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Original Scientific Paper

Determination of cyclodextrin production by cyclodextrin glycosyltransferase from alkalophilic *Bacillus circulans* strain B-65

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ABSTRACT: New alkalophilic *Bacillus* sp. producers of cyclodextrin glycosyltransferase (CGTase) were isolated from 54 different soil samples (Serbian springs and soils). Amylolytic activity of isolates was found to be in the range of 0.4 to 12.2 U/mL and cyclodextrinogenic activity in the range of 0.02 to 0.53 U/mL. The isolate designated as B-65 showed the highest amylolytic and cyclodextrinogenic activity. On the basis of morphological, physiological and biochemical characteristics, isolate B-65 was identified as an alkalophilic *Bacillus circulans* strain. Cyclodextrinogenic and amylolytic activity gradually increased over 96 h of incubation in a laboratory bioreactor. After 30 h of incubation, CGTase converted 40% of starch to cyclodextrins (CDs), whose final concentration reached 20 g/L. Analysis by HPLC revealed b-, a- and g- cyclodextrins with relative abundances of 95,3 and 2%, respectively.

Keywords: cyclodextrins, cyclodextrin glycosyltransferase, *Bacillus circulans*

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INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is an extracellular enzyme produced mainly by several species of the genus *Bacillus*, but also by *Klebsiella pneumoniae*, *Thermoanaerobacterium thermosulfurigenes*, *Thermococcus* sp., *Brevibacterium* sp. and some other microorganisms (Mori *et al.* 1995; Tonkova 1998; Win *et al.* 1998; Tachibana *et al.* 1999; Gawande & Patkar 2001; Tereda *et al.* 2001). CGTase catalyzes the formation of cyclodextrins (CDs) from starch and related carbohydrates such as amylose, amylopectin, glycogen and maltooligosaccharides. CDs are cyclic non-reducing maltooligosaccharides composed of six and more glucose units joined by a-1.4 glycosidic bonds. The most common CDs are a-, b- and g-types consisting of six, seven and eight glucose units, respectively.

CGTases produced by different microorganisms show some similarities in characteristics, but there are great differences in specificity for formation of a-, b- or g-CDs. Alkalophilic microorganisms produce CGTases which form the b-CD as the main product, while enzymes producing primarily a- and g-CD are relatively rare. CGTases produced by *Paenibacillus macerans* IAM 1243, *Klebsiella pneumoniae M5al* and *Klebsiella oxytoca 19-1* form the highest yield of a-CD in comparison with other CDs (Goel & Nene 1995). On the other hand, *Bacillus subtilis* No. 313, alkalophilic *Bacillus* strain 290-3, *Bacillus* sp. AL-6 and *Brevibacterium* sp. No. 9605 are the only known γ-CD producers (Qi & Zimmermann 2005).

CDs are capable of forming inclusion complexes with a wide variety of inorganic and organic molecules. While incorporating themselves into the hydrophobic cavities of the molecules, CDs alter physical and chemical properties of those molecules. CGTase is known to catalyse reaction between CD or starch (glycosyl donors) and various acceptors in the process of production of new substances. As a result, interest in glycosylation reactions and CGTase

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producers has increased during the last decade (Qi & Zimmermann 2005). Isolation of microorganisms which produce CTGases, especially those that are capable of predominantly producing a particular type of CD, can reduce subsequent purification costs and is therefore commercially desirable. Purification of b-CD is easier and cheaper than purification of a- and g-CD. Consequently, β-CD has numerous possibilities of application in the pharmaceutical, cosmetic and food industries, particularly due to stability of its inclusion complexes. The aim of the present study was to isolate novel CGTase-producing alkalopilic bacteria from soil samples of different origin with emphasis on the ability to preferentially produce b-CD from starch.

MATERIALS AND METHODS

Reagents. CD was obtained from Sigma Chemical Co., MO, USA. Starch was purchased from local suppliers. Other chemicals were analytical grade and were purchased from Merck, Darmstadt, Germany.

Isolation of CGTase-producing bacteria. CGTase-producing alkalophilic bacteria were isolated from soil samples originating from southeastern Serbia. A suspension of soil (0.5 to 1.0 g) in 10 ml of sterile distilled water was heated at 80°C for 10 min. After heating, 0.1 ml of the soil suspension was spread on an agar plate with an alkaline (pH 10) phenolphthalein-methyl orange medium of the following composition: soluble starch, 1%; peptone, 0.5%; yeast extract, 0.5%; K_2HPO_4, 0.1%; $MgSO_4 \times 7\ H_2O$, 0.02%; Na_2CO_3, 1%; phenolphthalein, 0.03%; methyl orange, 0.01%; and agar, 1.5% (Park *et al.* 1989). Incubation was performed at 37°C for 48 h. Formation of a yellow halo zone around the colony, resulting from the phenolphthalein-CD inclusion complex, was an indication of CGTase activity. Colonies surrounded by the largest yellow halo zones were transferred to an agar slant and stored at 4°C after incubation.

A β-CD-specific CGTase-producing strain was isolated from the soil sample. This isolate was taxonomically identified as *Bacillus circulans* using "Bergey's Manual of Systematic Bacteriology" (Logan & De Vos 2009) and named *Bacillus circulans* B65.

Production of crude CGTase extract. Production of CGTase was carried out by growing the isolates in liquid Horikoshi II medium of the following composition: soluble starch, 1%; peptone, 0.5%; yeast extract, 0.5%; K_2HPO_4, 0.1%; $MgSO_4 \times 7\ H_2O$, 0.02%; and Na_2CO_3, 1% (Horikoshi 1971). A loopful of culture was transferred from the agar slant to 100-ml Erlenmeyer flasks containing 20 ml of Horikoshi II medium. The culture was grown aerobically at 37°C with continuous shaking at 200 rpm for 24 h. Overnight culture (1 ml) was inoculated in 20 ml of fresh Horikoshi II medium. Growing was carried out for four days under the same conditions as for inoculum preparation. The supernatant obtained after centrifugation (10000g, 10 min, 4°C) was used for determination of amylolytic and cyclodextrinogenic activity.

Amylolytic activity. Amylolytic activity was measured using a slightly modified version of the method described by Matzuzawa *et al.* (1975). The reaction mixture containing 0.3 ml of 0.2% soluble starch in 0.1 M Na-phosphate buffer (pH 6.0) and 0.05 ml of the crude enzyme preparation suitably diluted in distilled water was incubated at 40°C for 10 min. The reaction was stopped by sequential addition of 1 ml of 0.5 M acetic acid and 0.5 ml of iodine reagent (0.02% J_2 in 0.2% KJ) to the reaction mixture. The mixture was diluted to 10 ml with distilled water and its absorbance was measured at 700 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 10% reduction in intensity of the blue colour of the starch-iodine complex per min under the conditions described above.

Cyclodextrinogenic activity. Cyclodextrinogenic activity was measured using the method with phenolphthalein described by Kaneko *et al.* (1987). The reaction mixture containing 1 ml of 4% soluble starch in 0.1 M Na-phosphate buffer (pH 6.0) and 0.1 ml of the enzyme suitably diluted in distilled water was incubated at 40°C for 20 min. The reaction was stopped by addition of 3.5 ml of 30 mM NaOH solution followed by 0.5 ml of phenolphthalein solution (0.02% phenolphthalein in 5 mM Na_2CO_3). After 15-min incubation at room temperature, absorbance was measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that formed 1 mg of b-CD per min under the conditions described above.

Production of CGTase in a laboratory bioreactor. Production of CGTase was achieved in a laboratory bioreactor (Chemap AG, Switzerland). The bioreactor vessel was in the form of a cylinder with 22-cm diameter and total volume of 14 L. The vessel was supplied with two turbine stirrers (with a diameter of 8 cm). The stirrers (disks with four flat spades 2 × 2 cm) were placed 8 cm and 16 cm over the bottom of the vessel. The vessel was supplied with four buffers 3 cm distant from the wall. Air was blown in through the nozzle under the stirrer. The air flow rate was measured with a rotameter. The bioreactor was supplied with a system for tracking and control of temperature, pH and dissolved oxygen. A loopful of culture was transferred from an agar slant to 450 ml of liquid Horikoshi II medium for the process of CGTase production in the laboratory bioreactor. Incubation was carried out at 37°C with continuous shaking at 200 rpm for 48 h.

The laboratory bioreactor with 9 L of liquid Horikoshi II medium was inoculated with 450 ml of overnight culture, after which incubation was carried out at 37°C

Table 1. Bacterial isolates with amylolytic activity.

Amylolytic activity [U/mL]	Number of isolates*	Percent of total isolates
< 1	4	8.9
1 – 2	17	37.8
2 – 3	11	24.4
3 – 4	6	13.3
4 – 5	4	8.9
5 – 6	1	2.2
6 – 7	1	2.2
> 7	1	2.2

*Amylolytic activity was determined for a total of 45 isolates.

Table 2. Bacterial isolates with cyclodextrinogenic activity.

Cyclodextrinogenic activity [U/mL]	Number of isolates*	Percent of total isolates
< 0.05	21	51.2
0.05 - 0.10	12	29.3
0.10 - 0.15	5	12.2
0.15 - 0.20	2	4.9
> 0.20	1	2.4

*Cyclodextrinogenic activity was determined for 41 isolates. It was not determined for 4 isolates with amylolytic activity less than 1 U/mL.

with a flow of sterile air of 0.5 vvm at a velocity of rotation of the stirrer of 300 min^{-1} for 96 h. During growth, samples were taken every 24 h for determination of amylolytic and cyclodextrinogenic activity of the supernatant. After growing for 96 h, bacterial cells were separated by centrifugation (10000g, 10 min, 4°C), and extracellularly produced CGTase was concentrated by ultrafiltration. The CGTase produced in such a way was used for further work.

The amount of reducing sugar was determined according to the method described by Miller (1959).

Production of cyclodextrins. Soluble starch (5% w/v) was incubated with CGTase from isolate B-65. CGTase was added to the starch solution to a final concentration of 6.0 U (cyclodextrinogenic activity) /g starch. Incubation was carried out at 40°C and pH 6.5 for 30 h. Samples were taken at regular time intervals, reactions were stopped by heating at 100°C for 10 min and the concentration of CDs was determined. The amount of a-, b- or g-CD was determined by HPLC (the employed Pharmacia-LKB HPLC instrument was equipped with a Spectra Physics 6040 differential refractometer as detector) using a 250mm YMC-Pack Polyamine II column. Analysis was conducted with acetonitrile: water (62:38) as the eluent at a flow rate of 1 ml/min and sample size of 50 ml, the column being at room temperature in the process.

RESULTS AND DISCUSSION

Isolation of CGTase-producing bacteria. Out of 2000 analysed colonies that produced CGTase, 45 were chosen for further work on the basis of size of the zones around the colonies. Supernatants obtained after growth in liquid Horikoshi II medium were used for examination of CGTase production by determination of amylolytic and cyclodextrinogenic activity. Most of the isolates had amylolytic activity between 1 and 2 U/mL. The number of isolates that showed greater amylolytic activity gradually decreased and activity higher than 5 U/mL was determined for only three isolates (Table 1). The cyclodextrinogenic activity of isolates ranged from 0.02 to 0.53 U/mL but was up to 0.10 U/mL for most of the analysed isolates. Only isolate B-65 had cyclodextrinogenic activity greater than 0.20 U/mL (Table 2). This isolate showed amylolytic activity of 12.2 U/mL and cyclodextrinogenic activity of 0.53 U/mL, which was considerably higher than in other isolates.

On the basis of morphological, physiological and biochemical characteristics, isolate B-65 was identified as *Bacillus circulans.*

Production of CGTase by Bacillus circulans B-65 in a laboratory bioreactor. Results obtained in determining CGTase production, cell growth and pH changes during growth of *B. circulans* B-65 in a laboratory bioreactor are shown in Table 3. The level of amylolytic activity was 23.4 U/mL, while cyclodextrinogenic activity reached a value of 0.60 U/mL. Isolate B-65 produced CGTase to a greater extent after the phase of active cell growth. Similar results were obtained for *B. circulans* var. *alkalophilus* (ATCC 21783) after growth in Horikoshi II medium, where production of CGTase was approximately 40% of the total amount that appeared in the medium after 48 hours of growth (Makela *et al.* 1990). Stavn & Granum (1979) recorded maximum CGTase production by *B. macerans* after 5 days. Maximum CGTase-production was also observed after 48 h of growth for *B. lentus* by Sabioni & Park (1992) and for *B. macerans* by Pocsi *et al.* (1998). Gawande *et al.* (1998) reported maximum CGTase production for *B. firmus* after 80 h of growth. Stefanova *et al.* (1999) found that the peak of CGTase secretion by *B. stearothermophilus* was at 48 h of growth when prespores, mature spores within the sporangium and lysis of sporangia were observed.

In contrast to these results, maximum activity of CGTase was recorded after 24 h of growth of *B. firmus* (Goel & Nene 1995), *B. subtilis* no. 1-7 (Georganta *et*

Table 3. Production of CGTase and change of pH during growth of isolate B-65.

Time [h]	CGTase activity [U/mL]		Cell growth [610 nm]	pH
	Amylolytic activity	Cyclodextrinogenic activity		
0	/	/	0.12	10
24	7.3	0.20	1.68	9.0
48	9.7	0.25	1.48	9.3
72	14.9	0.38	1.49	9.2
96	23.4	0.60	1.49	9.2

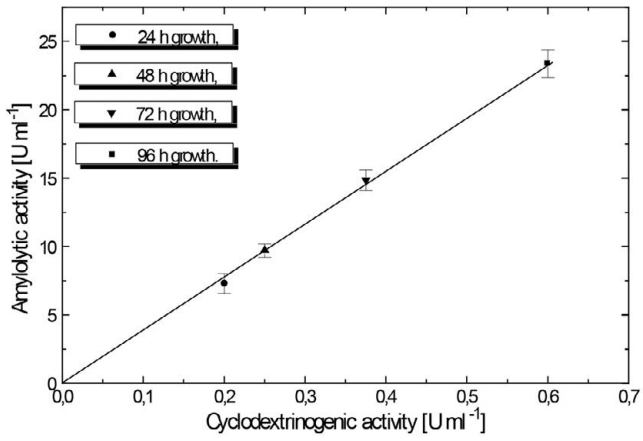

Figure 1. Amylolytic and cyclodextrinogenic activity during growth of *B. circulans* B-65.

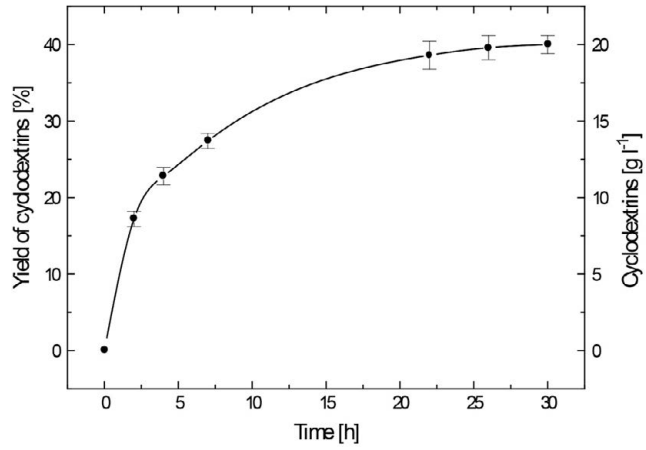

Figure 2. Cyclodextrin production by CGTase of *B. circulans* B-65. The yield of cyclodextrins corresponds to the percentage of starch converted to CDs.

al. 1993), *B. cereus* (Jamuna *et al*. 1993), *Bacillus* sp. BE101 (Lee & Kim 1991) and *B. macerans* (Lane & Pirt 1971).

Amylolytic and cyclodextrinogenic activity increase proportionally during growth of *B. circulans* B-65 (Fig. 1). On the basis of these results, it can be assumed that CGTase is the only amylolytic enzyme produced by *B. circulans* B-65. This was confirmed by determination of the content of CDs and reducing sugars during incubation of CGTase with starch. Our results showed that CDs were formed during the reaction, while the amount of reducing sugars did not increase, which would have happened in the presence of other amylolytic enzymes.

Production of cyclodextrins. Results obtained for production of cyclodextrins by *B. circulans* B-65 over the course of time are shown in Fig. 2. The enzymatic reaction of CD formation was faster at the outset of incubation, and the concentration of obtained CDs after 7 h of incubation was 13.7 g/L. With increase in the concentration of CDs in the reaction mixture, the rate of the enzymatic reaction of CD formation decreased, and the concentration of CDs after 22 h increased only insignificantly. After incubation for 30 h, CGTase converted 40% of starch to CDs, and the obtained CD concentration was 20 g l^{-1} in the reaction mixture. With a starch concentration of 5% (w/v), the yield of CDs obtained with CGTase from *B. cereus* (Jamuna *et al*. 1993) and *B. firmus* (Goel & Nene 1995) was 45 and 49%, respectively. When the starch concentration was 4.5% (w/ v), the yield of CDs was 37.5% with CGTase from *B. circulans* E192 (Bovetto *et al*. 1992). Analyses of produced CDs by HPLC showed that CGTase from *B. circulans* B-65 formed mainly b-CD. In the obtained CDs, b-CD was represented by 95%, while a-CD and g-CD were represented by 3 and 2%, respectively. This is a higher percentage of produced b-CD than values indicated by data from literature available to us (Qi & Zimmermann 2005; Szerman *et al*. 2007; Zhekova *et al*. 2009). It follows that using CGTase from *B. circulans* B-65 has advantages for production of b-CD in comparison with other CGTases characterised so far.

CONCLUSIONS

Our results show that CGTase from *B. circulans* B-65 is highly efficient in converting starch to CDs, even under non-optimized conditions. Also, this CGTase has high specificity for b-CD formation, producing about 95% b-CD. Furthermore, *B. circulans* B-65 does not produce

any amylolytic enzymes other than CGTase. The presence of these enzymes in CGTase preparations in the process of CD production would negatively affect the final yield of CDs through starch degradation.

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REZIME

Određivanje produkcije ciklodekstrina ciklodekstrin glikoziltransferazom iz alkalofilnog soja *Bacillus circulans* B-65

Slaviša Stanković, Dragan Pešić, Tanja Berić i Draga Simić

Iz 54 uzorka zemljišta izolovani su novi alkalofilni proizvođači enzima ciklodekstrin glikoziltransferaze iz roda *Bacilus*. Amilolitička aktivnost izolata je bila u opsegu od 0,4 do 12,2 U/mL, dok se efikasnost formiranja ciklodekstrina kretala od 0,02 do 0,53 U/mL. Izolat B-65 je pokazao najveću amilolitičku aktivnost i najveću efikasnost u formiranju ciklodekstrina. Prema morfološkim, fiziološkim i biohemijskim karakteristikama izolat B-65 je identifikovan kao *Bacillus circulans*. Maksimumi amilolitičke i aktivnosti formiranja ciklodekstrina postignuti su nakon 96 časova inkubacije *B. circulans* B-65 u laboratorijskom bioreaktoru. Nakon samo 30 časova inkubacije ciklodekstrin glikoziltransferaza je konvertovala 40% skroba u ciklodekstrine, u finalnoj koncentraciji 20 g/L. HPLC analiza dobijenih ciklodekstrina je pokazala da su β-ciklodekstrini bili zastupljeni sa čak 95% dok su ostatak činili α- i γ-ciklodekstrini.

Ključne reči: ciklodekstrini, ciklodekstrin glikoziltransferaza, *Bacillus circulans*