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Enzymatic Sorbitol Production with *Zymomonas mobilis* Immobilized in κ-Carrageenan

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The production of sorbitol by permeabilized cells of *Zymomonas mobilis* immobilized in κ-carrageenan was investigated. Cetyltrimethylammoniumbromide (CTAB) permeabilized cells were treated with glutaraldehyde prior to immobilization for cross-linking of enzymes, glucose-fructose oxidoreductase (GFOR) in cells. Rigidity of the immobilized beads was increased two-fold with 90% conversion efficiency by the additions of 40% (w/v) polyols (glycerol 25 g + propylene glycol 15 g) to 60% (w/v) distilled water containing 2.5% (w/v) κ-carrageenan as a final concentration, prior to immobilization. κ-Carrageenan beads entrapping permeabilized cells were dried to improve bead rigidity and storage stability. During semi-batch process for 72 h with dry beads, there was an improvement of the loss of enzyme activity (less than 10%). In batch process, the kinetic results of $K_{m,fructose}$ value for the free cells, wet beads and dry κ-carrageenan beads were 71.7, 72.4 and 116.7 g/l, respectively. Higher productivity was obtained with two-stage continuous packed bed reactors with both wet and dry κ-carrageenan beads at 25.00 and 21.15 g/l/h, respectively, when measured at second stage.

Zymomonas mobilis is able to convert glucose and fructose to gluconic acid and sorbitol simultaneously (6, 16). The enzyme complex, which is responsible for glucose oxidation and fructose reduction described (8, 17) as glucose-fructose oxidoreductase (GFOR), is strongly bound to its cofactor, NADP (2, 12). GFOR constitutes up to 1% of the soluble protein in *Z. mobilis* and is located in the periplasmic space (8). The enzyme shows the highest activity in *Z. mobilis* when equimolar concentrations of glucose and fructose are used as substrates, and gluconic acid is produced. The gluconic acid is phosphorylated by gluconate kinase to produce ethanol via the Entner-Doudoroff pathway (15). According to earlier observation, only permeabilized cells were able to produce gluconic acid and sorbitol (7). Cells with unaltered membrane permeability would convert glucose and fructose to ethanol, CO_2 and some sorbitol via the Entner Doudoroff pathway. When cells were permeabilized with appropriate concentrations of toluene, essential cofactors like ATP, Mg^{++} or phosphate, which are key compounds for fermentation of glucose and fructose are lost through the cell membrane by washing, while GFOR which is required for conversion of glucose and fructose remains inside the cell. In the absence of these cofactors, it is unlikely that gluconate accumulated via 6-phosphogluconolactone would be further metabolised to ethanol via the Entner Doudoroff pathway. When cells of *Z. mobilis* were treated with toluene or cetyltrimethylammoniumbromide (CTAB), glucose and fructose were only converted to gluconic acid and sorbitol instead of undergoing further conversion to ethanol (7, 10, 11, 14). It was reported that there was no significant mass transfer limitation when permeabilized cells were immobilized with κ-carrageenan (7). Also, it was shown that there was stable enzyme activity in the continuous process for up to 30 days and further reaction caused loss of enzyme activity. The loss of enzyme activity with a continuous stirred tank reactor (CSTR) due to breakage of bead was significantly increased in the long term process. Therefore, further treatment to the immobilized cells was required to increase bead rigidity and enzyme stability. In this paper, we investigate the efficiency of the immobilized dried-biocatalyst under different operating conditions, such as dilution rate, two-stage reactors and substrate concentration. A number of different process including batch, semi-batch and continuous processes using free, immobilized or dry κ-carrageenan beads were studied, and their kinetics were compared.

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Key words: sorbitol, *Zymomonas mobilis*, immobilization

MATERIALS AND METHODS

Chemicals

The Cetyltrimethylammoniumbromide (CTAB) was purchased from Fluka, Switzerland. The κ-carrageenan was purchased from the Korean Carrageenan Company. All other chemicals used were reagent grade.

Microorganisms and Growth Conditions

All experiments were performed with *Z. mobilis* ZM4 (ATCC 31821). They were maintained and grown as described previously (7).

Preparation of Permeabilized Cells and Glutaraldehyde Cross-linking

Cells were harvested in the late exponential phase 20 h after inoculation by centrifugation (4,000 rpm) prior to treatment with CTAB (0.2% w/v) at 4°C, pH 6.0-6.2. After gently stirring with CTAB for 10 min, cells were washed twice with saline buffer as reported previously (7). The yield of cells from the fermentor was approximately 2.0-3.0 g (dry weight) per liter. Cross-linking with glutaraldehyde involved suspending the permeabilized cells in 0.3% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) and stirring at 4°C for 10 min. The above suspensions were then centrifuged at 4000 rpm for 5 min and washed twice with the same buffer.

Procedure for Cell Immobilization

To evaluate sorbitol production, the cells were immobilized with κ-carrageenan. For immobilization with κ-carrageenan, CTAB permeabilized cells (12-13 g, wet weight) were also treated with glutaraldehyde before immobilization, and then mixed with 100 ml of κ-carrageenan solution (3.2%, w/v). The κ-carrageenan beads were produced in 0.1 M potassium phosphate buffer (pH 6.2) solution containing 20 g/l KCl and 0.15 g/l $CaCl_2$.

Dry κ-Carrageenan Bead

The beads immobilized with κ-carrageenan were dried for two weeks under the conditions of 20°C and 60% of humidity. The value of water content from the immobilized dry bead was determined by the total weight. Water content was represented as percentage. The change in bead size, volume and weight during drying was also measured. For the production of sorbitol with dry beads, rehydration of beads was carried out in glucose-fructose mixtures with stirring for 30 min under various temperatures. Following rehydration, temperature was increased to 39°C and sorbitol production with dry beads was investigated for 24 h at pH 6.2. For storage experiments, dry beads prepared by the procedure mentioned above were divided into 3 equal portions. One part was assayed for enzyme activity without storage while 2 portions were stored at 4°C for 14 and 40 days, respectively.

Reactor Operation

The reactor used for free and immobilized cells was a continuous stirred tank reactor (CSTR) with working volume of 186 ml. For the immobilized process, the liquid volume was maintained at 102 ml. The continuous process for sorbitol production was performed in a packed bed column reactor as shown in Fig. 1. The total working volume was 136 ml: 41 ml for the main column reactor and 95 ml for the pH-adjusting unit including connecting tubes. Dilution rates were determined using total working volume unless otherwise stated. The temperature and pH during the operation were maintained at 39°C and 6.2 respectively. For the packed bed column, pH and temperature were controlled in the recycle vessel. Various concentrations of sugar solution (equimolar glucose and fructose) were used for the continuous reaction under the controlled recycle rate and dilution rates.

Evaluation of Kinetic Constants

Productivity of sorbitol with glucose-fructose oxidoreductase was measured at 39°C. The pH was kept constantly at 6.2 with 2N KOH. To evaluate the kinetic constants for free, immobilized and dry κ-carrageenan beads, the initial reaction rates were measured at various concentrations of 100 ml substrate solution (equimolar glucose + equimolar fructose). A Lineweaver-Burk plot was obtained with a rate versus the fructose concentrations; 20, 30, 50 g/l. Free cells were prepared after permilization of cells with 0.2% (w/v) CTAB and 0.3% (w/v) glutaraldehyde treatment. Wet beads were obtained by the treatment of free cells with κ-carrageenan. Further drying procedures was performed to the wet beads for 2 weeks at 20°C.

Analytical Methods

Glucose, fructose, sorbitol and ethanol concentrations

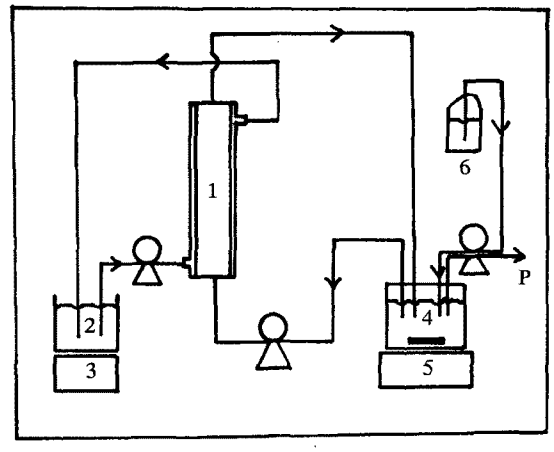

Fig. 1. Diagram of the experimental packed bed bioreactor for continuous production of sorbitol and gluconic acid.
1, packed bed column; 2, water reservoir for temperature control; 3, hot plate; 4, reservoir for pH control; 5, magnetic stirrer; 6, feed reservoir; p, product stream.

were determined by using a Waters Model HPLC at 85°C with a BioRad (Richmond, CA, USA) Aminex HPX-87C column (with a flow rate of 0.6 ml/min) and a refractive detector. The biomass concentrations for the free cells were determined as dry weights following oven drying at 104°C for 24 h. For the experiments with immobilized cells, a known mass of cells was used.

RESULTS AND DISCUSSION

In the results discussed below, we examined the effect of materials and conditions such as dryness and addition of polyols into beads on enzyme activity and stability. All runs were performed at 39°C and the pH of media was maintained between 6.0-6.2 unless stated otherwise.

Improvement of Hardness of κ-Carrageenan Beads

In order to improve the hardness of the κ-carrageenan beads, κ-carrageenan was mixed with a solution of 40% (w/v) polyols and 60% (w/v) distilled water as Casas *et al.* reported previously (4). It was suggested that glycerol imparts a higher resistance, while propylene glycol confers better characteristics with respect to smoothing and rehydration effects, so that the combined effect of the two results in a more resistant and elastic gel (4). In order to study the effects of polyols (glycerol + propylene glycol) on the rigidity of beads, different quantities of glycerol and propylene glycol were added to κ-carrageenan-cell mixture, and investigated at 15 h of batch process (Table 1). Of the various methods, one with 25% (w/v) of glycerol and 15% (w/v) of propylene glycol gave the least bead loss during the batch process and showed a conversion efficiency of over 90% for fructose to sorbitol during 10 h operation (Fig. 2). Furthermore, in this condition, the volume of beads appeared to be 48% of κ-carrageenan beads without use of polyols. The control experiment (without glycerol or propylene glycol) showed approximately 60% of beads loss. Although the conversion of sorbitol using wet beads with the use of polyols was lower than that of wet beads without the use of polyols, addition of polyols into beads caused increased bead hadrness so that a more stable process was possible.

Table 1. The effect of polyols (40% w/v) on the rigidity of κ-carrageenan beads.

κ-Carrageenan [glycerol + propylene glycol] (g/100 ml)		Bead loss(%)[a]	Conversion efficiency (%)
0	0	58.3	92
25	15	30.7	91
20	20	32.4	90
15	25	100.0	90

[a]Bead loss was determined by dividing weight of bead before reaction by weight of bead after 15 h reaction. Batch process was performed with immobilized beads on substrate containing 100 g/l of glucose and 100 g/l of fructose as a substrate.

Effect of Dryness of Immobilized Bead on Moisture Content of Beads and Enzyme Activity

A further improvement of bead rigidity was noted using the dry process. Part of the wet beads had an irregular shape, or were longer, however, the physical appearance of the bead was changed to a more circular shape during the dry process. Fig. 3 shows how the moisture content varied during the drying process. It is apparent that a critical point appeared at 60 h at which time more than 80% of the moisture was removed and the level of the water content was then maintained for longer than 120 h. It has been reported that beads are designed to overcome mass transfer limitations, such as size, and to give optimal values of the kinetic constant and that beads of 1.00-1.15 mm in diameter offer a maximum reaction rate (V_{max}) and apparent saturation constant (K_s)

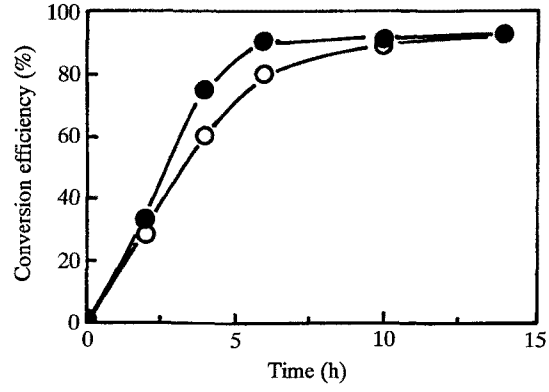

Fig. 2. The effect of addition of polyols [25% (w/v) glycerol plus 15% (w/v) propylene glycol] on the conversion efficiency of fructose to sorbitol at batch process: without polyols (●), and with polyols (○).

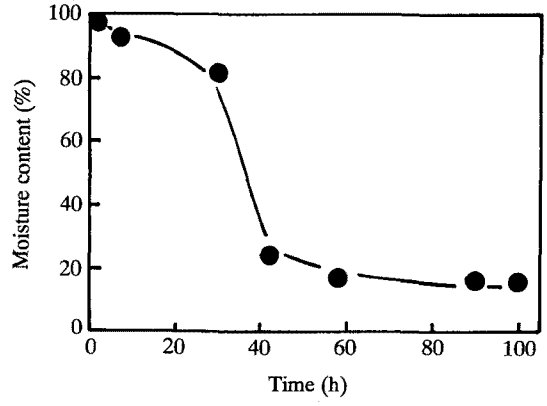

Fig. 3. Change of moisture content of immobilized κ-carrageenan beads during drying.
Temperature and relative humidity were 20°C and 60%, respectively. Cell loading was 14 g (dry cell weight).

(13). On the other hand, different data was reported that showed that bead size was one of the critical parameters to decide extent of cell leakage from beads, and small size bead (less than 2.0 mm in diameter) containing growing lactic acid bacteria minimised leakage of immobilized cells from κ-carrageenan bead (1). Sizes of wet and dry beads used in present experiment were 2.7-3.3 mm and 1.2-1.3 mm, respectively (Table 2). Slight increase in the bead size from 1.2-1.3 to 1.9-2.1 mm was observed after 3 time semibatch process. After 24 h reaction with dry beads, the volume of beads increased from 4.3 to 12-13 ml and the weight of beads increased from 1.87 to 9.5-10.0 g. To optimise enzyme activity from dry beads, the following factors which have been reported to be considerations during rehydration were tested: drying temperature, drying rate and the final moisture content in beads (9). The rehydration temperature did not seem to affect the production of sorbitol at a temperature between 30 and 40°C (Table 3). Therefore, the beads were rehydrated at 39°C at which the activity of GFOR was reported to be optimal (8, 17). Although specific sorbitol production rates (q_p) and conversion efficiency showed decreased values for dry beads than wet beads (Table 2), the reaction with dry beads had advantage in the size with regard to the dry beads. In the continuous process using wet beads, we have reported that GFOR activity was maintained for 30 days. However, some of substrates remained un-utilized, indicating that the conditions had been unfavorable to achieve full conversion (7). Using dry beads, we could increase the cell mass and enzyme presence in the reactor, which will result in an increase in the conversion efficiency from fructose to sorbitol in the continuous process.

Table 2. Comparison of physical and kinetic parameters of wet and dry beads.

Parameters	Wet beads	Dry beads	
		24 h	24 h×3[a]
Beads size (diameter, mm)[b]	2.7-3.2	1.8-2.0	1.9-2.1
Total beads volume (ml)[b]	35	14	15
Biomass concentration (g/l)[b]	18.8	18.8	18.8
Specific initial production rate ($q_{p.sorbitol}$) (g/g/h)[c]	0.72	0.27	0.21

[a]Data were obtained after 3 times semi-batch processes. Each process taken 24 h. [b]Data obtained before reaction. [c]Data obtained after reaction. Batch process was performed using wet and dry beads on 200 ml of sugar solution (50 g/l of glucose and 50 g/l of fructose) at 39°C, pH 6.2.

Table 3. Comparison of the physical parameters of wet and dry beads at various rehydration temperatures.

Parameters	Wet[a] beads	Rehydration temperature (°C) for dry beads		
		20	30	40
Conversion efficiency (%)	82	64	70	68
Specific initial production rate ($q_{p.sorbitol}$) (g/gl)	0.79	0.35	0.37	0.34
Biomass concentration (g/l)	11.0	10.8	10.8	10.8

[a]Wet beads were prepared by protocol mentioned in Materials and Methods, and used as control. Batch process was performed using wet and dry beads on 200 ml of sugar solution (50 g/l of glucose and 50 g/l of fructose). Reaction with dry beads was carried out after rehydration of dry beads in the sugar solution for 30 min at various temperatures.

Effect of Dryness of Beads on the Stability of Enzymes

The stability of GFOR with dry beads in κ-carrageenan was evaluated by means of extended semi-batch culture experiments and storage trials. For semi-batch process, the supernatant in the bioreactor was removed after 24 h of reaction and 200 ml of fresh sugar solution (50 g/l of glucose and 50 g/l of fructose) added. This procedure was repeated three times, and in each case the fructose and sorbitol concentrations were determined (Fig. 4). The results showed a number of interesting aspects with regard to the production of sorbitol; [1] the activity of enzyme was maintained long period when dry beads were used. During the semi-batch process with dry beads, there was a small loss of enzyme activity (approximately 10%) after 72 h operation (Fig. 4). These results show a decreased loss of enzyme activity in loss in comparison to observations previously reported by Choi *et al.* (5), who showed a loss of 71% of enzyme activity when immobilized toluene-treated cells of *Z. mobilis* without drying were used. Leakage of enzyme

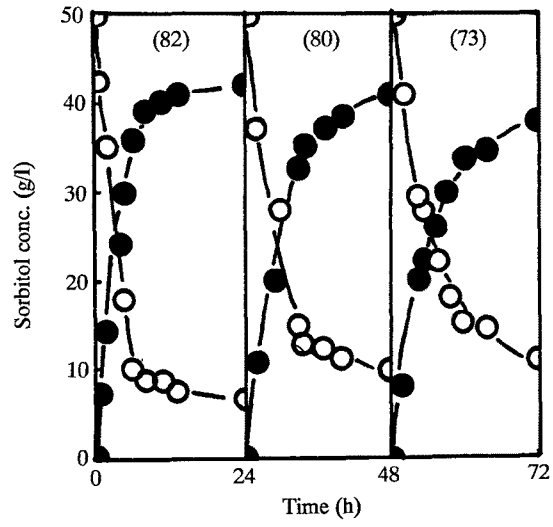

Fig. 4. Production of sorbitol in semi-batch process with CTAB-treated cells of *Z. mobilis* immobilized in κ-carrageenan: sorbitol (●), and fructose (○).
Numbers in parenthesis represent conversion efficiency of fructose to sorbitol. Three cycles of a batch process were carried out using dry beads at 39°C, pH 6.2. Cell loading was 18.8 g/l.

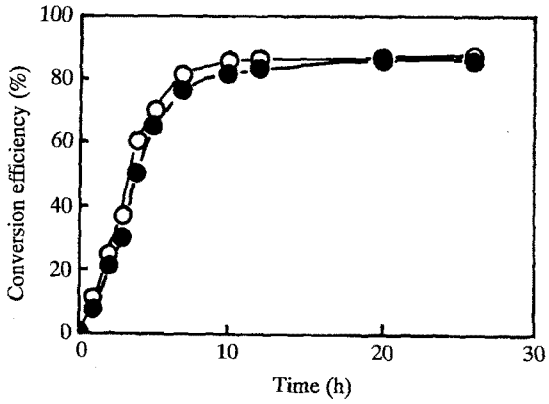

Fig. 5. The effect of long term storage on the conversion efficiency of fructose to sorbitol: no storage (○), and with storage for 40 days at 4°C (●).
Batch process was performed using dry beads on 200 ml of sugar solution (50 g/l of glucose and 50 g/l of fructose) at 39°C, pH 6.2. Cell loading was 10 g/l.

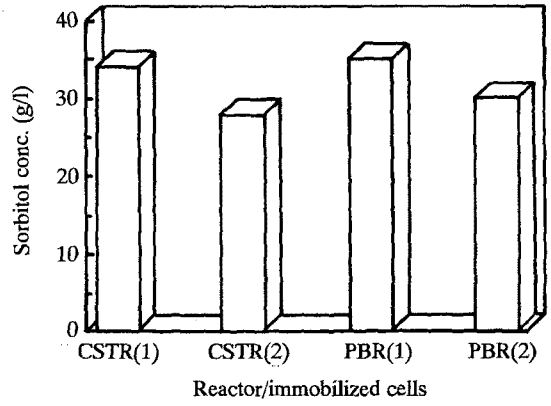

Fig. 6. Production of sorbitol in continuous process (CSTR, packed bed reactor) with CTAB-permeabilized cells of *Z. mobilis* immobilized in κ-carrageenan.
Continuous process was performed using wet beads and dry beads on 50 g/l each of glucose and fructose at dilution rate 0.1 h^{-1}: with wet bead (1), and with dry bead (2). PBR represents packed bed reactor. CSTR represents continuous stirred tank reactor.

was reported to be serious with permeabilized cells of *Saccharomyces carlsbergensis* (3). These authors found that more than 50% of the total hexokinase activity at the first stage of the recycle and 85% at the final stage leaked off of the toluene-treated permeabilized cell (2). There was slight expansion in the volume of the dry beads during the semi-batch process compared to the wet beads, indicating that more beads could be packed into the reactor. As shown in Fig. 5, there was no great difference in terms of enzyme activity, when dry beads were stored for 40 days and tested at various time intervals. The above result suggests that a dry process could be useful in increasing stability of enzyme immobilized in κ-carrageenan and it could be applied to increase the length of the continuous process.

Enzyme Stability with Dry Beads in a Continuous Process

Industrial production of sorbitol is governed by two factors, high productivity and enzyme stability. In this respect, CSTR and packed bed reactor were compared for sorbitol production in a continuous process. The immobilized beads were shown to be stable during 30 day operation (7). Similarly, when dry beads were used for continuous process, sorbitol production was found to be uniform for over 1 week. Fig. 6 showed the results of sorbitol production at the stationary stage in the process with either CSTR or packed bed reactor showing that the sorbitol concentration of the continuous process with wet and dry beads at the dilution rate 0.1 h^{-1} was between 28.8 to 35.0 g/l with 22 g/l of loaded cells. Above results show a slighter increase in conversion efficiency of fructose to sorbitol with wet beads and a packed bed reactor.

Kinetics of Permeabilized Beads in a Batch Process

Using a Lineweaver-Burk plot, the conversion for fructose to sorbitol with free cells, wet beads and dry κ-carrageenan beads were calculated and the values of $K_{m.fructose}$ were 71.7, 72.4, and 116.7 g/l, respectively (Table 4). The value of V_{max} for dry κ-carrageenan beads was found to be half of that for free cells which indicates that enzyme leakage from dry beads could be taking place either during drying, or deactivation of oxidoreductase, or decreasing in mass transfer with dry beads, or in all these cases. Similar levels of $K_{m.fructose}$ (77.2 g/l) and V_{max} (1.7 g/g/h) for CTAB-permeabilized cells was reported (11). Purified GFOR was found to have remarkably low affinities ($K_{m.glucose}$ from 1 to 5.4 g/l; $K_{m.fructose}$ from 72 to 180 g/l) but high specificity to its substrate (6, 17). Higher $K_{m.fructose}$ of 250 g/l were reported for toluene-permeabilized cells (10).

Table 4. Comparison of kinetic parameters for free cells, wet beads and dry beads.

Kinetic parameters	Free cells	Wet beads	Dry beads
$K_{m.\ fructose}$ (g/l)	71.7	72.4	116.7
V_{max} (g/l/h)	2.9	2.5	1.5

Batch process was performed using 3.2 g/l of cell.

Increasing Sorbitol Productivity in a Continuous Process by Varing Conditions

A high conversion of fructose to sorbitol can be obtained at lower dilution rates or using a multi-stage reactor system. To improve productivity with the immobilized cells, various sugar concentrations were used in the two-stage continuous packed bed reactors with both wet and dry beads. The results obtained from the two-stage continuous process are shown in Table 5.

It appeared that slightly higher productivity was ob-

Table 5. The effect of substrate concentration on productivity of sorbitol in a two-stage continuous packed bed reactors process using wet and dry beads.

Feed Substrate concentration (g/l) [glucose+fructose]	Stage 1		Stage 2	
	D (l/h)	Productivity[a] (g/l/h)	D (l/h)	Productivity[a] (g/l/h)
100+100 (wet beads)	0.12	8.40	0.13	9.20
100+100 (dry beads)	0.09	6.38	0.10	6.98
200+200 (wet beads)	0.13	14.50	0.14	19.04
200+200 (dry beads)	0.13	16.30	0.14	18.90
300+300 (wet beads)	0.10	16.53	0.14	25.00
300+300 (dry beads)	0.12	15.30	0.13	21.15

[a]Productivity value was calculated as P×D, where P=sorbitol concentration (g/l) at stationary stage and D=dilution rate used in the each experiment. Cell loading in the first and second stage were 8.4 and 15.6 g/l, respectively. In this experiment, two columns (25 cm×2.5 cm i.d., for the first column; 30 cm×2.5 cm i.d., for the second column) connected were used. Recycle rate were controlled at 1,500 ml/h in the first reactor and 800 ml/h in the second reactor. Dilution rate (D, h^{-1}) and productivity of sorbitol ($g_{sorbitol}$/l/h) were shown in the table.

tained with two-stage process in comparison to the single reactor process. However, at the high sugar concentration (60% w/v), conversion efficiency of the substrate was reduced dramatically in both the single and two-stage process, and a significant portion of reducing sugars remained un-utilized. This could be attributed to the inhibiting effect by high sorbitol concentration produced at the higher substrate concentration (10). Similar result were reported where high conversion of fructose in a continuous process can only be obtained at long residence times or in multi-stage reactor systems (11). The productivity increased with increases in dilution rates and sugar concentrations, showing that a two stage process could be used effectively for the production of sorbitol.

From these data, it is evident that operational stability could be enhanced by either drying the beads or by the addition of polyols into κ-carrageenan beads. The addition of polyols, during immobilization of the κ-carrageenan beads or dryness of beads has the advantage of obtaining stable process for sorbitol and gluconic acid production. Experiments conducted by semi-batch process revealed that enzyme activity was maintained longer than that for non-dried κ-carrageenan bead. Also, enzyme stability in the dry beads was maintained during storage for 40 days at 4°C implying that they could be stored for longer periods. Although the conversion of sorbitol with dry beads was lower than that of non-dry bead, the dry process was found to be satisfactory since it gave an increase in physical rigidity and a decrease in volume of the bead. This would help the long term operational stability of the system and the increase conversion efficiency of the process.

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