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Encapsulation of Whole Cell CGTase from Concentrated Broth Solution

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Abstract Most of the Cyclodextrin glucanotransferase (Gtases) which have been produced from *B. subtilis* were found to be excreted from the cells during cultivation. Immobilized whole cell CGTase from *B. subtilis* was prepared by encapsulating the broth solution which had been concentrated ten times with a rotary vacuum evaporator. Cyclization activity of CGTase was reduced by about 10% during the concentrating process, however, its transglycosylation activity, to convert xylitol to glucosyl-xylitol, using dextrin as glucosyl donor, increased by a factor of 3 or 5.

Keywords: *Bacillus macerans*, concentrated broth solution, encapsulated whole cell CGTase, glucosyl-xylitol

CGTase catalyzes, the cyclization of starch to cyclodextrin, the hydrolysis of starch and cyclodextrin, the coupling of cyclodextrin, and the disproportionation of malto-oligosaccharides. Oligoglucosyl-sugar alcohols, which are produced from sugar alcohols, such as, sorbitol, mannitol, maltitol, xylitol, inositol by the transglycosylation, are considered to be more resistant to microbial degradation than conventional sugars, and the hygroscopic and less cariogenic-like sugar alcohols [1-5]. The maltosylinositol synthesized from myoinositol as an acceptor and β-cyclodextrin as a glucosyl donor, using the transglucosylation reaction of CGTase from *Bacillus ohbensis*, exhibited a growth stimulating effect on *Bifidobacterium* [1,2]. The glucosyl-xylitol synthesized by the transglycosylation reaction of sucrose phosphorylase from *Leuconostoc mesenteroides* reduced the amount of water insoluble glucan synthesized by *Streptococcus mutans* and maintained a neutral pH in the cell suspension [3]. The transglucosylated xylitol, which was obtained by attaching one or two glucose molecules to a xylitol with the aid of CGTase, showed an increased stimulating effect on the growth of *Bifidobacterium breve* compared to xylitol, indicating the possibility that it could be utilized as a new alternative sweetners with bifidogenic effects [4,5].

Enzymes are isolated from broth solutions by complex separation processes, and the purified enzymes should be immobilized for easy handling and recovery. The *in-situ* whole cell enzyme immobilizing method was developed and generally allows a high recovery of enzymatic activity [6]. Although low volumetric activity is inevitable in the whole cell enzyme immobilization process, the total cost for preparing immobilized enzymes might be reduced by shortening the isolation and purifying processes, because the cost of isolating enzymes from the cell broth solution represents about 70 percent of the total enzyme production cost. The whole cell immobilized CGTase which was prepared by immobilizing *Bacillus macerans* by the gel entrapment method, using calcium alginate, agar, gelatin, and polyacrylamide did not catalize as effectively as free cell enzyme and, furthermore, immobilized cells caused liquefaction of gelatin beads at all gelatin concentrations used [7]. *Bacillus macerans* inocculated into a calcium alginate capsule could not grow to high cell density [8] though the inoculated *E. coli*, *C. glutamicum*, and *S. cerevisiae* grew inside the capsule and the dry cell densities reached 100 or 300 g/L on the basis of inside space of the capsule [9-14]. Most of the enzymes that have been used to catalyze transglucosylation are extracellular although 80 percent of the fructotransferase from *Aureobasidium pullulans* was distributed inside the cell membrane [15]. The immobilized whole cell fructotransferase prepared by immobilizing *Aureobasidium pullulans* (KFCC 10245) in a calcium alginate bead was used to catalize transfructosylation for 100 days and 65 percent of the enzyme activity was extracellularly sourced [16]. In this study, we prepared encapsulated whole cell CGTase using a broth solution of *Bacillus macerans* which was concentrated by a factor of ten in a rotary vacuum evaporator and then investigated the reduction of CGTase activity in terms of synthesizing β-CD and converting xylitol to glucosyl-xylitol during the concentration procedure.

Bacillus macerans (IFO 3490) was cultured to produce cyclodextrin glucanotransferase (CGTase). The cells were cultivated in the growth medium containing organic nitrogen sources, tryptone and yeast extract, because inorganic nitrogen sources do not participate in the production of CGTase from *B. macerans* [17]. The

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composition of growth medium was; 20 g soluble starch, 10 g tryptone, 10 g NaCl, 5 g yeast extract, and 5 g $CaCl_2$ per liter of medium solution. The pH of the medium was controlled by 1 M NaOH addition. The cells inocculated with a 10 %(v/v) broth solution were cultivated at 37°C, 200 rpm for 24 h in a shaking flask incubator. Broth solution was concentrated in a rotary vacuum evaporator (EYELA, Japan). The glass evaporator containing 500 mL of broth solution was rotated at 100 rpm and at 40°C. The concentrated broth solution was then mixed with 3.2(w/v)% $CaCl_2$ solution containing 0.52 (w/v)% Xanthan gum at the volumetric mixing ratio of 1:1. This mixture was added dropwise into a swirling 0.6 (w/v)% sodium alginate solution as described in the literature [9,18] in order to make the calcium alginate capsules containing the concentrated broth solution as a liquid core. The activity of CGTase in terms of synthesizing β-CD was determined using the primitive and the concentrated broth solution as follows; a mixture of 0.4 mL of 50 mM tris-maleate-NaOH buffer solution and 0.5 mL of 5% dextrin solution was added into 0.1 mL of primitive broth solution or one that had been diluted by a factor of ten with distilled water after the concentration process. The cyclization of dextrin was catalyzed at 50°C for 30 min and colorized with phenolphthalein. The concentration of the β-CD was calculated by measuring the transmittance of the solution at 550 nm. A standard correlation curve was prepared with commercial β-CD (MW 1135.0, Simga Chemical Co., USA). One unit of activity was defined as the amount of enzyme required to produce 1 μM of β-CD per min. Glucosyl-xylitol was synthesized by the transglucosylation reaction using CGTase in the broth solution. The glucosyl acceptor was xylitol (MW 152.1, Sigma Chemical Co., USA) and the donor was dextrin (Shindongbang, Dex 150, Korea). The size distribution of the commercial dextrin is shown in Table 3. 3 g of dextrin and 3 g of xylitol were solved in 60 mL of tris-maleate-NaOH buffer solution prior to mixing this solution with 40 mL of the broth solution. Calcium alginate capsules containing 5 mL of the concentrated broth solution were added to 100 mL of the reaction mixture, which was prepared by solving 5 g xylitol and 10 g dextrin in the tris-maleate-NaOH buffer solution (pH 6.0, 20 mM). The transglucosylation reaction was carried out at 50°C and 200 rpm for 12 h. 0.5 mL of the sample solution was then mixed with 4.5 mL of distilled water and heated for 10 min in order to stop the reaction. The conversion of xylitol to glucosyl-xylitol was monitored by measuring the con-centration of xylitol before and after the reaction by high performance liquid chromatography (HPLC Model-305, Gilson Medical Electronics, Inc., France). The solvent and sample solution were prefiltered. Cosmosil $5NH_2$ packed column (Nacalai Tesque, Inc., Japan) and RI detector were used and the flow rate of the solvent mixture of acetonitril and water (65:35) was set at 1.0 mL/min.

The amount of CGTase distributed in the medium and inside the cell membrane is described in Table 1, for inoculated cells cultivated in a shaking incubator at 37°C, and 200 rpm for 3 days with the pH of the medium at 5, 6, 7, 8, and 9. The total amount of CGTase produced during cultivation increased as pH of the medium increased, and the portion of extracellular enzyme also increased reaching 90 percent. If the portion of the extracellular enzyme is as large as this, the in-situ whole cell enzyme encapsulation method may not be useful even though the intracapsular dry cell densities of *S. cerevisiae*, *E. coli*, and *C. glutamicum* were as high as 100-300 g/L. We try to encapsulate the concentrated broth solution of *B. macerans* as an alternative immobilization method for the whole cell enzyme. The encapsulation process avoids the enzyme isolation and purification steps and retains the benefits of whole cell enzyme immobilization. The broth solution which had been obtained after 1 day of culture changed from a yellow liquid to a brown gel when it was concentrated ten times. The reduction of CGTase activity to catalyze the conversion of dextrin to β-CD during the concentration process was about 10 percent, as shown in Table 2.

Table 1. The distribution of CGTase activity to produce β-CD

pH of the growth medium	Total CGTase (unit/mL)	Intracellular CGTase (unit/mL)	Extracellular CGTase (unit/mL)
5	0.105	0.055	0.050
6	0.230	0.052	0.178
7	0.395	0.062	0.333
8	0.835	0.075	0.760
9	0.783	0.070	0.713

Table 2. The production of β-CD by CGTase which remains in the broth solution before and after concentration

	β-CD production (mg/mL)	
	Broth solution	Concentrated broth solution
Sample 1	0.97	0.86
Sample 2	1.07	1.00
Sample 3	1.01	0.82
Average	1.02	0.90

The optimum conditions for making spherical capsules containing the concentrated broth solution were a little different from those required for making capsules containing inoculated cells [9], because the concentrated broth solution was in the gel state. The core solution was prepared by mixing 3.2% (w/v) $CaCl_2$ solution containing 0.52% (w/v) xanthan gum and the concentrated broth solution in a 1:1 (v/v) ratio. 100 mL of the 0.6% (w/v) sodium alginate solution containing the nonionic surfactant nonoxynol, was stirred at the speed which introduced a cavity of depth about 45% of the height of the alginate solution in the 250 mL beaker. The constant air flow rate of 5 L/min at the annulus of the concentric double needles, which is described in the literature [14], kept the capsule size as 2-2.4 mm.

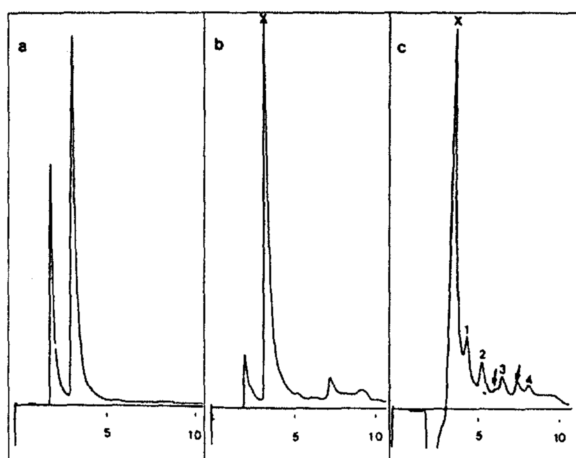

Fig. 1. HPLC chromatogram of the product solution obtained by the transglycosylation reaction, which was carried out using xylitol as the glucosyl acceptor and dextrin as the glucosyl donor by the broth solution containing extracellular CGTase. Reaction condition : 50°C, 200 rpm, 100 mL reaction mixture/250 mL flask, 5% (w/v) xylitol, 5% (w/v) dextrin. Frame a: xylitol solution, b: before reaction, c: after 15 h reaction peak 1: glucose, 2: maltose, 3: maltotriose, 4: maltotetraose x: xylitol, ✓: glucosyl-xylitol(G2-X, G3-X).

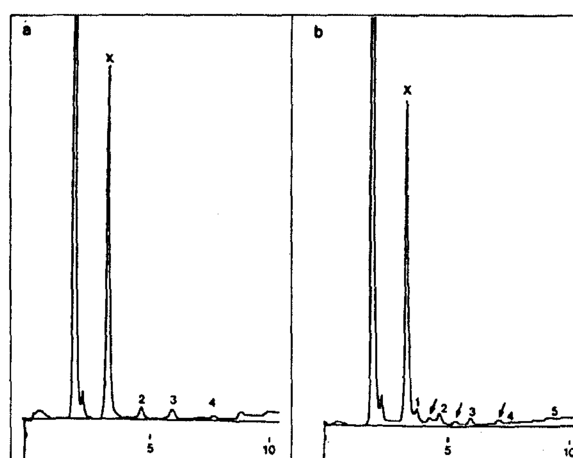

Fig. 2A. HPLC chromatogram of the product solution obtained by the transglycosylation reaction which was carried out using xylitol as the glucosyl acceptor and dextrin as the glucosyl donor by capsules containing the concentrated broth solution. Reaction condition: pH 6, 50°C, 150 rpm, 100 mL reaction mixture/250 mL flask; Frame a: after 20 min reaction, b: after 12 h reaction; Peak 1: dextrose, 2: maltose, 3: maltotriose, 4: maltotetraose, 5: maltopentaose, x: xylitol, ✓: glucosyl-xylitol(G1-X ,G2-X, G3-X) glucosyl donor: 2.5% (w/v) dextrin, acceptor: 5% (w/v) xylitol.

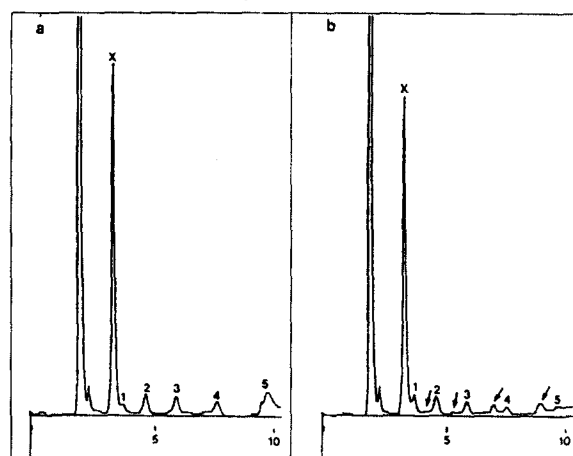

Fig. 2B. Glucosyl donor: 5.0% (w/v) dextrin, acceptor: 5% (w/v) xylitol.

Encapsulated whole cell CGTase was applied to catalyze a transglucosylation reaction in which xylitol was used as the glucosyl acceptor and dextrin as the glucosyl donor. The production yield of glucosyl-xylitol which was produced using encapsulated whole cell CGTase was compared with that produced by the free CGTase in the primitive broth solution which was obtained after 2 days of culture. The HPLC chromatogram of the reaction mixture in which the CGTase of the primitive broth solution catalyzed the transglucosylation reaction is shown in Fig. 1. The peaks of di- and tri-glucosyl xylitol in the right frame are distinguished from those of xylitol and dextrin in the middle frame. The G1 peak (glucosyl unit 1: glucose), which was not found in the HPLC chromatogram of dextrin appeared after transglucosylation because of the alternative hydrolysis function of CGTase. The conversion of xylitol to glucosyl xylitol during the transglucosylation reaction for 15 h was 2 percent. The chromatogram of the reaction mixture after the glucosylation reaction, which was carried out for 12 h using encapsulated whole cell CGTase in the reactant solutions, and which contained xylitol and dextrin at different ratios, is shown in Fig. 2. The chromatogram in the left frame is that of the reaction mixture which was obtained after the reaction was carried out for 20 min using encapsulated whole cell enzyme. Conversion of the xylitol was much higher than that obtained when the free CGTase was used. Nine and half percent of the xylitol was converted to glucosyl-xylitol when the reactant mixture was 5% dextrin and 5% xylitol. The use of the encapsulated whole CGTase in creased the conversion of xylitol by a factor of 5, that means that the concentration process of the broth solution increased the activity of CGTase to catalyze the transglucosylation by a factor of more than 5 than that of the free CGTase in the broth solution, if we consider the mass transfer resistance through the capsule membrane [14]. The increase of CGTase activity for the transglucosylation might be caused by the geometrical modification of the enzyme during the concentration process, this is partially related to the phenomena that the kinetic constants of CGTase to convert starch to cyclodextrin were changed in super-

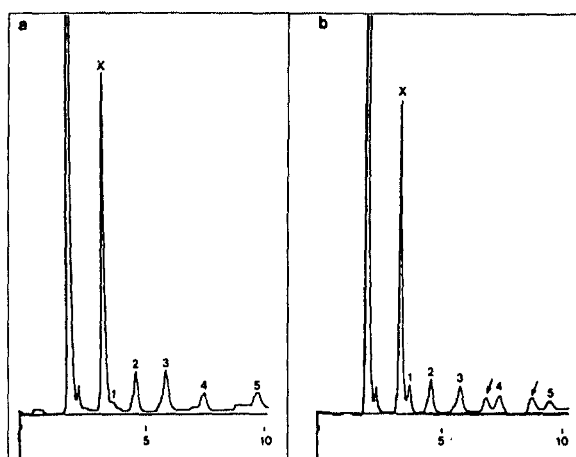

Fig. 2C. Glucosyl donor: 10(w/v)% dextrin, acceptor: 5(w/v)% xylitol.

Table 3. The size distribution of the commercial dextrin used in the cycling and transglycosylation reactions

unit	G1	G2	G3	G4	G5	G6	G7	G8	G9	>G10	Total
%	1.63	6.28	9.55	5.67	6.90	11.56	8.66	3.85	4.98	40.92	100%

Table 4. The increase of the transglycosylation activity of CGTase in the concentrating process

Broth solution			Capsules containing concentrated broth solution		
Xylitol (g/L)	Dextrin (g/L)	Conversion of xylitol (%)	Xylitol (g/L)	Dextrin (g/L)	Conversion of xylitol (%)
50	50	2.0	50	25	6.6
			50	50	9.5
			50	100	7.8

critical carbon dioxide($SC-CO_2$) (19): some enzymes maintain their activity in $SC-CO_2$ at 100 atm (20-22): activities of other enzymes increased a little in $SC-CO_2$ (23). As shown in Table 4, when the xylitol concentration was fixed at 50 g/L and dextrin was changed from 25 to 100 g/L, the conversion of xylitol increased and the maximum value was obtained at a mixing ratio of 1:1 (g of xylitol: g of dextrin) as reported in the case of maltitol to glucosyl maltitol conversion [4]. The conversion of xylitol were 6.6 percent in a reactant solution composed of 2.5% dextrin and 5% xylitol and 7.8 percent in a solution of 10% dextrin and 5% xylitol. The number of attached glucosyl units of glucosyl-xylitol (Gn-X, Gn: glucosyl unit, X: xylitol) increased as the ratio of the dextrin to xylitol increased. When the concentration of dextrin was 2.5%, the peaks G1-X, G2-X, G3-X were found, and G3-X and G4-X were dominant products for the 5 and 10% dextrin solutions. The concentrated broth solution can be easily encapsulated in the calcium alginate membrane with high intensity of CGTase concentration because β-cyclodextrin producing activity was slightly lower and that for transglucosylating xylitol was increased some 4-5 times over the concentration process. This new technology to encapsulate whole cell extracellular enzymes using the concentrated broth solution provides an alternative method for whole cell enzyme immobilization and the enhancement of CGTase activity using the vacuum evaporation process should be investigated under other experimental situations.

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