Screening and Isolation of Alkalophilic Cyclodextrin Glucanotransferase (CGTase) Producing Bacteria from Soil

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บทคัดย่อ

จากการคัดเลือกแบคทีเรียที่ผลิตเอ็นไซม์ไซโคลเด็กซ์ทรินกลูคาโนทรานสเฟอเรส จากดินจำนวน 60 ตัวอย่าง ที่เก็บจากบริเวณมหาวิทยาลัยขอนแก่น โดยการชั่งดิน 1 กรัมในน้ำกลั่นปลอดเชื้อ 9 มล. และ เกลี่ยโดยวิธี spread plate ลงบนอาหารเลี้ยงเชื้อ Horikoshi-II, pH 10.3 ที่เติม 0.03% phenolpthalein และ 0.01% methyl orange เป็นอินดิเคเตอร์ บ่มที่อุณหภูมิ 37 °C เป็นเวลา 24-72 ชั่วโมง ทำการคัดเลือกแบคทีเรียจำนวน 16 ไอโซเลต ที่สามารถเปลี่ยนสีอาหารจากสีแดงเป็นสีเหลือง ที่เกิดจาก complex ระหว่าง phenolphthalein และไซโคลเดกซ์ทริน และนำมาทดสอบต่อด้วยวิธี cyclodextrin (CD)-trichoroethylene assay (TCE) assay พบว่ามีแบคทีเรีย 5 ไอโซเลต คือ ไอโซเลตที่ 2, 6, 10, 26 และ 37 ที่สามารถสร้างเอ็นไซม์ CGTase โดยให้ค่า dilution limit สูงสุด เท่ากับ $1:2^4$, $1:2^5$, $1:2^6$, $1:2^3$ และ $1:2^3$ ตามลำดับ และพบว่าแป้งมันสำปะหลัง สามารถเหนี่ยวนำให้แบคทีเรียผลิตเอ็นไซม์ CGTase ได้ดีกว่า potato starch และ soluble starch จากการนำตะกอนที่ได้จาก CD-TCE assay มาวิเคราะห์ด้วยวิธี paper chromotography พบว่าไอโซเลตที่ 2, 26 และ 37 ผลิตไซโคลเดกซ์ทรินทั้งชนิดแอลฟาและเบต้า ในขณะที่ ไอโซเลตที่ 6 และ 10 ผลิตไซโคลเดกซ์ ทรินชนิดเบต้าเพียงชนิดเดียว

Abstract

Sixty soil samples in Khon Kaen University were collected and screened for alkalophilic CG Tase-producing bacteria. Each soil sample of 1 g was suspended in 9 mL of sterilized water and 0.1 mL of the suspensions was spread on Horikoshi-II medium agar, pH 10.3, containing 0.03% phenolphthalein and 0.01% methyl orange, and incubated at 37°C for 24-72 h. Yellow zones were observed around the CG Tase-producing colonies due to phenolphthalein inclusion complexes inside cyclodextrin. Five isolates were selected and further analyzed by cyclodextrin (CD)-trichoroethylene (TCE) assay. The results showed that isolates # 2, 6, 10, 26 and 37 gave CGTase activities as dilution limit at $1:2^4$, $1:2^5$, $1:2^6$, $1:2^3$ and $1:2^3$, respectively. The cassava starch was the most effective inducer in comparison to other soluble- and potato starches. The identification of cyclodextrins by paper chromatography indicates that isolates # 2, 26 and 37 produce α-CD and β-CD while isolate # 6 and 10 produce only β-CD.

คำสำคัญ: เอ็นไซม์ไซโคลเดกซ์ทรินกลูคาโนทรานสเฟอเรส ไซโครเดกซ์ทริน แบคทีเรียทนด่าง
Keywords: Cyclodextrin glucanotransferase, cyclodextrin, alkalophilic bacteria.

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Introduction

Many bacteria use starch as a carbon and energy source for their activities and growth. To utilize the carbon source, they produce a number of extracellular enzymes to convert the large molecules into utilizable small molecules. A large numbers of starch-hydrolyzing enzymes have been identified and characterized (Komiyama and Bender, 1984), including cyclodextrin glucanotransferase (CGTase). CGTase (α-1,4-glucan-4-glycosyltransferase: E.C.2.4.1.19) is a member of the α-amylase family and a multifunctional enzyme, which hydrolyses α-1,4-glycosidic bonds of starch to produce cyclodextrins (CDs, closed-ring structures composed mainly of 6, 7 and 8 glucosyl units, named α, β and γ-cyclodextrin, respectively) via an intramolecular transglycosylation reaction. In addition, CGTase can display coupling (opening of the rings of CDs and transfer of the linear maltooligosaccharide formed to acceptors), disproportionation (transfer of linear maltooligosaccharide formed to acceptors) and hydrolyzing activities. (French, 1957; Tonkova, 1998). Currently, bacteria are still regarded as an important source of CGTase. Since the discovery of *Bacillus maceran* as the first source that is capable of CGTase production (Takano et al., 1986), a wide variety of bacteria have been determined as CGTase producers, namely aerobic mesophilic-, aerobic thermophilic-, anaerobic thermophilic-, aerobic alkalophilic, and aerobic halophilic bacteria (Tonkova, 1998).

CD molecules have a unique structure with a hydrophobic cavity and hydrophilic regions at their outer surface and therefore can form inclusion complexes with a wide variety of hydrophobic guest molecules (Komiyama and Bender, 1984; Szejtli, 1988; Tonkova, 1998). The formation of inclusion complexes leads to a change in the chemical and physical properties of the guest molecules. These altered characteristics of encapsulated compounds have led to various applications of CDs in agriculture, analytical chemistry, biotechnology, food, pharmacy and cosmetics (Saenger, 1980; Pczczola, 1988; Schmid, 1989; Starnes, 1990; Allegre and Deratani, 1994; Szejtli, 1994 and 1997; Luong et al., 1995).

In this study, we have screened and isolated alkalophilic CGTase-producing bacteria with high enzyme activity from soil in Khon Kaen University.

Materials and Methods

Microorganisms

The 60 soil samples were collected in the Khon Kaen University area. To isolate alkalophilic CGTase producing microorganisms, each soil sample of 1 g was suspended in 9 mL of sterilized water and 0.1 mL of the suspension was spread on Horikoshi-II medium agar containing 1% cassava starch, 0.5% yeast extract, 0.5% peptone, 1% Na_2CO_3, 0.1% KH_2PO_4, 0.02% $Mg_2SO_4.7H_2O$, 0.03% phenolphthalein and 0.01% methyl orange at pH 10.3, and incubated at 37 ^{o}C for 24-72 h. The colonies, which were surrounded by a yellowish halo, were selected for further examination (Park, et al., 1989; Salva et al., 1997).

Enzyme assay

The bacterial cells were grown at 37 ^{o}C for 24, 48 and 72 h in Horikoshi-II medium broth except for the exclusion of dyes (phenolphthalein and methyl orange). Then the bacterial cells were removed by centrifugation at 6,000 rpm for 10 min. The pH of the supernatant was measured by a pH meter and CGTase enzyme was determined by

phenolphthalein-methyl orange inclusion complex test (PMIC) (Park et al., 1989) and CD-trichloroethylene (TCE) complex precipitation (Nomoto et al., 1984).

For the phenolphthalein-methyl orange inclusion complex test, the enzyme solution (10, 20, or 30 μL) was applied on Horikoshi-II medium containing inclusion dyes. After 24 h, the yellowish halo was observed and its diameter was determined. For CD-TCE complex precipitation, the enzyme solution was sequentially 2-fold diluted in 0.2 M phosphate buffer, pH 6.0 to adjust the enzyme from 1:2 to $1:2^n$ dilutions. The enzyme was incubated in the same buffer containing 2% soluble starch at 40 °C for 24 h. 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 as a white pellet on the interface between TCE and enzyme solution.

Determination of inducer for CGTase production

To determine the inducer for CGTase production, the bacterial cells were grown at 37 °C for 24-72 h in Horikoshi-II medium containing 2% (w/v) of different starch types including soluble-, potato-, and cassava starch but excluding phenolphthalein and methyl orange. Then the cell-free medium was determined for pH, CGTase activity by PMIC test (Park et al., 1989) and CD-TCE complex precipitation (Nomoto et al., 1984).

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 μm filter. Then the 10, 20 and 30 μL 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

Screening and isolation of the CGTase-producing microorganisms

During the selection of CGTase-producing microorganisms, a total of 45 colonies from 60 soil samples showing yellowish halo were selected and re-point-inoculated on fresh Horikoshi-II medium agar (Figure 1). Only five isolates, including isolates # 2, 6, 10, 26, and 37 of alkalophilic bacteria which showed the yellowish halo with a diameter of 7-20 mm were selected for further study.

Effect of inducer and incubation time on CGTase production

The induction of CGTase production was studied using three different types of starch, including soluble-, potato-, and cassava starch. CGTase activity of cell-free medium after incubation at 24, 48, and 72 h were determined by PMIC test using enzyme solutions of 10, 20, and 30 μL and CD-TCE complex precipitation (Table 1). The CD-TCE complex precipitates were further identified by paper chromatography (Figure 2).

Discussion

We have isolated the alkalophilic CG Tase-producing bacteria from soil collected in Khon Kaen University. The results show that all 45 isolates of alkalophilic bacteria produced CGTase only when cassava starch was present in the medium (Figure 1). These results indicated that starch is an

inducer for CGTase production and is in agreement with the knowledge that CGTase is an inducible enzyme in which the starch acts as an inducer (Schmid, 1989).

In PMIC test of 5 selected bacteria, the initial pH of media was 9.0 and pH of culturing medium was in the range of 8.55-9.64 indicating that the yellowish halo came from CD-phenolphthalein complex. The results also showed that the diameter of the yellowish halo was not correlated to CD-TCE activity (Table 1). For the effect of starch inducer, the PMIC test showed that potato starch induced the synthesis of CGTase only by the isolate # 2, 6, and 10 within 24 h. The maximum CD-TCE activity of isolate # 2, 6, and 10 are $1:2^2$, $1:2^2$, and $1:2^3$ dilutions, respectively at 48 h. For soluble starch, all isolated bacteria have ability to use this starch as an inducer and produced CGTase within 24 h, with the exception of isolate # 26 which produced the enzyme within 48 h. The maximum CD-TCE activity of isolates # 2, 10, and 26 are $1:2^4$, $1:2^4$, and $1:2^1$ dilution, respectively at 48 h. In contrast, the maximum CD-TCE activity of isolates # 6 and 37 are $1:2^5$ and $1:2^3$ dilution respectively at 72 h (Table 1). Cassava starch was shown to be the most effective CGTase inducer among the tested starch for all selected bacteria. In addition, the highest CGTase activity was obtained from isolate # 10 in the presence of cassava starch with CD-TCE activity of $1:2^6$ dilution, at 72 h.. Furthermore, the identification of CDs by paper chromatography indicated that isolates # 2, 26 and 37 produced α-CD and β-CD while isolates # 6 and 10 produced only β-CD (Figure 2).

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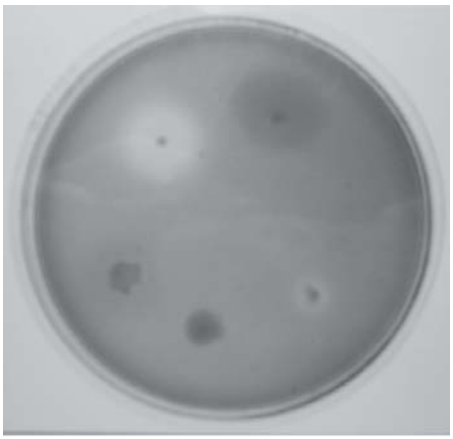

Figure 1 Isolation and screening of CGTase-producing bacteria. After re-point-inoculation on Horikoshi-II agar, the yellowish halo of PMIC was formed surrounding the colonies of alkalophilic CGTase-producing bacteria

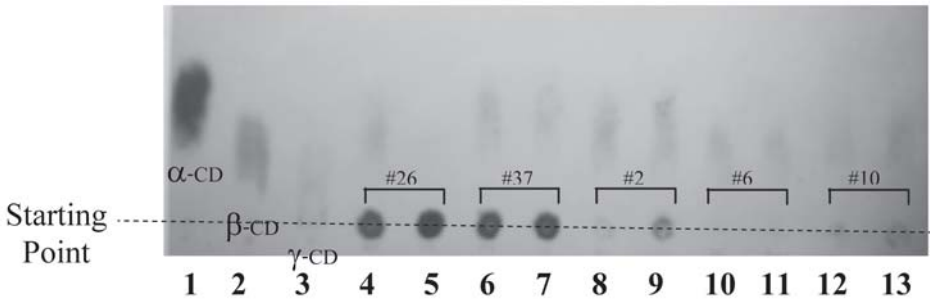

Figure 2 Paper chromatography analysis of CD-TCE complex precipitate. Lanes 1-3, standard α-, β-, and γ-CD (Rf : 0.16, 0.11, and 0.05, respectively); Lanes 4-5: isolate # 26 (Rf: 0.11, 0.16), Lanes 6-7: isolate #37 (Rf: 0.11, 0.17), Lanes 8-9: isolate #2 (Rf: 0.11, 0.17), Lanes 10-11: isolate #6: (Rf: 0.12) and Lanes 12-13: isolate #10 (Rf: 0.12) grown in the medium containing cassava starch and soluble starch as carbon source and inducer respectively for 72h.

Table 1 Effect of starch inducer on the synthesis of CGTase by five isolates of alkalophilic bacteria. The activity of CGTase was measured by PMIC test on Horikoshi-II agar and CD-TCE complex precipitation.

Inducer	Isolate #	pH[a]	PMIC test Yellowish halo diameter (mm)			CD-TCE Dilution limit
			10[b]	20[b]	30[b]	
24 h Growth						
Potato Starch	2	9.64	8	10	13	$1:2^{2}$
	6	9.28	8	11	13	$1:2^{1}$
	10	9.41	6	10	12	$1:2^{1}$
	26	8.55	7	11	12	-
	37	8.82	7	11	12	-
Soluble Starch	2	9.50	-	ND	ND	$1:2^{2}$
	6	8.65	9	ND	ND	$1:2^{3}$
	10	8.73	10	ND	ND	$1:2^{3}$
	26	8.73	-	ND	ND	-
	37	8.81	9	ND	ND	$1:2^{1}$
Cassava Starch	2	9.64	8	9	13	$1:2^{2}$
	6	8.49	-	10	10	$1:2^{3}$
	10	8.85	8	11	14	$1:2^{2}$
	26	8.70	8	13	14	-
	37	8.64	8	11	12	-

Table 1 (Continue)

Inducer	Isolate #	pH[a]	PMIC test Yellowish halo diameter (mm)			CD-TCE Dilution limit
			10[b]	20[b]	30[b]	
48 h Growth						
Potato Starch	2	9.33	7	9	10	$1:2^{2}$
	6	9.38	8	11	12	$1:2^{2}$
	10	9.40	8	10	13	$1:2^{3}$
	26	9.08	12	12	15	–
	37	9.06	8	12	17	–
Soluble Starch	2	9.56	10	ND	ND	$1:2^{4}$
	6	8.97	11	ND	ND	$1:2^{4}$
	10	8.99	12	ND	ND	$1:2^{4}$
	26	9.02	10	ND	ND	$1:2^{1}$
	37	9.08	11	ND	ND	$1:2^{2}$
Cassava Starch	2	9.16	11	16	16	$1:2^{2}$
	6	8.85	13	14	14	$1:2^{3}$
	10	8.88	12	14	14	$1:2^{4}$
	26	8.94	11	14	17	$1:2^{2}$
	37	8.98	10	14	16	$1:2^{2}$
72 h Growth						
Potato Starch	2	9.46	9	11	13	–
	6	9.42	10	10	13	$1:2^{1}$
	10	9.55	12	14	14	$1:2^{1}$
	26	9.33	12	14	16	–
	37	9.26	13	15	17	–
Soluble Starch	2	9.59	9	ND	ND	$1:2^{3}$
	6	9.32	13	ND	ND	$1:2^{5}$
	10	9.11	13	ND	ND	$1:2^{4}$
	26	9.23	12	ND	ND	–
	37	9.32	13	ND	ND	$1:2^{3}$
Cassava Starch	2	9.31	13	17	18	$1:2^{4}$
	6	9.19	13	15	20	$1:2^{5}$
	10	9.17	12	16	20	$1:2^{6}$
	26	9.20	13	13	15	$1:2^{3}$
	37	9.20	13	15	16	$1:2^{3}$

[a] pH of enzyme solution; [b] volume of enzyme solution in mL

ND, Not determined