Immobilization of Cyclodextrin Glucanotransferase on Amberlite IRA-900 for Biosynthesis of Transglycosylated Xylitol

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Abstract Cyclodextrin glucanotransferase (CGTase) from *Thermoanaerobacter* sp. was adsorbed on the ion exchange resin Amberlite IRA-900. The optimum conditions for the immobilization of the CGTase were pH 6 0 and 600 U CGTase/g resin, and the maximum yield of immobilization was around 63% on the basis of the amount ratio of the adsorbed enzyme to the initial amount in the solution. Immobilization of CGTase shifted the optimum temperature for the enzyme to produce transglycosylated xylitol from 70°C to 90°C and improved the thermal stability of immobilized CGTase, especially after the addition of soluble starch and calcium ions. Transglycosylated xylitol was continuously produced using immobilized CGTase in the column type packed bed reactor, and the operating conditions for maximum yield were 10%(w/v) dextrin (13 of the dextrose equivalent) as the glycosyl donor, 10%(w/v) xylitol as the glycosyl acceptor, 20 mL/h of medium flow rate, and 60 °C. The maximum yield of transglycosylated xylitol and productivity were 25% and 7.82 $g \cdot L^{-1} h^{-1}$, respectively. The half-life of the immobilized CGTase in a column type packed bed reactor was longer than 30 days.

Keywords transglycosylated xylitol, transglycosylation, cyclodextrin glucanotransferase, immobilization, Amberlite IRA-900, *Thermoanaerobacter* sp.

INTRODUCTION

Xylitol is a natural sugar alcohol composed of five carbons, and sweet as sugar with no after-taste. It exhibits higher heat stability and endothermic heat during solubilization due to its structural lack of a free carbonyl group compared to other mono- or di-saccharides. It is also more resistant to microbial degradation and is extremely hydroscopic. It has 40% less calories than sugar, and thus can be safely used for diabetics [1]. The most beneficial aspect of xylitol utilization can be found in its effect on dental health because plaque bacteria, *Streptococcus mutants,* cannot utilize xylitol. *Streptococcus pneumoniae,* the bacterium causing ear and respiratory infections, is also unable to utilize xylitol. Accordingly, xylitol is currently used in chewing gum, confectionery, pharmaceuticals, oral hygiene products, and diabetic foods [2].

Cyclodextrin glucanotransferase (CGTase) is an enzyme that catalyzes the transglycosylation reaction, which transfers various glycosyl units into glycosyl acceptors, such as sugars, glucose, sucrose [3], xylose,

sorbose [4], stevioside, ascorbic acid [5], and hesperidin [6]. These glycosyl products are attractive in two viewpoints; one is related to the improvement of physiochemical properties and the other is the possibility of obtaining functional oligosaccharides [7]. CGTase has also been used for the transglycosylation of sugar alcohols such as the oligoglucosyl-inositol from myo-inositol [8], which exhibits a growth stimulating effect on *Bifidobacterium* showing physiological functionality [9]. In the previous studies by the current authors [10,11], two glycosyl-sugar alcohols, *i.e.,* glycosyl-maltitol and transglycosylated xylitol, were synthesized with the aid of CGTase. The structural characteristics of the transglycosylated xylitols were analyzed by NMR and these bioproducts showed a growth stimulation effect on *Bifidobacterium breve,* thereby showing their potential to be alternative sweeteners.

CGTase has been immobilized by the adsorption method; the adsorption of the CGTase from alkalophilic *Bacillus* sp. on Diaion HP-20 [12] and its characterization in a column type bioreactor were investigated for the optimization of the operation conditions for cyclodextrin (CD) production [13]. The CGTase from *Bacillus macerans* was adsorbed on IRA-93 and immobilized in a polysulfone capillary membrane for CD production [14,15]. In the previous studies by the current authors, the CGTase from *Bacillus macerans*

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was adsorbed on Amberlite IRA-900, and the perform-ance of the column type bioreactor containing immobi-lized enzymes was evaluated with respect to the pro-duction cost of CD [16,17]. The utilization of immobi-lized CGTase for a coupling reaction to transfer the glycosyl units of the donor molecules to the accepter molecules by inter-transglycosylation has been rarely examined although many literatures report the produc-tion of CD by the immobilized CGTase. Only one study reports the CGTase from *Bacillus macerans* ad-sorbed on Diaion™ HPA75 used for the production of transglycosylated stevioside [18].

In this work, the CGTase from *Thermoanaerobacter* sp was adsorbed on Amberlite IRA-900 for the produc-tion of transglycosylated xylitol and the optimum conditions of the CGTase immobilization, such as the pH of the solution and the mixing ratio of the enzyme to the Amberlite resin, were determined Stability and activity of the immobilized enzyme were also investi-gated. The effect of the variation of operating condi-tions of the column type packed bed reactor containing immobilized CGTase on the yield of transglycosylated xylitol was investigated along with the stability of the immobilized enzyme in a column type packed bed reac-tor. This research will not only facilitate in the develop-ment of an effective method for the immobilization of CGTase used for an inter-transglycosylation reaction but also improve the process for the efficient produc-tion of transglycosylated xylitol.

MATERIALS AND METHODS

Enzyme and Substrates

The crude CGTase from *Thermoanaerobacter* sp. (102 units/mg of protein, Novo-Nordisk Co., Denmark) was further purified using an ultrafiltration kit (MWCO 50,000, Amicon Co., USA) to remove impuri-ties. The purified CGTase showed a specific activity of 132 units/mg of protein. Xylitol (Sigma Chemical Co., USA) was used as the glycosyl acceptor and soluble starch (Sigma Chemical Co., USA) and dextrins (Al-drich Co., USA and Sindongbang Co., Korea) were used as the glycosyl donor.

Measurements of CGTase Activities

The coupling activity of CGTase was determined by a modified Yamamoto's method [19]. one milliliter of 5 mM β-CD, 25 mM sucrose and 0.1 mL of 10 mM Tris-Malate-NaOH buffer (pH 6.0) solution containing the purified CGTase were mixed and incubated at 50°C for 30 min. The CGTase was removed by centrifugal ultra-filtration, then the reaction mixture was mixed with 5 units of amyloglucosidase (Sigma Chemical Co., USA) and further incubated for 30 min at 50 °C. The amount of glucose formed was measured using a glucose assay kit (Dongbang Co., Korea), and one unit of activity was defined as the amount of enzyme producing 1 mM of glucose per min.

Soluble starch was used as the substrate to measure the cyclization activity of CGTase. one milliliter of 10 mM Tris-Malate-NaOH buffer (pH 6.0) solution con-taining 10%(w/v) soluble starch was mixed with 0.1 mL of the purified CGTase solution, then incubated at 50°C for 30 min. The produced CDs were analyzed with HPLC, and one unit of activity was defined as the amount of enzyme producing 1 μM of CDs per min.

The hydrolysis activity of CGTase was determined by the method of Kitahata [20]. 10 μL of the purified CGTase solution was mixed with starch (1.0% w/v) dissolved in 1.0 mL of 20 mM Tris-Malate-NaOH buffer (pH 6.0), and incubated for 30 min at 37°C. One unit of activity was defined as the amount of CGTase that increased 1.0% in transmittance at 660 nm per min.

Immobilization of CGTase

Amberlite IRA-900, the strong basic anion exchanger on polystyrene, was washed with 0.5 N HCl solution and 0.5 N NaOH alkaline solution to activate the resin itself, then suspended in 100 mL of 10 mM Tris-Malate-NaOH buffer (pH 6.0) for equilibration. In order to immobilize enzyme on the resin, 10 g of the activated Amberlite IRA-900 was mixed with 6,000 units of the CGTase dissolved in 100 mL of 20 mM Tris-Malate-NaOH buffer (pH 6.0) and incubated at 30°C and 150 rpm for 2 h. The optimum pH for the CGTase immobi-lization was investigated using the following buffer solutions: 10 mM Glycine-HCl (pH 3.0), 10 mM Cit-rate (pH 4.0-5.0), 10 mM Tris-Malate-NaOH (pH 6.0), 10 mM KH_2PO_4-K_2HPO_4 (pH 7.0-8.0), 10 mM Tris-HCl (pH 9.0), and 10 mM Glycine-NaOH (pH 10.0). The mixing ratio of the CGTase to the resin in the buffer solution varied from 200 to 1,200 units of CGTase per g of resin.

pH and Thermal Stabilities of Immobilized CGTase

The pH stability was investigated by measuring the residual coupling activities of the immobilized and the free CGTases in various buffer solutions at 50°C for 1 h. To measure the thermal stability of an enzyme, the residual activity was measured just after a 30-min pre-incubation at temperature ranging from 50 to 100°C. Additives of 10 mM calcium ions and 0.1% (w/v) solu-ble starch were used in the expectation of an increase in the thermal stability.

Operation of Column Type Packed Bed Reactor

The column type packed bed reactor was composed of a water-jacketed glass column (2.2 cm ID, 33.0 cm height), flow-rate controller, and water bath. The glass column was packed with 80 g of Amberlite IRA-900 adsorbing 354 units of CGTase per g of resin itself. Just after packing resins in the glass column, 10 mM Tris-Malate-NaOH buffer (pH 6.0) was made to pass through the column to eliminate any unadsorbed CGTases. A

substrate solution containing 10% (w/v) dextrin and 10% (w/v) xylitol dissolved in a buffer passed through the column type packed bed reactor at a flow rate ranging from 10-50 mL/h, and the transglycosylated xylitol produced in the column at 60°C was detected by HPLC.

Analytical Methods

Xylitol and transglycosylated xylitol were analyzed by HPLC system (Gilson Medical Electronics, Inc., France): Cosmosil-packed column 5NH$_2$ (Nacalai Tesque, Inc , Japan), acetonitrile/water (65/35), 1 0 mL/min, and RI detector. The protein concentration was analyzed by the Bradford method [21].

RESULTS AND DISCUSSION

Immobilization of CGTase on Amberlite IRA-900

The CGTase purified by ultrafiltration was adsorbed on Amberlite IRA-900 resins. The well adjusted pH of the solution during immobilization may improve the yield of immobilization and also the stability of immobilized CGTase. The effect of the pH control on the immobilization of the free CGTase and the activity of the adsorbed CGTase was examined by varying the pH of the solution from 3.0 to 10.0 and is illustrated as the data in Fig. 1(a). The amount of the adsorbed CGTase increased proportionally up to pH 6.0, due to the increment the negative charge of CGTase according to the pH value, and thereafter, the adsorbed protein reached plateau at 4.2 mg of protein per gram of resin. The activity of the adsorbed CGTase also increased proportionally up to pH 6 0 with the increase in the adsorbed CGTase; however, it decreased rapidly at pHs higher than this level, due may be to the denaturation of the CGTase at the high pH because the optimal pH of enzyme activity is 5.8 [22]. The optimum pH for the immobilization of the CGTase without denaturation was about 6.0.

The mixing ratio of CGTase to Amberlite IRA-900 was also found to affect the yield of immobilization and Fig. 1(b) illustrates how the mixing ratio of the CGTase to the Amberlite IRA-900 affects on the activity of the adsorbed CGTase and the adsorption yield. 10 g of Amberlite IRA-900 resin was mixed with various units of CGTase ranged from 2,000 to 12,000 The activity of the adsorbed CGTase increased proportionally with the amount of the loaded CGTase up to 600 units per gram of resin, and thereafter remained at a constant level. From the yield data shown in Fig. 1(b), it can be easily found that the amount of adsorbed CGTase was constant and the resin was saturated with adsorbed enzyme at a mixing ratio higher than 600 units per gram of resin. The saturation of the resin with enzymes resulted in the constant level of enzyme activity. It can be easily concluded from the linear relationship between the activity of the immobilized enzyme and the enzyme loading at the mixing ratio lower
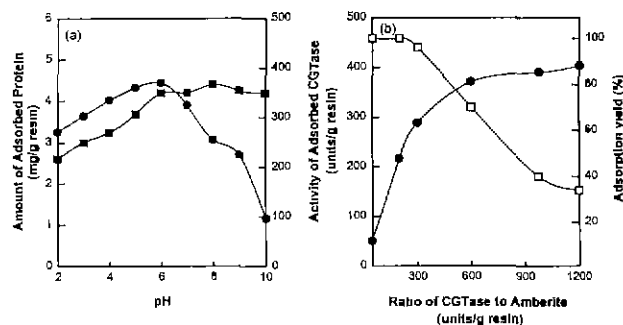

Fig. 1 Effect of pH (a) and the mixing ratio of CGTase and Amberlite IRA-900, (b) on immobilization of thermostable CGTase on Amberlite IRA-900 (a) 6,000 units of CGTase were incubated with 10 g of Amberlite IRA-900, equilibrated with various buffers of different pHs, at 30°C for 2 h, and the amounts of protein and CGTase activities in the resins were measured. (b) various amounts of CGTase ranging from 200 to 1,200 units of CGTase per g of resin were immobilized on 10 g of Amberlite IRA-900, equilibrated with 10 mM Tris-Malate-NaOH buffer (pH 6 0), at 30°C for 2 h, and the enzyme activities of the resins were measured. (■) amount of adsorbed protein, (●) activity of adsorbed CGTase, (□) adsorption yield.

than 300 units per gram of resin that there was no significant mass transfer resistance of substrate on the resin surface. The optimum mixing ratio of CGTase to Amberlite IRA-900 for the maximum activity of immobilized CGTase was determined as 600 units of CGTase per gram of resin and the corresponding adsorption yield was 63%.

Enzymatic Properties of Immobilized CGTase

The activities and stabilities of the free and immobilized CGTase at different pHs ranged from 3.0 to 9.0 were measured and shown in Fig. 2. We found that the optimum pH and pH stability did not change significantly even after immobilization. The effect of the reaction temperature on the three kinds of enzyme activities of the immobilized CGTase was also examined. As shown in Fig. 3, the activities of immobilized CGTase for three kinds of reactions, i.e., coupling, cyclization, and hydrolysis, varied according to temperatures ranged from 50°C to 100°C. The optimum temperature for the coupling reaction shifted from 70°C to 90°C after immobilization, however, those for the hydrolysis and cyclization reactions increased from 80°C to 90°C. The increase in the optimum temperature may be caused by the fact that there is a temperature gradient in the resin and the temperature inside the resin is correspondingly lower than the bulk temperature. However, the optimum temperature for the coupling reaction was shifted remarkable and this may be caused by an improvement in the thermal stability and the enforcement of the coupling activity of CGTase due to the conformational change through the immobilization of CGTase on the strong anion exchange resin. The
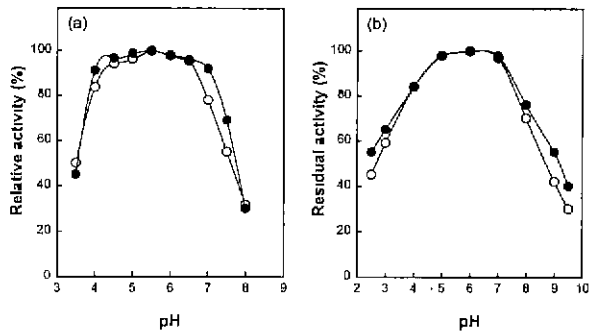
Fig. 2. Effect of pH on activity (a) and pH stability (b) of soluble and immobilized CGTase. (a) The coupling activities of the soluble and immobilized CGTase were measured at various pHs. (b) The residual coupling activities of the soluble and immobilized CGTase were measured after incubation in various pHs at 50°C for 1 h. (O) soluble CGTase; (●) immobilized CGTase.
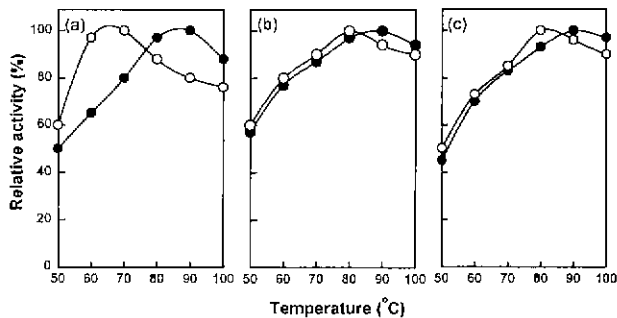

Fig. 3 Effect of temperature on coupling (a), cyclization (b), and hydrolysis (c) activities of the soluble and immobilized CGTase. The coupling, cyclization and hydrolysis reactions were measured at different temperatures from 50°C to 100°C. (O) soluble CGTase, (●) immobilized CGTase.

CGTase from *Bacillus macerans* was previously immobilized by a similar method and used to produce CD; however, its optimum temperature decreased from 55°C to 50°C after immobilization [15], showing the strong dependence on the origin of the CGTase.

Thermal stabilities of the free and immobilized CGTase treated at different temperatures ranged from 50°C to 100°C were compared and are presented in Fig. 4. An improvement of the thermal stability of the CGTase after immobilization at the temperature above 70°C is of interest. For example, the immobilized CGTase retained more than 80% of its initial activity at 80°C. However, the soluble CGTase retained only 55% of its initial activity. The increased thermal stability after immobilization seems to add to the advantages of the column type packed bed reactor used to produce transglycosylated xylitol, such as improved productivity and the increased reaction rate caused by the high dilution rate of the medium in the reactor.

Two studies report that the addition of the calcium ions and soluble starch showed a stabilizing effect on
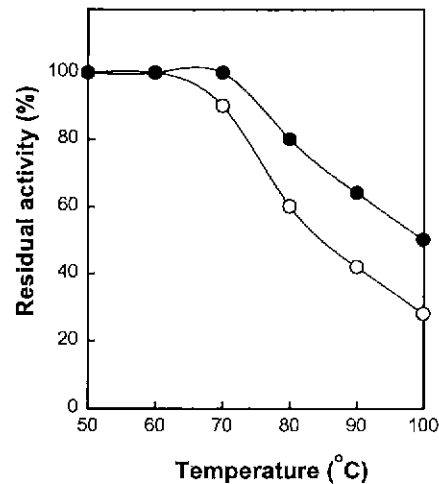

Fig. 4. Comparison of thermostability of soluble and immobilized CGTase. The residual coupling activities of the soluble and immobilized CGTase were measured after preincubating at different temperatures for 30 min. (O) soluble CGTase; (●) immobilized CGTase.
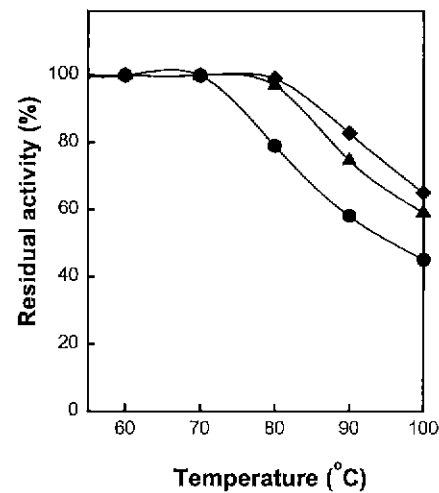

Fig. 5. Effect of soluble starch and Ca^{2+} ions on thermostability of immobilized CGTase 10 mM Ca^{2+} and 0.1%(w/v) of soluble starch were added into the suspended solution containing 100 units of immobilized CGTase, respectively, and the residual coupling activities were measured after preincubation at different temperatures for 30 min. (●) control; (▲) 0 1%(w/v) of soluble starch; (◆) 10 mM Ca^{2+}.

the conformation of the CGTase [23,24]. 10 mM calcium ions and 0.1% (w/v) soluble starch were added to the suspended solution of the immobilized CGTase, respectively, in the expectation that the thermal stability of the immobilized CGTase will be increased. As shown in Fig. 5, the thermal stability of the immobilized CGTase increased significantly. For example, the residual coupling activity increased from 60% to 85% by the addition of calcium ions and from 60% to 80% with soluble starch at the temperature of 90°C.

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Table 1. Effect of dextrose equivalent of glycosyl donor on transglycosylation yield and productivity of immobilized CGTase

D E value of donor	Transglycosylation yield (%)	Productivity $(g \cdot L^{-1} \cdot h^{-1})$
β-Cyclodextrin	31 1	9.72
Soluble starch	22 2	6.94
Dextrin (D.E.[a] = 6)	23.8	7 44
Dextrin (D.E = 11)	23.7	7.40
Dextrin (D.E. = 13)	24 2	7.56
Dextrin (D.E. = 19)	18.6	5.81

Substrate solution of 10% (w/v) donor and xylitol dissolved in 10 mM Tris-Malate-NaOH buffer solution (pH 6.0), respectively, was applied to the column type packed bed reactor (2.2 × 33 cm) containing immobilized CGTase (354 units/g resin) at a flow rate of 20 mL/h and 60°C

[a] D E values of Dextrins (Aldrich Co., USA and Sindongbang Co, Korea) were determined by Lee's method [16].

Production of Transglycosylated Xylitol in a Column Type Packed Bed Reactor Containing Immobilized CGTase

We investigated how the variations of the dextrose equivalent (D.E.) value of dextrin used as the glycosyl donor, substrate concentration, and the flow rate in the column type packed bed reactor would affect the transglycosylation yield The degree of polymerization (D.P.) of the glycosyl donor such as starch, dextrin, and maltooligosaccharides, is already recognized as an important factor affecting the efficiency of the transglycosylation reaction catalyzed by CGTase [7,10,25]. Table 1 shows the effect of the D.E. value, indirectly representing the D P of the glycosyl donor, on the efficiency of the transglycosylation reaction in the column type packed bed reactor containing immobilized CGTase. The transglycosylation yields and productivities were maintained as about 23-24% and 7.4-7.6 $g \cdot L^{-1} \cdot h^{-1}$, respectively, at a wide range of D.E. values from 6 to 13. However, they decreased significantly to 18 6% and 5.8 g $L^{-1} \cdot h^{-1}$, respectively at a D E value higher than 19. The optimum D.E. level of dextrin was determined as 13 in this reaction system, which exhibited the maximum productivity of 7.6 $g \cdot L^{-1} \cdot h^{-1}$.

These results indicate that the D.E. value of dextrin is also an important factor for the transglycosylation reaction catalyzed by immobilized CGTase, due to the donor specificity of the enzyme. Another study also reports the donor specificity of CGTase in that simple or short chain sugars, such as glucose, maltose, and maltotriose, are unsuitable as the glycosyl donor in the transglycosylation reaction catalyzed by the CGTase from *Thermoanaerobacter* sp. [10]. However, glucose and maltose were considered to be better glycosyl acceptors rather than glycosyl donors in the transglycosylation reaction of the CGTase from *Bacillus* sp. [7, 25].

Fig. 6 shows the effect of variation of the substrate concentration from 1.0 to 15%(w/v) on the transgly-
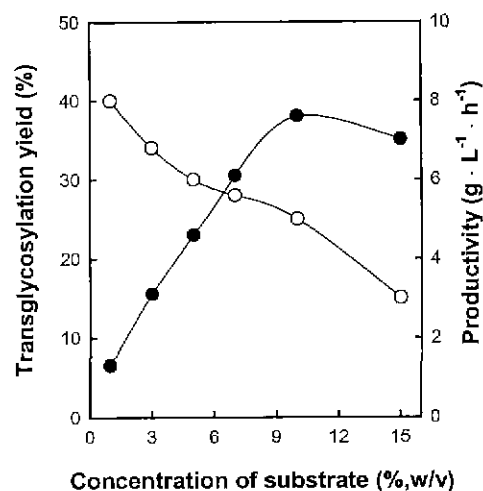

Fig. 6. Effect of substrate concentration on transglycosylation yield and productivity of the column type packed bed reactor containing immobilized CGTase Substrate solutions composed of the same concentrations of xylitol and dextrin (D E = 13) ranging from 1.0-15%(w/v) in 10 mM Tris-Malate-NaOH buffer (pH 6.0) were passed through the column type packed bed reactor (2.2 × 33 cm) at 20 mL/h and 60°C. (O) transglycosylation yield; (●) productivity.

cosylation yield and productivity of the column type packed bed reactor containing immobilized CGTase. The transglycosylation yield decreased as the substrate concentration increased, but the productivity increased as the concentration of substrate increased up to 10% (w/v) and reached 7.8 $g \cdot L^{-1} \cdot h^{-1}$. The yield of transglycosylation by a free enzyme [26] and that of the CD production by immobilized CGTase contained in the column type packed bed reactor [17] were also found to be dependent according to the substrate concentration. This phenomena is trivial because the reaction mode in the packed bed reactor is similar to that in the batch reactor and the reaction was progressed by the first order reaction at low substrate concentration and by the zero order reaction at high concentration if the transglycosylation reaction was catalyzed by CGTase following the Michaelis-Menten kinetic mode.

Fig. 7 shows the effect of the substrate flow rate (dilution rate) on the productivity of the column type packed bed reactor and the transglycosylation yield. The transglycosylation yield decreased slightly down to a flow rate of 20 mL/h and thereafter, decreased rapidly. In contrast, the productivity increased with a flow rate up to 20 mL/h, and thereafter, decreased gradually. As the flow rate of the substrate increased, the retention time of the substrate in the reactor was shortened and the transglycosylation yield decreased correspondingly. However, the productivity increased and reached the maximum value of 7.8 $g \cdot L^{-1} \cdot h^{-1}$ as the substrate flow rate increased up to 20 mL/h because the mass transfer rate through the boundary layer decreased with the flow rate. It seemed that the controlling step is the reaction by the immobilized enzyme on the surface of the resin at the flow rate lower than 20 mL/h
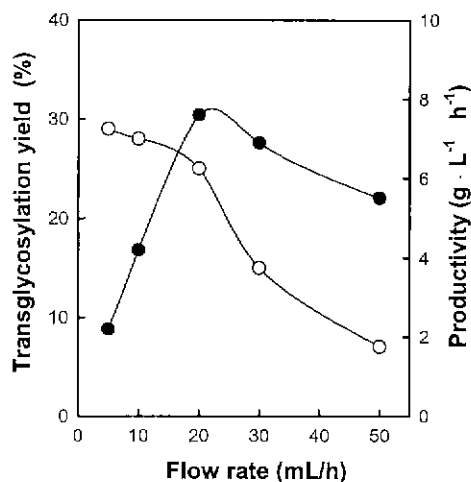
Fig. 7 Effect of flow rate on transglycosylation yield and productivity of the column type packed bed reactor containing immobilized CGTase. 10%(w/v) substrate solutions in 10 mM Tris-Malate-NaOH buffer(pH 6 0) were passed through the column type packed bed reactor (2.2 × 33 cm) at different flow rates from 5 mL/h to 50 mL/h at 60°C. (O) transglycosylation yield, (●) productivity

Fig. 8. Operational stability of immobilized CGTase on Amberlite IRA-900 in the column type packed bed reactor Substrate solution composed of 10%(w/v) dextrin (DE ≒ 13) and xylitol in 10 mM Tris-Malate-NaOH buffer (pH 6.0) was applied to the column type packed bed reactor (2 2× 33 cm) at 20 mL/h and 60°C for 40 days.

and a conformational problem of the enzyme due to the shear stress becomes serious at a flow rate faster than 20 mL/h. Accordingly, the optimum flow rate was determined as 20 mL/h and was then applied for a long time operation for the production of transglycosylated xylitol.

Operational Stability of Immobilized CGTase Packed in Column Bioreactor

The operational stability of the immobilized CGTase used for the production of transglycosylated xylitol in the column type packed bed reactor during a 40-days continuous operation is illustrated in Fig. 8. The half-life of the immobilized CGTase used for this operation was 30 days, which was a little extended compared with 21 days of immobilized CGTase from *Bacillus macerans* [14], 23 days of *Bacillus macerans* CGTase immobilized on Amberlite IRA-900 [16], and 25 days of alkalophilic *Bacillus* sp CGTase immobilized on Diaion HP-20 [13]. The high operational stability of this reaction system is one of the advantages in the overproduction of transglycosylated xylitol.

CONCLUSION

The *Thermoanaerobacter* sp CGTase immobilized on Amberlite IRA-900 showed a resistance against thermal denaturation and a shift to a higher optimum temperature. Moreover, the immobilized CGTase in a column type packed bed reactor demonstrated a higher operational stability compared to other CGTases from different species immobilized on different materials. With these improved merits, the method we used in the
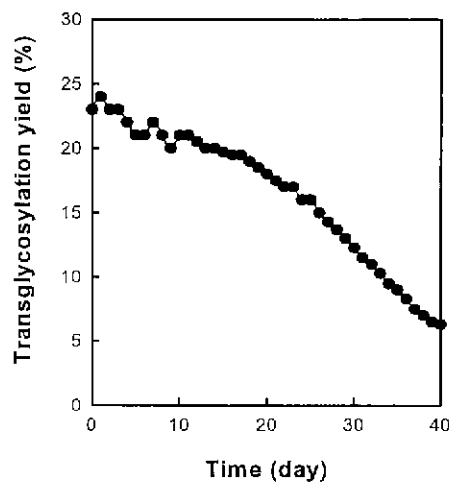
current study appears to be effective in the overproduction of transglycosylated xylitol. Further studies on the kinetics of a column type packed bed reactor containing immobilized CGTase and an efficient separation method to purify the bioproduct are required for the mass production of transglycosylated xylitol.

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