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Full Paper

Repeated-batch production of galactooligosaccharides from lactose at high concentration by using alginate-immobilized cells of *Sporobolomyces singularis* YIT 10047

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We investigated the production of galactooligosaccharides (GOS) from lactose by alginate-immobilized cells of *Sporobolomyces singularis* YIT 10047 (IM-SS). β-Galactosidase activity was stable at 30 to 50°C but decreased dramatically between 50 and 60°C and disappeared at 70°C in acetate buffer. The enzyme activity remaining was no more than 20% of that of unheated samples after incubation in distilled water at 55°C, whereas its value was about 60% at the same temperature under buffered condition. However, activity was maintained more than 80% with 10% to 50% (w/w) lactose after incubation at 55°C without buffer. In a single-batch reaction, GOS yield was 41.0% with free cells and 40.4% with IM-SS. We attempted a repeated-batch reaction using IM-SS with 600 g L^{-1} lactose. IM-SS produced GOS stably for 20 batches (22 h/batch, 440 h in total) at 55°C and pH 5.0 or 6.0. IM-SS produced GOS at 242 g L^{-1}, at a rate of 8.72 g L^{-1} h^{-1}. Both GOS yield and production rate were higher than those in published experiments on GOS production using immobilized biocatalysts. The repeated-batch reaction with IM-SS would be an ideal system for GOS production because of its stability and high productivity.

Key Words——alginate; galactooligosaccharide; β-galactosidase; immobilization; lactose; *Sporobolomyces singularis*

Introduction

Galactooligosaccharides (GOS) are well-known, nondigestible carbohydrates that are resistant to gastrointestinal digestive enzymes and are fermented by specific colonic bacteria (Sako et al., 1999). GOS benefit their hosts by selectively stimulating the growth or activity of one, or a limited number of, bacterial species (defined as "prebiotics") in the colon (Gibson and Roberfroid, 1995). GOS intake enhances the growth of bifidobacteria in the intestine and suppresses that of harmful bacteria such as clostridia (Bouhnik et al., 1997; Fooks et al., 1999; Oku, 1996; Tanaka et al., 1983).

GOS can be produced from lactose by transgalactosylation catalyzed by β-galactosidase (Prenosil et al., 1989). β-Galactosidase or β-glucosidase, or microorganisms that harbor these enzymes, can produce GOS (Akiyama et al., 2001; Bodun et al., 2001; Burvall et al., 1979; Huber and Wallenfels, 1976; Lamoureux et al., 2002; Onishi et al., 1995; Roy et al., 2002; Toba et al., 1985). Among these microorganisms, *Sporobolo-*

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Abbreviations: GOS, galactooligosaccharides; HPLC, high-performance liquid chromatography; IM-SS, alginate-immobilized cells of *Sporobolomyces singularis* YIT 10047.

myces singularis, a basidiomycetous yeast, possesses an interesting property with respect to GOS production. Gorin et al. (1964a) reported that *S*. *singularis* can produce a large amount of GOS from lactose by fermentation or an enzymatic reaction. They also revealed that the main product was a trisaccharide, β-D-Gal-(1-4)-β-D-Gal-(1-4)-β-D-Glc (4′-galactosyllactose). Shin and Yang (1998) reported the enzymatic production of GOS by β-galactosidase of *Bullera singularis* (now known as *S*. *singularis*), where the initial concentration of lactose did not affect the yield of GOS. Even though it is difficult to increase the production rate, it is possible to increase the total amount of GOS if a high concentration of lactose is supplied initially. Shin et al. (1998) also reported the continuous production of GOS by chitosan-immobilized, partly purified β-galactosidase of *B*. *singularis*. Using an immobilized-enzyme column as a reactor, they conducted continuous production of GOS from 10% (w/v) lactose at 45°C and pH 4.8. Under these conditions, GOS were produced stably for up to 350 h, at a rate of 4.4 g L^{-1} h^{-1}. A greater GOS yield could be expected with the use of a higher initial lactose concentration, but this has not yet been attempted with this type of reactor.

An immobilized enzyme might be useful for GOS production because it is reusable and easy to separate from the reaction mixture. However, because the enzyme purification process is quite complicated and leads to a loss of enzyme activity, it is desirable to apply whole cells instead of purified enzymes. An entrapping method is frequently used to immobilize whole cells. Generally, the materials used to entrap biocatalysts are polymeric, for example, κ-carrageenan or polyacrylamide. The calcium alginate method is a readily available entrapping method for immobilizing whole cells (Kierstan and Bucke, 1977). Calcium alginate easily forms a gel particle when dropped into a calcium ion solution, and its particles have good physical strength. In addition, alginate is a natural polysaccharide extracted from seaweed and is safe for use in the food industry.

Sporobolomyces singularis YIT 10047, previously reported as *S*. *singularis* 7B6 (Ishikawa et al., 2005), is a 2-deoxy-D-glucose-resistant mutant of *S. singularis* ATCC 24193. Its β-galactosidase activity is about 10 times that of the parent strain, which has been used in previous studies (Gorin et al., 1964a, b; Shin et al., 1998). Use of this mutant strain may improve GOS production rates, especially if whole cells are immobilized. We therefore investigated GOS production by alginate-immobilized *S*. *singularis* YIT 10047 cells (IM-SS). We revealed that lactose preserved β-galactosidase activity at high temperatures. In repeated-batch reactions, IM-SS produced large amounts of GOS from high initial lactose concentrations.

Materials and Methods

Chemicals. Sodium alginate (Kimica Algin I-1M) was obtained from Kimica (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Yeast strain and culture conditions. *S*. *singularis* YIT 10047 was originally reported as *S*. *singularis* 7B6 (Ishikawa et al., 2005). This strain has been deposited in the International Patent Organism Depositary (IPOD, Tsukuba, Japan) as *S. singularis* FERM P-18817. It was cultivated in a medium containing 10 g yeast extract (Difco Laboratories, Detroit, MI, USA), 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 50 g lactose or glucose in 1 L distilled water (pH 5.0). The cells were inoculated into 100 ml of fresh medium in a 300-ml Erlenmeyer flask and incubated at 27°C on a rotary shaker (180 rpm) for 3 days. Then 5 ml of the culture was inoculated into 1.5 L of fresh medium in a 2.0-L glass vessel fermenter (MDL-200, B.E. Marubishi, Tokyo, Japan) and incubated at 27°C with air (1 volume per volume per minute). The cells were harvested by centrifugation (9,000 × *g*, 20 min, 4°C) and washed twice with distilled water, and the pellet was then collected as wet cells.

Preparation of IM-SS. Wet cells and 4.2% (w/w) sodium alginate were mixed at a ratio of 4 : 6 (w : w). The mixture was dropped from the pipette tip (ϕ 1 mm) into a 5% (w/w) calcium chloride solution to make gel particles of IM-SS (ϕ 3–4 mm). The gels were stabilized in the calcium chloride solution for 1 h. The amount of IM-SS was determined by the total weight of the wet cells plus the sodium alginate solution applied for immobilization.

β-Galactosidase assay. β-Galactosidase activity was defined as the activity catalyzing the hydrolysis of lactose-releasing glucose molecules and was determined by the amount of glucose released during the reaction. The thermal stability of β-galactosidase activity was investigated as follows. Four grams of wet cells or 10 g of IM-SS (containing 4 g of wet cells) was incubated in 50 ml of 50 mmol L^{-1} calcium acetate buffer (pH 5.5) at 30 to 80°C for 30 min. After incubation, the

suspensions were cooled on ice for 10 min. Each suspension was added to 50 ml of 200 g L^{-1} lactose in the same buffer (100 g L^{-1} final concentration), and incubated at 40°C for 90 min. Then the amount of glucose released was determined by the Glucose C-II test (Wako).

pH stability was investigated by the following procedure. Four grams of wet cells or 10 g of IM-SS was incubated in 50 ml of 50 mmol L^{-1} calcium acetate buffer without lactose (pH 3.5 to 6.0) at 30°C for 30 min. After incubation, the suspensions were cooled on ice for 10 min. Each cooled suspension of free cells was centrifuged (3,000×*g*, 30 min, 4°C), and the pellet was resuspended in 50 ml of 50 mmol L^{-1} calcium acetate buffer (pH 5.5). In the case of IM-SS, the buffer was discarded by decantation and 50 mmol L^{-1} calcium acetate buffer (pH 5.5) was added up to 50 ml. Each suspension was added to 50 ml of 200 g L^{-1} lactose in the same buffer and incubated at 40°C for 90 min. The amount of glucose released was then determined as described above.

The preservative effect of lactose on β-galactosidase activity was investigated as follows. Free cells: 5 g of wet cells was incubated at 55°C for 30 min in 0% to 50% (w/w) lactose dissolved in distilled water (no buffer). After the incubation, 10 ml of each suspension was added to 40 ml of saline (0.85% NaCl). The suspensions were centrifuged (3,000×*g*, 30 min, 4°C) and the supernatant was discarded. The collected cells were washed twice with saline, and 0.2 g of the cells was resuspended in 0.5 ml of saline. Then, 0.5 ml of the resuspended cells was added to 2 ml of 125 g L^{-1} lactose (100 g L^{-1} final concentration) in 50 mmol L^{-1} sodium acetate buffer (pH 5.5) and incubated at 40°C for 90 min. IM-SS: 5 g of IM-SS was incubated at 55°C for 30 min in 0% to 50% (w/w) lactose dissolved in distilled water. After incubation, the lactose solution was discarded by decantation. The IM-SS was washed twice with 0.85% (w/v) $CaCl_2$, and the $CaCl_2$ solution was added to the IM-SS to make up 10 ml. Then, 40 ml of 125 g L^{-1} lactose (100 g L^{-1} as final concentration) in 50 mmol L^{-1} sodium acetate buffer (pH 5.5) was added to the suspension of IM-SS and incubated at 40°C for 90 min. The amount of glucose released was then determined as described above.

Single-batch reaction. Lactose solution (600 g L^{-1}) was prepared by dissolving lactose in distilled water with boiling. The pH was adjusted to 6.0 with 5% (w/v) sodium carbonate. Five grams of free cells or 20 g of IM-SS (containing 8 g of wet cells) was added to 50 ml of lactose solution (containing 30 g of lactose) and incubated at 55°C for 14 h. In the case of IM-SS, the reaction was performed with stirring. To analyze the relationship between reaction time and the amount of IM-SS, 3.75 to 18 g of IM-SS (0.125 to 0.6 g of IM-SS per g of lactose) was added to 50 ml of 600 g L^{-1} lactose solution (containing 30 g of lactose) prepared as described above. The reactions were performed at 55, 60, or 65°C until the disaccharide rate reached 55% (45% lactose conversion) by monitoring results of high-performance liquid chromatography (HPLC).

Repeated-batch reaction. Lactose solution (600 g L^{-1}) was prepared as described above. Then, 50, 35, or 25 g of IM-SS (containing 20, 14, or 10 g of wet cells, respectively) was added to 330 ml of the lactose solution (containing 200 g of lactose) for reaction at 55, 60, or 65°C, respectively. The reactions were performed at each temperature mentioned above, and pH 6.0 for 22 h with stirring (80 to 100 rpm) in a 500-ml Erlenmeyer flask. To investigate the effect of pH on the repeated reaction, 50 g of IM-SS was added to 330 ml of lactose solution (containing 200 g of lactose). The pH value of the lactose solution was adjusted to 4.0, 5.0, or 6.0 and the solution was incubated at 55°C for 22 h with stirring (80 to 100 rpm) in a 500-ml Erlenmeyer flask. The reaction was terminated when the amount of GOS had decreased to less than 95% of that in the first batch.

Analysis of GOS. The amount of tetrasaccharide, trisaccharide, disaccharide, glucose and galactose produced in each reaction was determined by HPLC. The HPLC system consisted of a pump (Waters 510, Waters, Milford, MA, USA), an autosampler (Waters 717plus, Waters), a refractive index detector (RI-98, Labo System, Tokyo, Japan), and a column (Shodex SUGAR KS-802, 8×300 mm, Showa Denko K.K., Tokyo, Japan). Distilled water was supplied as an eluent at a flow rate of 0.5 ml/min. The column temperature was maintained at 80°C.

Calculation of trend lines. The least squares method was used to calculate the trend lines to show the relationship between lactose conversion rate and GOS production rate. The power curves were fitted as trend lines to show the relationship between reaction time and IM-SS amount depending on the amount of substrate lactose. Calculation of trend lines was performed by Microsoft® Excel 2003 (Microsoft Corporation, USA).

Results and Discussion

Increasing the concentration of substrate lactose may improve the total amount of GOS produced. However, lactose is poorly soluble in water and can easily crystallize at low temperatures (Herrington, 1934). Because the enzyme can catalyze only soluble substrate, crystallization leads to low rates of GOS production. The most practical way to avoid crystallization is to keep the solution at high temperature. Therefore, we first analyzed the thermal stability of the β-galactosidase activity of IM-SS and compared it with that of free cells (Fig. 1). Enzyme activity in both free cells and IM-SS was stable up to 50°C, but decreased dramatically between 50 and 60°C, and finally disappeared at 70°C. The activity was stable at a pH range between 3.5 and 6.0 (data not shown).

The enzyme activity in both free cells and IM-SS dropped to about 60% at 55°C under buffered (pH 5.5) conditions (Fig. 1). However, after incubation in distilled water at 55°C for 30 min with 0% lactose, in both free cells and IM-SS the β-galactosidase activity remaining was no more than 20% of that of unheated samples (Fig. 2). These findings suggest that acetate buffer protects β-galactosidase activity against heating. In contrast, in IM-SS the activity was maintained at more than 80% in lactose solution (30% to 50%, w/w) without acetate buffer (Fig. 2). In free cells the activity level remained higher than 90% in 10% to 50% (w/w) lactose and was maintained at almost 100% in 40% and 50% (w/w) lactose. These results revealed that lactose, which is a substrate for GOS production, preserved the β-galactosidase activity of free cells and IM-SS against heating, and suggest that IM-SS might be active at high temperatures in high concentrations of lactose.

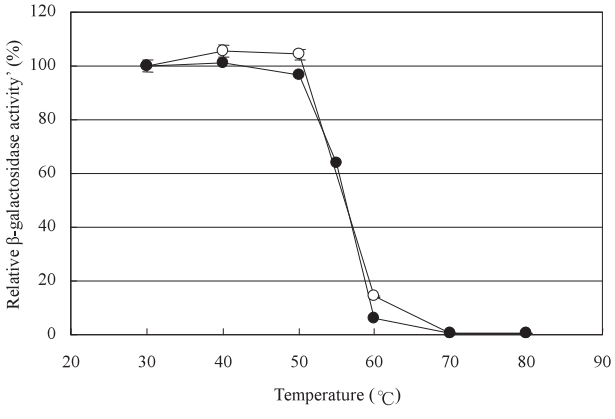

Fig. 1. Thermal stability of β-galactosidase activity of free cells and IM-SS in acetate buffer.

Activities were calculated relative to the values at 30°C (100%). ○, free cells; ●, IM-SS.

Single-batch reactions using free cells and IM-SS were performed at 55°C for 14 h in 600 g L^{-1} lactose (=50%, w/w) (Fig. 3). A total of 12.3 g of GOS (tri- and tetrasaccharides) was produced from 30 g of lactose (41.0% yield) by free cells. In the case of IM-SS, 12.1 g of GOS was produced (40.4% yield). GOS production by IM-SS was 1.51 g/g wet cells used; this value was 61% of the production by free cells (2.46 g/g wet cells used). The enzymes inside IM-SS would have had less opportunity than the enzymes on the free cells to reach and contact the lactose substrate; this could explain the reduction in GOS production rate with IM-SS. However, there was no difference in GOS yield between free cells and IM-SS.

We examined the profiles of GOS production and lactose conversion during a single-batch reaction (Fig. 4). The IM-SS profile was almost identical to that with free cells. These results suggested that the reaction pattern was not changed by immobilization. The production of GOS as a percentage of total saccharides was linear up to a value of 40%, at which time 60% of the lactose had been converted. The enzyme activity reached a stationary state at this point.

We then examined the relationship between volumetric GOS production rate and lactose conversion during a single-batch reaction (Fig. 5). Volumetric production rate decreased as the reaction proceeded.

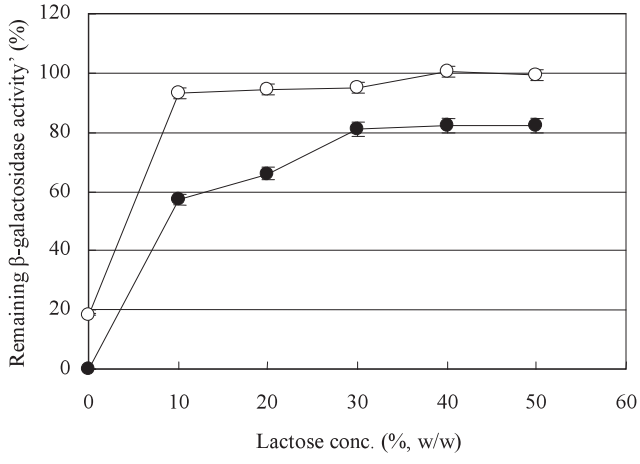

Fig. 2. Preservative effect of lactose on β-galactosidase activity at 55°C without buffer.

Remaining activities were calculated by comparison with those of unheated samples (=100%). ○, free cells; ●, IM-SS.

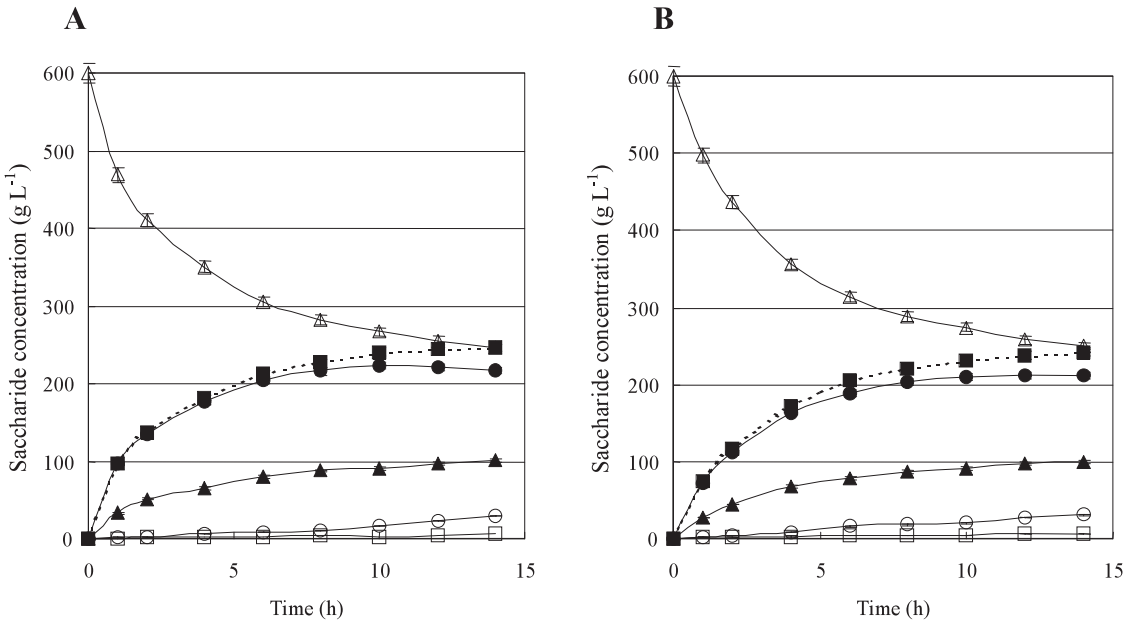

Fig. 3. Time course of GOS production by free cells and IM-SS during a single-batch reaction.

The concentration of each saccharide is plotted. Five grams of free cells or 20 g of IM-SS was added to 50 ml of 600 g L^{-1} lactose solution (containing 30 g of lactose) and incubated at 55°C for 14 h. A, free cells; B, IM-SS. ○, tetrasaccharide; ●, trisaccharide; △, disaccharide; ▲, glucose; □, galactose; ■, total GOS (tri- and tetrasaccharides).

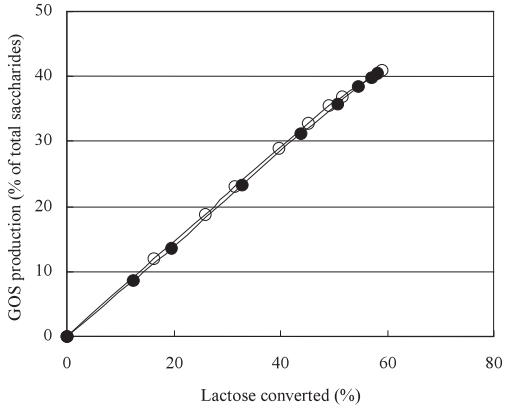

Fig. 4. Profile of GOS concentration and lactose conversion during single-batch reaction with free cells and IM-SS.

Five grams of free cells or 20 g of IM-SS was added to 50 ml of 600 g L^{-1} lactose solution and incubated at 55°C for 14 h. ○, free cells; ●, IM-SS.

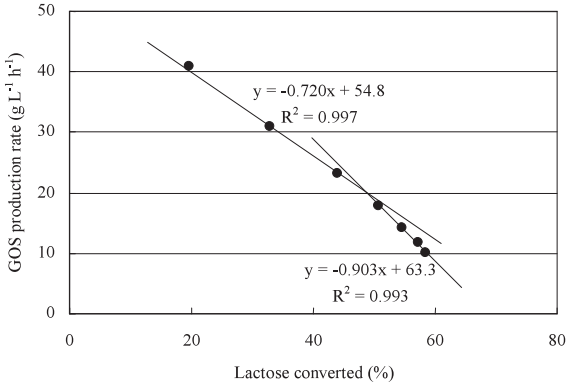

Fig. 5. Volumetric GOS productivity and lactose conversion during a single-batch reaction with IM-SS.

The trend lines were fitted by the least squares method.

The rate of decrease was stable up to 40% lactose conversion and then accelerated between 40% and 50%. The inflection point was calculated to be at 46.4% lactose conversion, by equation of two trend lines which are shown in Fig. 5. At this point, GOS yield was around 32% of the total saccharides; this GOS yield was about 80% of the maximum GOS yield (40%), which occurred at 60% lactose conversion. However, the volumetric GOS production rate was 21.4 g L^{-1} h^{-1}, which was more than twice that at 60% lactose conversion (9.1 g L^{-1} h^{-1}). In light of the efficiency of the repeated batch reaction it is not necessary to maximize the yield for each batch. For example, applying the result of Fig. 3B, 46.4% lactose conversion fell on 4.5 h and the resulting GOS amount was 9.6 g in 50 ml reaction mixture (containing 30 g of lactose). In contrast, IM-SS reached maximum GOS yield after 14 h,

producing 12.1 g of GOS. If we terminate at 46.4% lactose conversion for each batch, we may gain 28.8 g of GOS in 13.5 h by performing 3 batches (4.5 h×3 batches, assuming no enzyme activity loss). The amount is 2.4 times higher than the maximum amount for single-batch reaction even though the total reaction time is shorter. For such a reason, it would be better to maintain a high volumetric production rate. We therefore set a 45% lactose conversion rate, at a value just before the inflection point, as the end point for each batch.

We analyzed the relationship between reaction time and the amount of IM-SS, depending on the amount of substrate lactose, at different temperatures in a single-batch reaction (Fig. 6). The profile of GOS production was not changed by temperature or the amount of IM-SS (data not shown). As the temperature rose, the reaction proceeded faster. At the same reaction time, the amount of IM-SS required at 65°C was almost half that at 55°C. This result suggested that GOS productivity per gram of IM-SS could be improved by using a higher temperature. We therefore decided to examine the effect of temperature on the repeated-batch reaction.

To examine the effect of temperature on the repeated-batch reaction, we chose to fix the reaction time, not the amount of IM-SS, because it could have proved difficult to understand the temperature effect with different exposure times. For each batch the reactions were performed by 50, 35, and 25 g of IM-SS at 55, 60, and 65°C, respectively, with 600 g L^{-1} lactose (pH 6.0) for 22 h. IM-SS activity decreased dramatically at 65°C within three batches (Fig. 7). At 60°C it decreased more gradually. In contrast, the repeated reactions occurred stably at 55°C for 20 batches (440 h). Under these conditions, IM-SS still produced GOS at more than 98% of its initial value even on batch 20. Factory management and quality control are important factors in industrial GOS production. A decrease in enzyme activity may necessitate an extension of operation time and reduced productivity. Even though at 60 or 65°C we were able to reduce the amount of IM-SS needed in the single-batch reaction to less than that needed at 55°C, the former two temperatures were not ideal because the enzyme activity was less stable. For this reason, we concluded that the optimum temperature for repeated-batch reactions was 55°C. We then investigated the effect of pH (4.0, 5.0, and 6.0) by 50 g of IM-SS with 600 g L^{-1} lactose at 55°C for 22 h per batch. The repeated reactions proceeded stably at pH 5.0 and 6.0 (Fig. 8), whereas the GOS production decreased gradually at pH 4.0. After 11 batch reactions at pH 4.0, relative GOS production had declined to 94.8% of its value at the end of production of batch 1. These results suggested that acidic conditions could harm GOS production.

IM-SS produced 1,280 g of GOS from 4,000 g of lactose in a total of 20 batches at 55°C, pH 6.0. GOS

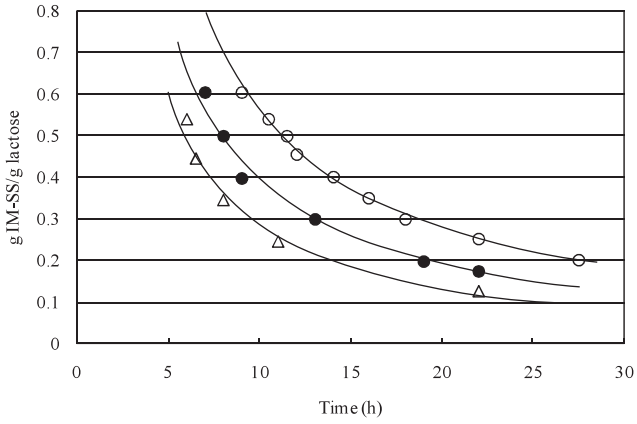

Fig. 6. Relationship between reaction time and IM-SS amount depending on the amount of substrate lactose, at different temperatures during a single-batch reaction.

Power curves were fitted as trend lines. The reactions were performed by 3.75 to 18 g of IM-SS (0.125 to 0.6 g of IM-SS per g of lactose) with 50 ml of 600 g L^{-1} lactose solution (containing 30 g of lactose), at 55, 60, or 65°C until the disaccharide rate reached 55% (45% lactose conversion). Each marker indicates the point at which 45% lactose conversion was reached. ○, 55°C; ●, 60°C; △, 65°C.

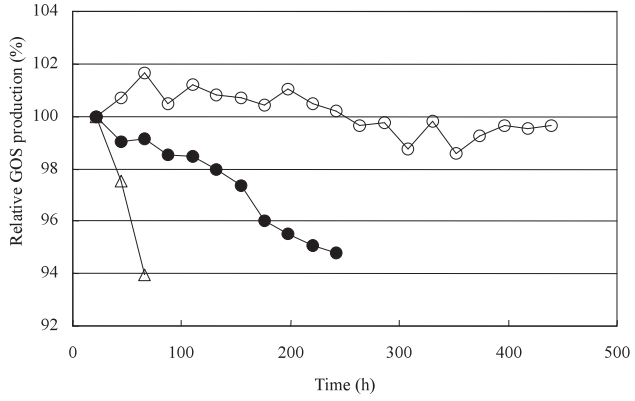

Fig. 7. Repeated-batch reactions by IM-SS at different temperatures.

The reactions were performed by 50, 35, or 25 g of IM-SS at 55, 60, or 65°C, respectively, with 330 ml of 600 g L^{-1} lactose solution (pH 6.0). Relative GOS production was calculated by comparing the amount of GOS produced to that produced in the first batch (100%). ○, 55°C; ●, 60°C; △, 65°C.

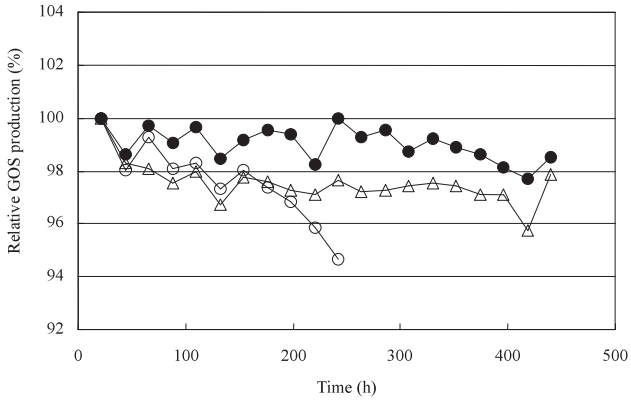

Fig. 8. Repeated-batch reactions by IM-SS at different pHs.

The reactions were performed by 50 g of IM-SS at 55°C, with 330 ml of 600 g L^{-1} lactose solution at pH 4.0, 5.0 or 6.0. Relative GOS production was calculated by comparing the amount of GOS produced to that produced in the first batch (100%). ○, pH 4.0; ●, pH 5.0; △, pH 6.0.

production by IM-SS was 64.0 g/g wet cells used; as mentioned earlier, in the single-batch reaction only 1.51 g/g wet cells was produced. This production rate was much higher than that of free cells in the single-batch reaction (2.46 g/g wet cells). These results indicate that repeated use of IM-SS can help to reduce the cost of sourcing additional enzyme. From the total amount of GOS produced through repeated reactions at 55°C, we calculated the GOS production rate as 8.72±0.07 g L^{-1} h^{-1} (mean±SD). Shin et al. (1998) reported a continuous production of GOS by immobilized enzymes of *B. singularis* of 4.4 g L^{-1} h^{-1}, so our result was almost twice theirs. We used a 6 times higher concentration of lactose (600 g L^{-1}) than theirs (100 g L^{-1}). The high lactose concentration may contribute to improve the volumetric GOS production rate.

Several reports on immobilized biocatalysts for GOS production have already been published. We compared GOS production using various immobilized biocatalysts (Table 1). Ozawa et al. (1989) investigated repeated-batch production of 4′-galactosyllactose by calcium-alginate-immobilized cells of *Cryptococcus laurentii*. Their approach was similar to ours. Their immobilized cells could perform for 20 batches (440 h) at 40 or 45°C with 200 g L^{-1} lactose. However, GOS pro-

Table 1. Comparison of GOS production by various immobilized biocatalysts.

Biocatalyst	Type of reaction	Total reaction time (reaction time/batch or flow rate)	Reaction conditions			Maximum GOS yield	Maximum GOS conc.	GOS production rate[a]	References
			Lactose conc. (g L^{-1})	Temperature (°C)	pH	(%)	(g L^{-1})	(g L^{-1} h^{-1})	
Aspergillus oryzae (enzyme)	Continuous	2 and 12 days (37 and 69 ml/h)[b]	400 and 200	40	4.5	26.6 and 21.7	106 and 43.4	3.9 and 3.0	Albayrak and Yang (2002)
Bacillus circulans (enzyme)	Continuous	240 h[c]	45.6, 120, 200	40	6.0	40	70.5	—	Mozaffar et al. (1986a, b)
Thermus aquaticus (enzyme)	Batch (single)	72 h (72 h/batch)	160	70	4.6, 6.0	34.8	55.7	0.77	Berger et al. (1995)
Cryptococcus laurentii (cells)	Batch (repeated)	440 h (22 h/batch)	200	40, 45	—[d]	40	80	3.6[e]	Ozawa et al. (1989)
Bullera singularis (enzyme)	Continuous	350 h (80 ml/h)	100	45	3.7	55[f]	55[f]	4.4	Shin et al. (1998)
Sporobolomyces singularis YIT 10047 (cells)	Batch (repeated)	440 h (22 h/batch)	600	55	5.0–6.0	40.4	242	8.72	This work

[a]We defined the production rate as gram of produced GOS per liter of reaction mixture per hour. Production rate was calculated as follows: continuous reaction: [lactose concentration (g L^{-1})] × [flow rate (ml/h)]/1,000 × [maximum GOS yield (%)]/100; batch reaction: [lactose concentration (g L^{-1})]/[reaction time/batch (h/batch)] × [maximum GOS yield (%)]/100. For the present study, the production rate was shown as the mean value of 20 batches, calculated by the GOS yield of each batch. [b]Initial conditions of the reaction were 400 g L^{-1} lactose and 37 ml/h. After 2 days, the lactose concentration and flow rate were changed to 200 g L^{-1} and 69 ml/h. [c]Flow rate was not specified. [d]The pH of the repeated-batch reaction was not specified. The results for free cells suggest that it was between 3.0 and 7.0. [e]4′-Galactosyllactose production rate. [f]Including disaccharides as GOS.

duction had decreased to approximately 80% of its initial value after 20 batch reactions. In contrast, we found that relative GOS production was at more than 95% of its initial value even after 20 batches at 55°C. We revealed that lactose preserved β-galactosidase activity against high temperatures (Fig. 2). We used 600 g L^{-1} lactose in our repeated-batch reaction, much more than in the study of Ozawa et al. (1989). This high concentration of lactose might have prevented the IM-SS from losing its enzyme activity.

Albayrak and Yang (2002) reported continuous GOS production by an immobilized enzyme, derived from *Aspergillus oryzae*, on cotton cloth. They applied 400 g L^{-1} lactose and achieved 106 g L^{-1} GOS. Their results for both substrate concentration and resulting GOS concentration were the highest among those in published reports. These results indicate that use of a higher substrate concentration may increase the amount of GOS through the reaction. However, the high lactose concentration that we used had not previously been tested. We achieved yields of 242 g L^{-1} of GOS from 600 g L^{-1} lactose. This GOS concentration was more than twice that in Albayak's study. The solubility of lactose in water is lower than that of other sugars such as glucose, and the high risk of crystallization makes using high lactose concentrations in GOS production difficult. We observed this crystallization at 50°C when the reaction failed to continue (data not shown). In contrast, we were able to perform repeated reactions without crystallization at 55°C. This is the first report of successful GOS production with lactose at an extremely high concentration (600 g L^{-1}), which could contribute to both increased enzyme stability and increased GOS production rate.

Our results revealed that alginate-immobilized cells of *S. singularis* YIT 10047 could produce a large amount of GOS from high concentrations of lactose in a repeated-batch reaction. Because of its long life and high productivity, this would be an ideal procedure for the industrial production of GOS.

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