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Production of cyclodextrin from starch by cyclodextrin glycosyltransferase from *Bacillus firmus* and characterization of purified enzyme

DG Yim[1], HH Sato[1], YH Park[2] and YK Park[1]

[1]*State University of Campinas, College of Food Engineering (UNICAMP), Campinas, SP, CEP 13081-970, Brazil;* [2]*Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea*

During screening for cyclodextrin-forming microorganisms, an alkalophilic *Bacillus* sp, which produced high activity of cyclodextrin glycosyltransferase, was isolated and identified as *Bacillus firmus*. The crude enzyme transformed starch to mainly β-and γ-cyclodextrin. The purified enzyme had an optimum pH of 7.5–8.5 and its optimum temperature was 65°C, which is the highest optimum temperature as compared to other cyclodextrin glycosyltransferases except that produced by *Bacillus amyloliquefaciens*.

Keywords: cyclodextrin; cyclodextrin glycosyltransferase; starch; alkalophilic bacillus; *Bacillus firmus*

Introduction

Cyclodextrins are cyclic oligosaccharides that consist of 6, 7, and 8 D-glycosyl units united by α-1,4-glucosidic linkages and are designated as α-, β-, or γ-cyclodextrins [4,20,21]. The center of cyclodextrins are relatively apolar cavities and therefore, the cavities are hydrophobic. Thus, cyclodextrins easily form inclusion complexes with organic and inorganic compounds. This property of cyclodextrins can be utilized for medicine, cosmetics and foods [7,19].

Cyclodextrins are produced from starch by cyclodextrin glycosyltransferase (EC 2.4.1.19) and the proportions of α-, β-, and γ-cyclodextrins are dependent on the sources of microbial cyclodextrin glycosyltransferase [3,5,19]. Only α- and β-cyclodextrins are produced on an industrial scale because of low yields of γ-cyclodextrin by microbial cyclodextrin glycosyltransferase [13].

The objective of this research was to isolate new strains of cyclodextrin glycosyltransferase-producing microorganisms from nature, to select one strain which produces the highest activity of the enzyme for the production of cyclodextrins and to study some of the characteristics of the purified cyclodextrin glycosyltransferase from the microorganism.

Materials and methods

Microorganisms

Cyclodextrin glycosyltransferase-producing microorganisms were isolated using the method described by Park *et al* [16]. Approximately 1 g of soil samples from rice, corn, potato and tapioca fields were suspended in 10 ml of sterilized water and one drop of the suspension was inoculated onto a plate containing a basal culture medium comprised of 1% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% Na_2CO_3 (separately autoclaved), 0.03% phenolphthalein, 0.01% methyl orange and 1.5% agar and incubated at 37°C. Colonies producing enzyme formed clear zones on the plate. They were transferred to slant cultures containing the same culture medium but without phenolphthalein and methyl orange. The isolated strains were inoculated into 50-ml conical flasks containing 20 ml of the basal culture medium without agar. After cultivation at 37°C for 2 days, the culture media were centrifuged to obtain supernatant phases which were examined for cyclodextrin glycosyltransferase activity.

Determination of cyclodextrin glycosyltransferase activity

The enzyme activity was determined by mixing 0.1 ml of the enzyme solution with 0.9 ml of 2% starch solution in 50 mM Tris-HCl buffer, pH 8 and incubating the mixture at 65°C for 1 h. The enzyme activity was stopped by adding 0.1 ml of 0.5 N HCl and the mixture filtered through a Millipore HA membrane (pore size, 0.45 μm) and the quantities of cyclodextrins formed were analyzed by HPLC. One unit of α-, β- or γ-cyclodextrin-forming activity is defined as the amount of enzyme forming 1 μmole of the respective cyclodextrins per min per ml of enzyme solution, or per mg of proteins.

Production of the enzyme

The selected strain was cultivated in 50 ml of a modified culture medium consisting of 1% starch, 5% corn steep liquor (v/v), 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 1% Na_2CO_3, incubating the culture at 40°C for 2 days with agitation at 180 rpm. Then the culture was used as inoculum for a 1-L mini-fermentor (New Brunswick Co, Edison, NJ, USA) containing 900 ml of the same culture medium and 2 ml of corn oil as an anti-foam agent, and fermented at 35 or 40°C with agitation at 200 rpm and aeration of 1 vvm. Samples were taken periodically for the analysis of cell growth and enzyme activities.

Correspondence: YK Park, State University of Campinas, College of Food Engineering (UNICAMP), Campinas, SP, CEP 13081-970, Brazil
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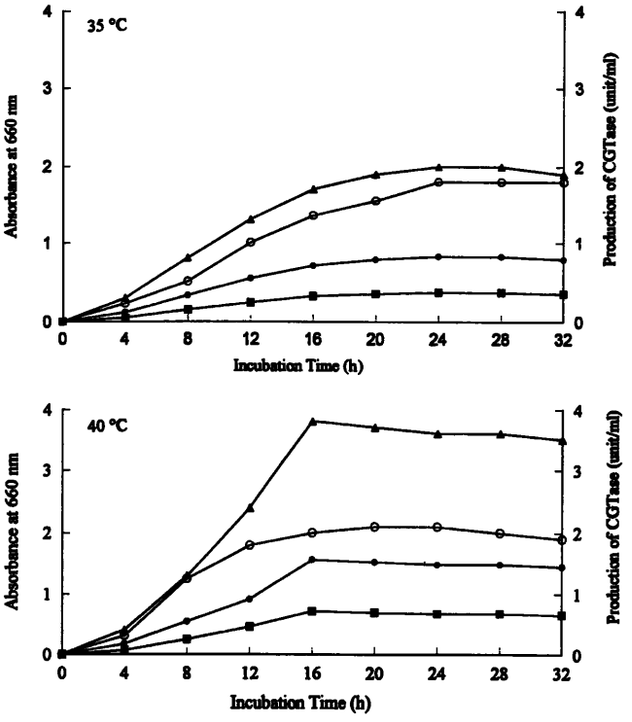

Figure 1 Time course of CGTase production by *B. firmus*. -○-, Cell growth; -■-, α-CGTase; -▲-, β-CGTase; -●-, γ-CGTase.

Table 1 Effect of starch concentration on the production of cyclodextrins by CGTase

Starch concentration (%)	Formation of cyclodextrins (%)			
	α-CD	β-CD	γ-CD	Total-CD
1	6.0	46.0	15.0	67
2	5.9	35.4	15.0	56.3
3	5.0	35.1	15.0	55.1
4	3.4	34.0	14.7	52.1
5	3.0	31.0	14.0	48.0
6	2.5	27.1	13.3	42.9

Cyclodextrins were quantified after 7 h enzyme reaction.

Analysis of cyclodextrins

Cyclodextrins were analyzed by HPLC with a differential refractometer detector (IR) and YMC-Pack polyamine-II column. Samples were applied to the column after a membrane filtration (MW cut-off YM 30 = 30 000 MW). The mobile phase was acetonitrile : water (75 : 25 v/v) with a flow rate of 1 ml min^{-1}.

Purification of the enzyme

The fermented culture medium (385 ml) was centrifuged to obtain the supernatant medium which was concentrated by ultrafiltration using the Micro-Thin Channel Ultra-filtration system-Model TCF2A (Amicon Co, Beverly, MA, USA). The concentrated supernatant phase was dialysed against deionized water and then against 0.05 M Tris-HCl buffer, pH 8.0. The dialysed supernatants were applied to a DEAE-Sephadex A-50 column, equilibrated with the same buffer. After this operation, all fractions which contained enzyme activity were combined and dialysed against the same buffer. The dialysed enzyme solution was applied to a DEAE-Sepharose CL-6B (Sigma Co, St Louis, MO, USA) column as described above and the enzyme fractions were dialyzed against deionized water, then freeze-dried.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out in a vertical slab gel apparatus according to the method of Laemmli [10]. Protein bands were stained with Coomassie blue R-250. Molecular weight marker proteins were phosphorylase b (94 000 Da), albumin (67 000 Da), ovalbumin (43 000 Da), carbonic anhydrase (30 000 Da), trypsin inhibitor (21 100 Da), and α-lactalbumin (14 400 Da).

Determination of protein concentration

Protein concentrations were determined by the method of Lowry *et al* [11].

Results and discussion

During screening for cyclodextrin-forming microorganisms, eight strains of alkalophilic *Bacillus* were isolated. Each was examined for production of cyclodextrin glycosyltransferase. Strain No. 324 produced the highest activities of β- and γ-cyclodextrin glycosyltransferases,

Table 2 Purification of CGTase from *B. firmus*

Purification step	Volume (ml)	Total protein (mg)	Total activity (units ml^{-1})			Specific activity (units mg^{-1} of protein)			Recovery of enzyme[a] (%)
			α-CGTase	β-CGTase	γ-CGTase	α-CGTase	β-CGTase	γ-CGTase	
Crude enzyme	385.0	2080	273.4	1463.0	604.5	0.1	0.7	0.3	100
UF-concentration	70.0	317	194.5	1041.1	430.2	0.6	3.3	1.4	71.2
DEAE-Sephadex	50.0	54	136.3	729.3	301.3	2.5	13.5	5.6	49.8
DEAE-Sepharose CL-6B	12.8	6.2	72.8	389.8	161.0	11.7	62.9	26.0	26.6

[a]Recovery of enzyme represented β-CGTase.

producing 0.57 and 0.19 units ml^{-1} respectively. This strain also produced a low level of α-cyclodextrin glycosyltransferase (0.05 units ml^{-1}) activity. Therefore, strain No. 324 was selected for further studies and was identified as *Bacillus firmus*.

Production of cyclodextrin glycosyltransferase was attempted by changing incubation temperatures and the nitrogen sources of the basal culture medium. The highest production of enzyme activity was obtained when 5% (v/v) corn steep liquor was substituted for yeast extract and peptone in the basal culture medium. Furthermore, the time course of enzyme production by *B. firmus* No. 324 was followed using the modified basal culture medium. Cultivation at 40°C produced high activity of cyclodextrin glycosyltransferases: 3.8 units β-, 1.6 units γ- and 0.6 units α-cyclodextrin glycosyltransferases (Figure 1). The maximum level of production of the enzyme was reached after 16 h of cultivation at 40°C while at 35°C it took 24 h to reach maximum production of the enzyme.

The effect of starch concentration on the production of cyclodextrins by the enzyme was examined by incubating the mixture of 90 ml of starch (1–6% based on 100 ml) in 0.05 M Tris-HCl buffer, pH 8 and 10 ml of crude enzyme containing 7.1 units α-, 38 units β- and 16 units γ-cyclodextrin glycosyltransferase, at 65°C with constant slow shaking. The results are demonstrated in Table 1. When the reaction mixture contained 1% soluble starch, conversion of starch was 67% which consisted of 6% α-, 46% β- and 15% γ-cyclodextrin; whereas 5% of soluble starch yielded only 48% cyclodextrins which consisted of 3% α-, 31% β- and 14% γ-cyclodextrins. According to these results, increased starch concentration reduced formation of α- and β-cyclodextrins, but formation of γ-cyclodextrin remained the same. The maximum production of β- and γ-cyclodextrins was attained after 7 h enzyme reaction. Furthermore, 5% starch substrate concentration produced 17 mg of β-, 7.5 mg of γ- and 1.5 mg of α-cyclodextrins per ml (Figure 2).

Purification of the enzyme

Enzyme purification led to a 90-fold purification of cyclodextrin glycosyltransferase with an enzyme recovery of 26.6% as shown in Table 2. The purified enzyme yielded a single band of protein by SDS-PAGE with an estimated molecular weight of 75 000 daltons. The purified enzyme had an optimum pH of 7.5–8.5 and it was stable between

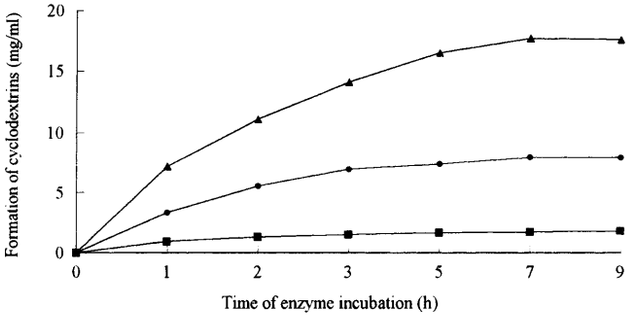

Figure 2 Production of cyclodextrins from starch by CGTase from *B. firmus*. -■-, α-CD; -▲-, β-CD; -●-, γ-CD.

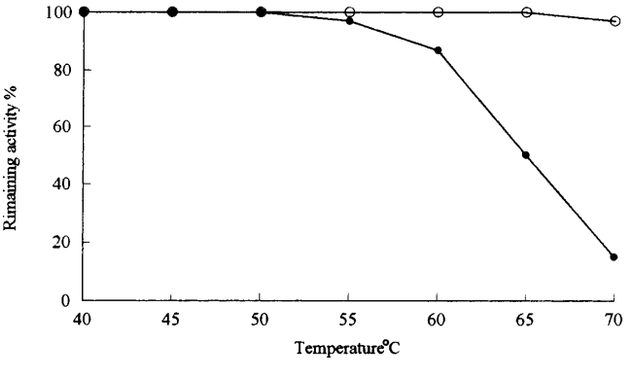

Figure 3 Temperature stability of CGTase activity. -●-, Control; -○-, 1 mM $CaCl_2$.

pH 6.5 and 9.0. The optimum temperature was 65°C. The temperature stability for the enzyme was 55°C but its thermostability was increased to 70°C by the addition of 1 mM $CaCl_2$ to the enzyme reaction mixture (Figure 3). As shown in Table 3, $HgCl_2$, $CuSO_4$, $CoCl_2$, or $ZnSO_4$ inhibited the enzyme when the reaction mixtures contained more than 1 mM of these compounds; 5 mM $FeSO_4$ also inhibited the enzyme. The other ten chemicals examined did not inhibit the enzyme at concentrations of 1–5 mM (Table 3).

Previously, production of cyclodextrin glycosyltransferase from *Bacillus lentus* was reported from our laboratory and the enzyme formed mainly β- and γ-cyclodextrins from starch [17]. Cyclodextrin glycosyltransferase production by *B. firmus* No. 324 was compared with the yield of the enzyme from *B. lentus*. *B. firmus* produced 0.08, 0.71 and 0.28 units ml^{-1} of α-, β- and γ-cyclodextrin glycosyltransferases respectively, whereas *B. lentus* produced 0.01, 0.26 and 0.1 units ml^{-1}. Thus, *B. firmus* produced approximately 2.7 times more β- and γ-cyclodextrin glycosyltransferases than *B. lentus*. Table 4 shows characteristics of cyclodextrin glycosyltransferases from 13 strains of bacteria. The enzyme from *B. firmus* No. 324 has a high optimum tem-

Table 3 Effect of metal ions and chemical reagents on CGTase activity

Metal ions and chemicals	Relative enzyme activity (%) at:	
	1 mM	5 mM
Control	100	100
$CoCl_2$	60	40
$MgSO_4$	100	100
$ZnSO_4$	20	15
$HgCl_2$	91	75
$CuSO_4$	80	50
Kcl	100	100
$FeSO_4$	100	83
$CaCl_2$	100	100
EDTA	100	100
p-Hydroxymercuribenzoate	100	100
Sodium bisulfate	100	100
L-cysteine	100	100
Sodium azide	100	100
2-Mercaptoethanol	100	100
N-Bromosuccinimide	100	100
Sodium arsenate	100	100

Table 4 Comparison of microbial CGTases

Microorganism	Optimum pH	Optimum temperature (°C)	Ratio of CD formed $\alpha : \beta : \gamma$	References
Bacillus macerans	5.2–5.7	55	2.7 : 1 : 1	[3,8]
Bacillus megaterium	5.2–6.2	55	1 : 2.4 : 1	[8]
Alkalophilic *Bacillus* sp	4.5–4.7	45	mainly β-CD	[12,14]
Bacillus ohbensis	5.5	50	mainly β-CD	[18]
Klebsiella pneumoniae	6.9	40	1 : 1.86 : 0.56	[1]
Bacillus sp HA 3-2-2	6.5–8.5	60	mainly β-CD	[15]
Bacillus amyloliquefaciens	6	70	mainly α-CD	[22]
Bacillus circulans	5.5	60	1 : 2.3 : 0.8	[2]
Bacillus sp BE101	6.0–6.5	45	1 : 7 : 2	[9]
Bacillus sp AL-6	8	60	mainly β e γ-CD	[6]
Bacillus lentus	6.5–7.5	55	mainly β-CD	[17]
Brevibacterium sp 9065	10	45	mainly β e γ-CD	[13]
Bacillus firmus	7.5–8.5	65	1 : 11 : 5	This work

perature (65°C) which is desirable for industrial process, and the enzyme is adequate for production of β- and γ-cyclodextrins.

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