อการทำงานของเอ็นไซม์ ในขณะที่เอ็นไซม์จะทำงานได้ดีที่อุณหภูมิ 50 องศาเซลเซียส เมื่อตรวจสอบชนิดของไซโคลเดกซ์ทรินด้วยวิธีโครมาโตรกราฟีแบบกระดาษพบว่าเป็นชนิด β-CD จากการศึกษาทางสัณฐานวิทยาและการทดสอบทางชีวเคมีของแบคทีเรียไอโซเลต #10 คาดว่าจัดอยู่ในสกุล *Bacillus* sp.

Abstract

A new strain of alkalophilic bacterial isolate #10 isolated from Khon Kaen University soil was studied for its optimal condition for CGTase production. The characterization of enzyme production showed an optimal temperature and incubation time of 37 °C and 72 hrs, respectively. The results showed that sticky-rice starch was the best carbon source for enzyme production at a concentration of 1% (w/v). The optimal temperature for the enzyme-catalyzed reaction was 50 °C. The sticky-rice starch was also the best substrate for enzyme activity at a concentration of 4% (w/v). The alkalophilic isolate #10 produced only β-CD as indicated by paper chromatography. From morphology and biochemical tests, this bacterium was identified as *Bacillus* sp.

คำสำคัญ: เอ็นไซม์ไซโครเดกซ์ทริน กลูคาโนทรานสเฟอเรส ไซโครเดกซ์ทริน แบคทีเรียทนด่าง
Keywords: Cyclodextrin-glucanotransferase, cyclodextrin, alkalophilic bacteria

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Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides of glucopyranose units with a lipophilic cavity in the center. The cyclodextrin natural product consists of a mixture of the various cyclodextrins, mainly α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, which consist of six, seven, and eight glucopyranose units, respectively. Cyclodextrins are capable of forming inclusion complexes with many drugs by taking up a whole drug molecule, or some part of it, into the cavity. This type of molecular encapsulation affects many of the physiochemical properties of the drugs (Szejtli, 1994). In addition, these altered characteristics of encapsulated compounds have led to various applications of CDs in agriculture, analytical chemistry, biotechnology, food, and cosmetics (Saenger, 1980; Allegre and Deratani, 1994; Luong et al., 1995).

CDs are produced by the action of a group of enzymes called cyclodextrin glucanotransferases (α-1,4-glucan-4-glycosyltransferase: EC 2.4.1.19, CGTase) on starch (Tonkova, 1998; Kanai et al., 2001). CGTase belongs to the α-amylase family which catalyzes transglycosylation reactions (glycosyl hydrolase family 13) (Herrissat, 1991). CGTase mainly catalyzes transglycosylation reactions by transferring a newly made reducing end oligosaccharide to an acceptor molecule. Four transferase reactions including cyclization, coupling, disproportionation, and hydrolysis can be distinguished depending on the nature of the acceptor molecule (Nakamura et al., 1993). Cyclization is the transfer of the reducing end to another sugar residue in the same oligosaccharide chain, resulting in formation of a cyclic compound or cyclodextrin. Coupling is the reverse reaction of cyclization where a cyclodextrin molecule is cleaved and combined with a linear oligosaccharide chain to produce a longer linear oligosaccharide. Disproportionation is the transfer of a part of a linear oligosaccharide chain to a linear acceptor chain, resulting in a mixture of smaller and longer oligosaccharides. Hydrolyzing or saccharifying activity is the transfer of the newly made reducing end sugar to water. Only the hydrolysis reaction thus results in an increased number of reducing ends (Nakamura et al., 1993; Penninga et al., 1995).

Bacillus species are the main producers of bacterial CGTase (Tonkova, 1998). *Bacillus macerans* produces CGTase after reaching the stationary phase between 168-188 h (DePinto and Campbell, 1968). Later, this cultivation time was reduced to 10-15 h by improving the growth conditions and medium content (Stefanova et al., 1999). In *Bacillus cereus*, CGTase synthesis starts from the early exponential phase and maximal CGTase activity is measured after 16-20 h of cultivation (Jamuna et al., 1993).

The main objective of this work was to improve the CGTase production from bacterial isolate #10 which is an alkalophilic CGTase producing microorganism and isolated from soil in Khon Kaen University (Siripornadulsil and Tantianu-nanont, 2008). This present paper also reports conditions which optimize enzyme production.

Materials and Methods

Microorganism identification

The bacterial isolate #10, alkalophilic CGTase producing microorganism, was isolated from soil in the Khon Kaen University area. The identification of the bacterial isolate #10 was performed using the

methods described in BERGY's Manual of Systematic Bacteriology (1986) and Gee et al. (1980). The media used for identification were adjusted to pH 8.5-9.0 with sterile Na_2CO_3.

Optimization of CGTase production and substrate specificity for CGTase

The bacterial isolate #10 was cultivated at 37 °C and 120 rpm in Horikoshi-II medium broth containing 1% starch, 1% Na_2CO_3, 0.5% yeast extract, 0.5% peptone, 0.1% K_2HPO_4 and 0.02% $MgSO_4.7H_2O$. Samples were withdrawn periodically at 24, 48, and 72 h to analyze pH and enzyme activity to determine the production of enzyme. Several attempts were made to improve the enzyme production. For culture condition, the effect of different types of starch as carbon source i.e. cassava, rice, sticky rice and wheat, concentration of carbon source, and different temperature were tested. During growth (0-120 h), the cells were counted and harvested by centrifugation at 6,000 rpm, 4 °C. The supernatants were assayed for CGTase activity using CD-Trichloroethylene (TCE) complex precipitation assay.

Enzyme assay

The bacterial cells were grown at 37 °C and 120 rpm for 120 h in Horikoshi-II medium broth. Then the bacterial cells were removed by centrifugation at 6,000 rpm, 4 °C for 10 min. The supernatant which contains CGTase enzyme was tested for CD-TCE complex precipitation (Nomoto et al., 1984). The enzyme solution was sequentially 2-fold diluted in 0.2 M phosphate buffer, pH 6.0 to adjust the enzyme concentration from 1:2 to $1:2^n$ dilutions. The effects of different starch types and their concentration, including incubation temperature were tested. The incubation time was 24 h at the indicated temperature. Then 1 mL of 100% TCE was added and vigorously mixed by vortex. The mixture solution was incubated in the dark for 12 h. The CD-TCE complex was precipitated on the inter-phase between TCE and enzyme solution. The CGTase/CD-TCE activity was expressed as the lowest dilution unit of the crude enzyme showing the white pellet of CD-TCE complex.

The hydrolyzing activity was assayed by measuring the increase in reducing power upon incubation of 1 mL of the enzyme with 5 mL of 1% soluble starch at 50 °C for 24 h (Bernfeld, 1955; Penninga et al., 1995). After addition of 1 mL of 3, 5-dinitrosalicylic acid and Rochelle salt (potassium sodium tartrate), the reaction was stopped by incubating the tubes for 5 min in a boiling waterbath. The absorbance at 540 nm of the contents of each tube was measured against water. A calibration curve for maltose was used to estimate the amount of reducing sugar.

Analysis of CD products

CD-TCE complex precipitate was dissolved with distilled water and boiled at 80 °C for 10 min. The solution was filtered through a 0.45 mm filter. The 15 mL filtrate was applied onto Whatman #1 paper (22 x 22 cm). The paper sheet was separately irrigated with 4:3:3 (v/v) of n-butanol-ethanol-water. The CDs on the paper sheet were located using 1% methanolic iodine (Szejtli, 1988).

Results

Identification of bacterial isolate #10

The selected bacterial isolate #10 is an alkalophilic bacterium showing a yellowish halo when grown on a medium containing phenolphthalein and methyl orange (Figure 1A). It is a Gram positive,

aerobic, and rod-shaped bacterium without spore (Figure 1B). It was able to grow over a wide range of temperatures (20 - 50 °C) with optimal growth at 37 °C and showed rapid growth at pH 9.0. The bacterium was not able to ferment glucose, sucrose, maltose, and lactose. It neither produced catalase and urease nor hydrolyzed gelatin and casein. Based on such biochemical characteristics, the bacterium was identified and designated as *Bacillus* sp. #10.

Optimization of CGTase

The effect of carbon sources on CGTase production by *Bacillus* sp. #10 was determined at 37 °C for 24 -72 h (Table 1). CGTase was synthesized during growth in the presence of all tested carbon sources. Rice and sticky rice starch were the most effective inducers followed by wheat, corn, and cassava starch. The optimal temperature for CGTase activity of *Bacillus* sp. # 10 was 50 °C compared with 40 and 60 °C (Table 1). Substrate specificity of the CGTase produced in Horikoshi-II medium containing either rice or sticky rice starch as carbon source at 50 °C for 24, 48, and 72 h was also studied. It was found that sticky rice starch was a more effective carbon source than rice starch at 48 and 72 h of culture time (Table 2). Sticky rice starch at a concentration of 1% (w/v) was the most effective for CGTase synthesis by *Bacillus* sp. #10 (Table 3) with the optimal temperature of cultivation at 37 °C when compared to 20, 30 and 50 °C (Table 4). The most effective substrate for CGTase activity was sticky rice starch at a concentration of 4% (w/v) (Table 5). CGTase synthesis of *Bacillus* sp. #10 started from the early exponential phase and maximal CGTase activity was found at the stationary phase at 120 h when reducing sugar was increased to 71.32 μg/mL (Table 5). The pH of cultivation medium during cell growth was 8.83 - 9.96 where the initial pH of the media was 10.0. The maximal CGTase activity by CD-TCE activity assay from optimal condition was $1:2^8$ dilution limit when 4% and 1% (w/v) sticky rice were used as carbon source and CGTase substrate, respectively (Table 5). The CD-TCE complex precipitates were further identified by paper chromatography. The result showed that bacterial isolate #10 produced only β-CD (Figure 2).

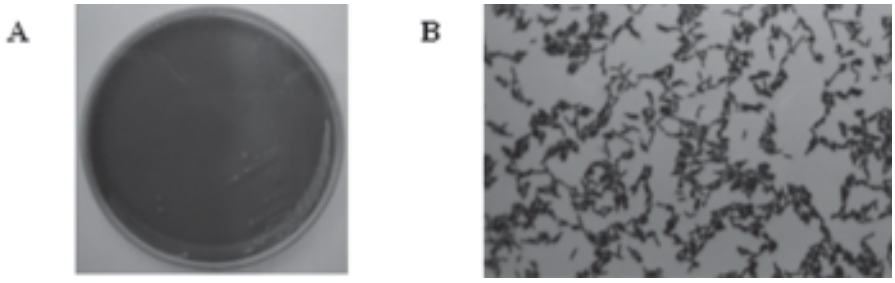

Figure 1. (A) The yellowish halo of bacterial isolate #10 when grown on Horikoshi-II medium agar containing phenolphthalein and methyl orange; (B) Gram stain of bacterial isolate #10.

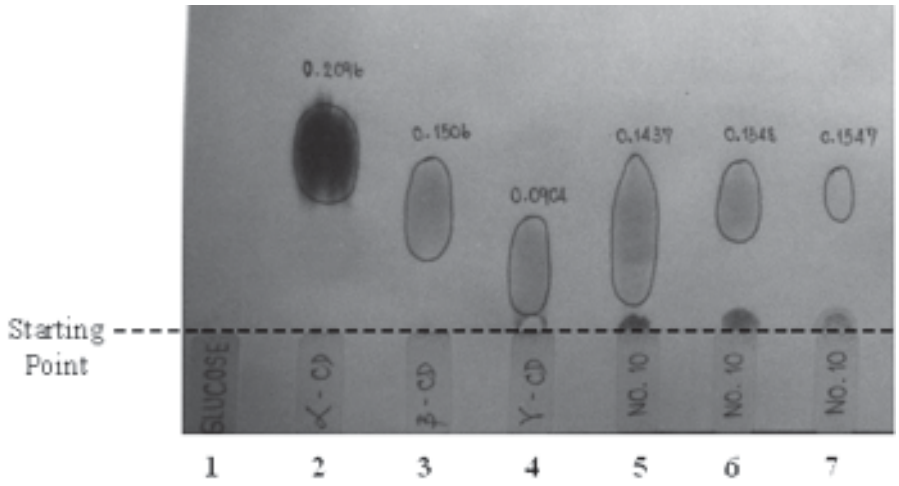

Figure 2. Paper chromatography analysis. Lane 1, glucose; Lane 2-4, standard a-, b-, and g-CD (R_f : 0.2096, 0.1506 and 0.0904, respectively); Lane 5-7, CD-TCE complex precipitate from *Bacillus* sp. #10 at $1:2^2$ (R_f: 0.1437), $1:2^3$ (R_f: 0.1548), and $1:2^4$ (R_f: 0.1547), respectively, when grown in a medium containing 1% (w/v) and 2% (w/v) sticky rice starch as carbon source and CGTase substrate, respectively.

Discussion

The characteristics of selected alkalophilic bacteria isolate #10 were studied. Even though no spore was found inside the bacterial cells, its biochemical characteristics were identical to those of *Bacillus* sp. described by Gee et al. (1980). We designated the selected bacteria as *Bacillus* sp. #10. The optimal conditions for CGTase production were studied including substitution of different starch as carbon source and CGTase inducer, concentration of starch, and temperature. It was found that rice and sticky rice starch were the most effective carbon sources of *Bacillus* sp. #10 for enzyme production at a concentration of 1% (w/v) (Table 1). The optimal temperature for cultivation was 37 °C (Table 4). Furthermore, CGTase synthesis of *Bacillus* sp. #10 started from the early exponential phase and maximal CGTase activity was found at stationary phase at 120 h (Table 6). These results are in agreement with the culture characteristic of *Bacillus circulans* (Nakamura and Horikoshi, 1976; Paloheimo et al., 1992) and *Bacillus stearothermophilus* (Stefanova et al., 1999) which exhibited maximum activity after 48-50 h of growth. The optimal temperature for enzyme-catalyzed reaction was 50 °C (Table 1). The sticky rice starch was also the most effective substrate for enzyme activity at a concentration of 4% (w/v) (Table 6). We have improved the CGTase production by alkalophilic *Bacillus* sp. #10 from $1:2^4$ to $1:2^8$ dilution limit of CGTase/CD-TCE activity assay when sticky rice was used as carbon source at 1% (w/v) and substrate at 4% (w/v) (Table 1, 6). The alkalophilic *Bacillus* sp. #10 produced only β-CD (Figure 2).

Table 1. Effect of various starches as carbon source on the synthesis of CGTase by *Bacillus* sp. #10.

Culture Time (h)	CGTase activity assay Temperature (°C)	CGTase/CD-TCE activity[a] in the presence of different starch inducer				
		Cassava	Rice	Sticky rice	Corn	Wheat
24	40	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^2$
	50	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^2$
	60	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^1$	$1:2^2$
48	40	$1:2^2$	$1:2^3$	$1:2^3$	$1:2^3$	$1:2^3$
	50	$1:2^2$	$1:2^3$	$1:2^3$	$1:2^2$	$1:2^3$
	60	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^2$	$1:2^2$
72	40	$1:2^2$	$1:2^3$	$1:2^3$	$1:2^3$	$1:2^3$
	50	$1:2^2$	$1:2^4$	$1:2^4$	$1:2^3$	$1:2^3$
	60	$1:2^2$	$1:2^3$	$1:2^3$	$1:2^2$	$1:2^2$

Culture condition: at 37 °C, 120 rpm, 1% starch as carbon source

[a] Enzyme assay condition: 2% potato soluble starch as substrate

Table 2. Substrate specificity on the CGTase activity of *Bacillus* sp. #10.

Culture Time (h)	C-Source (1% Starch)	CGTase/CD-TCE activity[a] in the presence of different substrate					
		Cassava	Rice	Sticky rice	Corn	Wheat	Soluble
24	Rice	$1:2^3$	$1:2^1$	$1:2^4$	$1:2^0$	$1:2^0$	$1:2^2$
	Sticky rice	$1:2^3$	$1:2^1$	$1:2^4$	$1:2^1$	$1:2^1$	$1:2^1$
48	Rice	$1:2^4$	$1:2^2$	$1:2^4$	$1:2^2$	$1:2^1$	$1:2^2$
	Sticky rice	$1:2^4$	$1:2^2$	$1:2^5$	$1:2^2$	$1:2^1$	$1:2^2$
72	Rice	$1:2^5$	$1:2^3$	$1:2^5$	$1:2^3$	$1:2^2$	$1:2^3$
	Sticky rice	$1:2^5$	$1:2^3$	$1:2^6$	$1:2^3$	$1:2^2$	$1:2^4$

Culture condition: at 37 °C, 120 rpm, 1% starch either from rice or sticky rice

[a] Enzyme assay condition: at 50 °C, 24 h, each starch substrate concentration was 2%.

Table 3. Effect of sticky rice starch concentration as carbon source on the CGTase synthesis by *Bacillus* sp. #10.

Culture Time (h)	CGTase/CD-TCE activity[a] in the presence of sticky rice starch concentration (w/v)				
	0%	0.5%	1.0%	1.5%	2.0%
24	-	$1:2^3$	$1:2^4$	$1:2^3$	$1:2^3$
48	-	$1:2^3$	$1:2^5$	$1:2^4$	$1:2^3$
72	-	$1:2^3$	$1:2^6$	$1:2^4$	$1:2^4$

Culture condition: at 37 °C, 120 rpm, 0 - 2.0% sticky rice starch as carbon source

[a] Enzyme assay condition: at 50 °C, 24 h, 2% sticky rice starch substrate

Table 4. Effect of cultivation temperature on the CGTase synthesis by *Bacillus* sp. #10.

Culture Time (h)	CGTase/CD-TCE activity[a] at different cultivation temperature (°C)			
	20	30	37	50
24	$1:2^2$	$1:2^2$	$1:2^4$	$1:2^2$
48	$1:2^2$	$1:2^3$	$1:2^5$	$1:2^1$
72	$1:2^2$	$1:2^4$	$1:2^6$	$1:2^1$

Culture condition: at 120 rpm, 1% sticky rice starch as carbon source

[a] Enzyme assay condition: at 50 °C, 24 h, 2% sticky rice starch substrate

Table 5. Effect of sticky rice starch concentration as substrate on the CGTase activity of *Bacillus* sp. #10.

Culture Time (h)	CGTase/CD-TCE activity[a] in the presence of sticky rice			
	1.0%	2.0%	3.0%	4.0%
24	$1:2^3$	$1:2^4$	$1:2^5$	$1:2^6$
48	$1:2^3$	$1:2^5$	$1:2^6$	$1:2^8$
72	$1:2^3$	$1:2^6$	$1:2^7$	$1:2^8$

Culture condition: at 37 oC, 120 rpm, 1% sticky rice starch as carbon source

[a] Enzyme assay condition: at 50 °C, 24 h, 1.0 – 4.0% sticky rice starch substrate

Table 6. Growth and CGTase synthesis by *Bacillus* sp. #10.

Culture Time (h)	Cell number (cfu/mL)	pH	CD-TCE[a]	Reducing sugar[b] (μg/mL)
0	2.65×10^7	9.96	-	0.00
12	1.75×10^9	9.23	$1:2^2$	23.77
24	4.50×10^9	8.83	$1:2^4$	41.60
48	1.09×10^9	9.18	$1:2^5$	40.12
72	9.85×10^8	9.40	$1:2^6$	56.46
96	4.17×10^8	9.77	$1:2^4$	65.38
120	3.80×10^8	9.93	$1:2^4$	71.32

Culture condition: at 37 °C, 120 rpm, 1% sticky rice starch as carbon source [a]

Enzyme assay condition: at 50 °C, 24 h, 2% sticky rice starch substrate

[b] Hydrolyzing activity assay condition: at 50 °C, 24 h, 1% soluble starch

Acknowledgement

This study was supported by the Research Fund of the Faculty of Science, Khon Kaen University.

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