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Semibatch Production of Fructo-Oligosaccharides from Sucrose by Immobilized Cells of *Aureobasidium pullulans*

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ABSTRACT

Aureobasidium pullulans cells with fructosyltransferase activity were immobilized in 2% calcium alginate beads, and the production of fructo-oligosaccharides from sucrose was studied in a stirred tank bioreactor. It was found that cells of *A. pullulans* were entrapped evenly on the alginate matrix of 2.2 mm in diameter, and an effectiveness factor of the beads was determined to be 0.3. By comparison with the system of free cells in batch operation, the total amount of fructo-oligosaccharides produced by immobilized cells was similar although the composition of fructo-oligosaccharides was found to be different. In semibatch operation with immobilized cells, reproducible results up to 60 cycles were obtained at 50°C and this operation resulted in no loss of activity of immobilized cells.

Index Entries: Fructo-oligosaccharides; semibatch process; immobilized cells; *Aureobasidium pullulans;* effectiveness factor.

INTRODUCTION

In recent years, a number of new sweeteners, including palatinose (*1,2*), galacto-oligosaccharides (*3,4*), and fructo-oligosaccharides (*5*), have been reported. The fructo-oligosaccharides, in which 1–4 fructose

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units are bound at the beta (2→1) position of sucrose, are mainly composed of GF_2, GF_3, and GF_4. With the sweetness 0.2–0.4 times of sucrose, the nondigestible fructo-oligosaccharides are found in many kinds of plants, such as onion and asparagus root (*6*). However, the supply is rather limited owing to limited content of fructo-oligosaccharides in plants. Therefore, the industrial production of fructo-oligosaccharides, which may be used in so-called health foods, may be carried out from sucrose by the action of fructosyltransferase. This enzyme has been found in fungi, such as *Aspergillus* sp. (*5*), *Fusarium* sp. (*7,8*), and *Aureobasidium* sp. (*9*).

In our previous work (*10*), conditions for the production of fructosyltransferase from *Aureobasidium pullulans* were studied, and the importance of magnesium sulfate on the ratio of intra- to extracellular enzyme production was demonstrated. Based on enzyme kinetic studies with various substrates, such as GF, GF_2, and GF_3 (*11*), it was found that the formation of fructo-oligosaccharides occurred from a consecutive set of disproportionation reactions (viz. $GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1}$). In the present investigation, semibatch production of fructo-oligosaccharides by immobilized cells of *A. pullulans* was studied in a stirred tank bioreactor over a period of 2 mo. For comparative purposes, continuous production was also carried out at a dilution rate of 0.05 h^{-1} (residence time of 20 h).

MATERIALS AND METHODS

Cell Preparation

A. pullulans KFCC 10245 was grown at 28°C for 36 h under 400 rpm in a 100-L pilot fermentor containing 70 L of the following medium composition: 20% sucrose, 2% yeast extract, 0.5% K_2HPO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 1.5% $NaNO_3$. The cells were then harvested by centrifugation and washed twice with deionized water prior to use.

Cell Immobilization

Cells were mixed thoroughly with 2% sodium alginate at room temperature. The mixture was extruded as small beads using various size of syringe needles into 1% calcium chloride solution. The beads were cured at room temperature for 2 h and then hardened overnight at 4°C.

Enzyme Assay

Fructosyltransferase activity in both free and immobilized cells was determined by measuring the release of glucose by the method reported previously (*10*). One fructosyltransferase unit is defined as the amount of enzyme activity required to produce 1 μmole of glucose/min under the specified conditions described.

Bioreactor Operation

Unless otherwise specified, 25 U of immobilized cells were transferred to a bioreactor (BIOSTAT M, B. Braun, Melsungen, West Germany) containing 1 L of 77% sucrose (Brix 60), and then batch operation was started at 300 rpm for 20 h at 50°C and pH 5.5. In a semibatch process (*12,13*), the whole reaction product was drawn off within 5 min, and the bioreactor was filled quickly with fresh feed substrate and then the operation was repeated with the same immobilized cells. Continuous operation was also carried out at a dilution rate of 0.05 h^{-1} for comparative purposes.

Analytical Method

Enzyme reaction products were analyzed by high pressure liquid chromatography (HPLC, Waters Associates Model 244, equipped with a differential refractometer RI-401 detector), using the μ Bondapak carbohydrate column (0.4×30 cm). A mixture of acetonitrile/distilled water (75/25, v/v) was used as the mobile phase at a flow rate of 1.5 mL/min.

RESULTS AND DISCUSSION

Batch Culture Kinetics of *A. pullulans*

A typical time course for the growth of *A. pullulans* on 20% sucrose medium is shown in Fig. 1. As can be seen from Fig. 1, both cell concentration and intracellular fructosyltransferase activity were increased almost linearly with time up to 40 g/L and 200 U/mL, respectively. Also shown in Fig. 1 are sugar concentration profiles. During the early period of growth (12 h), sucrose concentration declined very rapidly, and an accumulation of GF_2, GF_3, and GF_4 was observed. However, these fructo-oligosaccharides were utilized slowly toward the end of batch culture. The cells of *A. pullulans* obtained were used as a fructosyltransferase enzyme source throughout the course of this work.

Production of Fructo-Oligosaccharides from Sucrose by Free Cells

Batch reaction kinetics with 77% sucrose (Brix 60) is shown in Fig. 2. For this particular experiment, free cells equivalent to 5 U/g sucrose were employed. The fructo-oligosaccharides formed from this time-course reaction were found to be GF_2, GF_3, and GF_4, and the liberation of glucose occurred simultaneously, as shown in Fig. 2. Similar reaction patterns were found by us with a fructosyltransferase enzyme system. The fructo-oligosaccharides formed accounted for 55–57% of the total sugars after 24 h reaction.

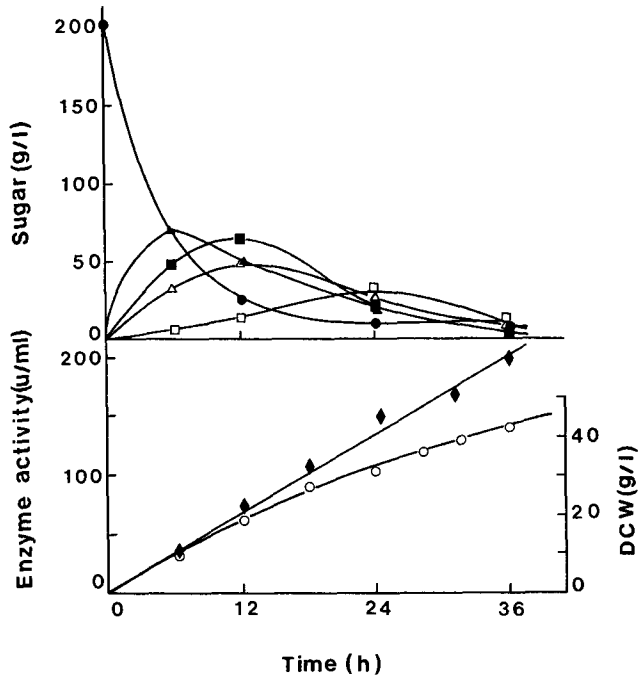

Fig. 1. Batch culture kinetics of *A. pullulans* on 20% sucrose medium at 28°C. (△), G; (●), GF; (▲), GF_2; (■), GF_3; (□), GF_4; (◆), enzyme activity; and (○), dry cell weight.

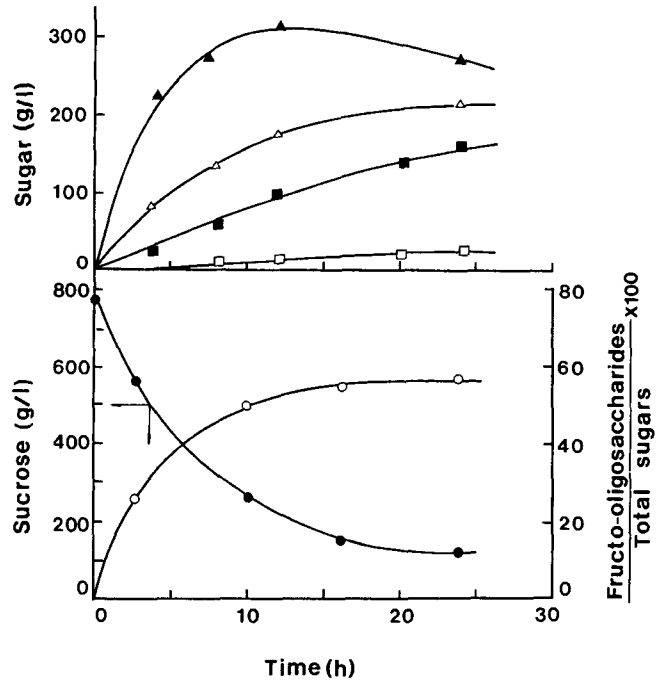

Fig. 2. Batch reaction kinetics with 77% sucrose by the use of 5 U free cells at 55°C and pH 5.5. (△), G; (●), GF; (▲), GF_2; (■), GF_3; and (□), GF_4.

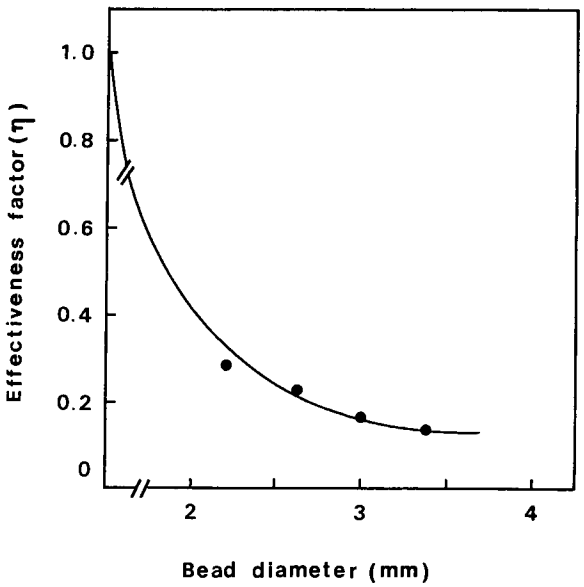

Fig. 3. The influence of bead diameter on the effectiveness factor.

Measurement of Effectiveness Factor for Immobilized Cells

The effect of alginate bead size on effectiveness factor was studied, as shown in Fig. 3. The effectiveness factor was determined by measuring the ratio of the observed initial rate of sucrose disappearance to that with free cells (*14*). The effectiveness factor for the bead diameter of 2.2 to 3.3 mm was in the range 0.1–0.3. The beads, which have an effectiveness factor of 0.3, were used in later kinetic studies.

The internal structure of the immobilized cells has been examined by scanning electron microscopy (Jeol 35 CF, Tokyo, Japan), as shown in Fig. 4. Clearly, cells of *A. pullulans* were entrapped evenly throughout the alginate matrix.

Production of Fructo-Oligosaccharides from Sucrose by Immobilized Cells

In Fig. 5, a typical time-course kinetic study is shown. The peak concentration of GF_2 was reached after 5 h. Thereafter, the concentration declined, presumably because GF_2 was being converted more rapidly to GF_3, GF_4, and GF_5 than it was being formed. In Fig. 6, the production of fructo-oligosaccharides with 77% sucrose with different immobilized cell dosages at pH 5.5 and 55°C is compared. It is clear that increasing the amount of immobilized cell dosage resulted in an increased initial reaction

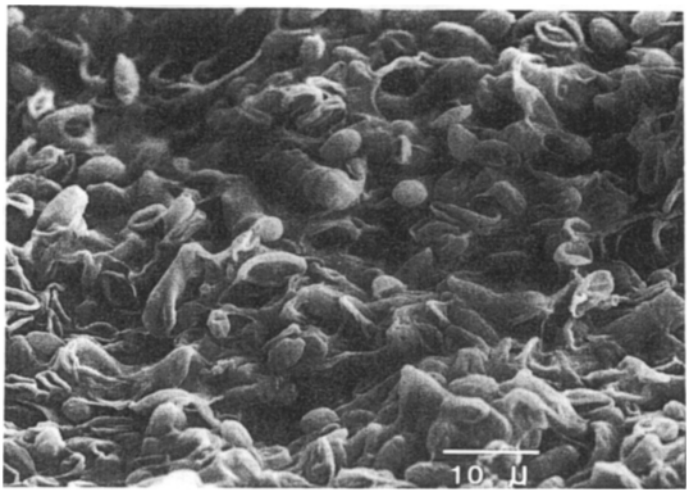

Fig. 4. A scanning electron micrograph of immobilized cells.

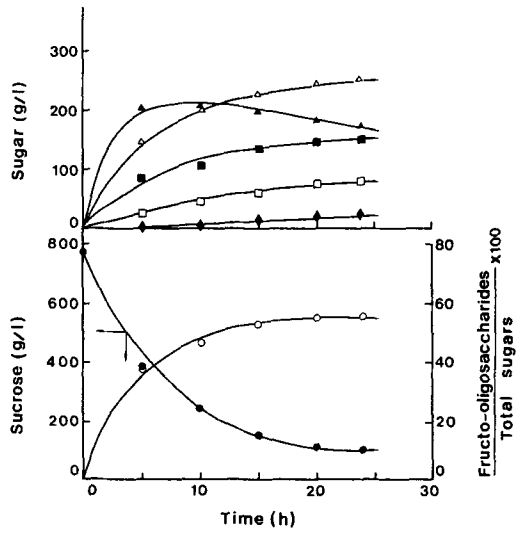

Fig. 5. A typical time-course batch reaction kinetics with 77% sucrose by the use of 25 U immobilized cells at 55°C and pH 5.5. (△), G; (●), GF; (▲), GF_2; (■), GF_3; (□), GF_4; and (◆) GF_5.

rate of fructo-oligosaccharides production. The reaction rate declined significantly at low immobilized cell dosage (5 U) compared to 5 U of free cells. By comparison with the system with free cells, the total amount of fructo-oligosaccharides produced by immobilized cells was similar when 25 U of immobilized cells were used although GF_5 was formed besides GF_2, GF_3, and GF_4, as illustrated in Table 1.

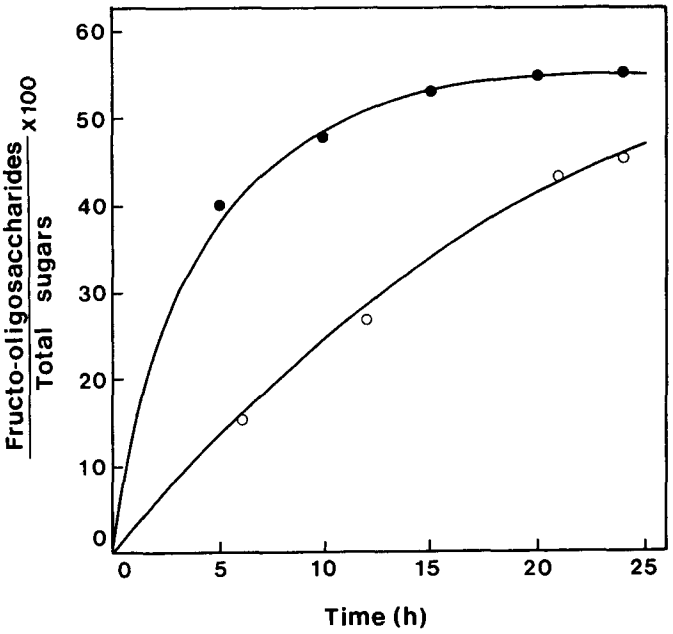

Fig. 6. Effect of immobilized cell dosage on the production of fructo-oligosaccharides: (●), 25 U; (○) 5 U.

Table 1
Comparison of Typical Composition of Reaction Products with Free Cells and Immobilized Cells

Sugars		Free cells, %	Immobilized cells, %
Mono- and disaccharides	F	1.0	1.0
	G	26.6	29.0
	GF	15.0	15.0
	F+G+GF	42.6	45.0
Fructo-oligosaccharides	GF_2	35.0	26.0
	GF_3	19.0	19.5
	GF_4	3.4	7.8
	GF_5	-	1.7
	$\sum_{n=2}^{5} GF_n$	57.0	55.0
Total		100.0	100.0

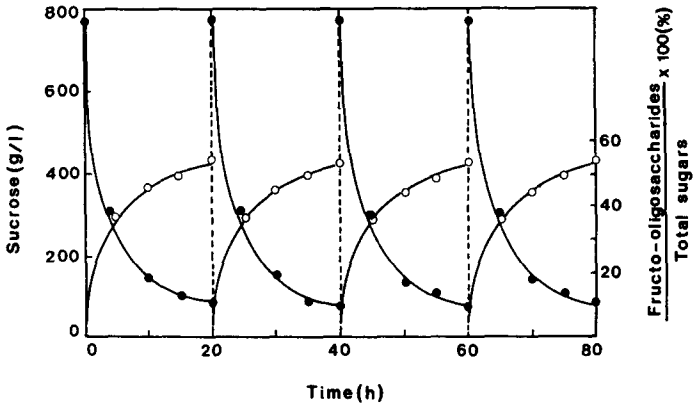

Fig. 7. A typical profile of semibatch production of fructo-oligosaccharides at 50°C.

Semibatch Production of Fructo-Oligosaccharides with Immobilized Cells

Semibatch production of fructo-oligosaccharides was investigated in a stirred tank bioreactor. In the semibatch process, reaction was carried out in batch mode. The whole reaction product was drawn off within 5 min, and the reactor was filled quickly with fresh feed substrate and the operation repeated with the same immobilized cells. Initial studies on 77% sucrose feed at 55°C showed that it was not possible to obtain reproducible cycles that are essential for sustained semibatch process, probably owing to deactivation of immobilized cells caused by prolonged exposure to high temperatures. However, it was possible to maintain steady-state cycles at 50°C, as shown in Fig. 7. To check the long-term operational stability of semibatch process, the semibatch operation was repeated up to 60 cycles (over a period of 1200 h). As shown in Fig. 8, there was no evidence for the deactivation of immobilized cells at 50°C and, therefore, the total amount of fructo-oligosaccharides at the end of each cycle (expressed by Relative Productivity in Fig. 8) was not changed.

For comparative purposes, continuous production of fructo-oligosaccharides was also carried out in a stirred tank bioreactor at a dilution rate of 0.05 h^{-1} (residence time of 20 h). Unlike the semibatch process, it was found that a high concentration of glucose (77% feed × 0.27 = 21%) was steadily maintained in the bioreactor with the conventional continuous process. As illustrated by Table 2, it is clear that the performance of the semibatch process is superior to the continuous process for the production of fructo-oligosaccharides. This was owing to the decrease in reaction rate associated with competitive inhibition caused by a high concentration of glucose in the latter case (*11*).

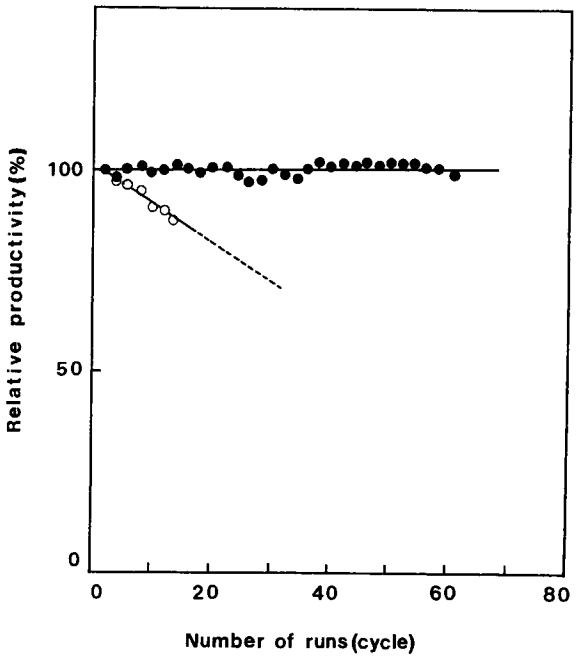

Fig. 8. Effect of operational temperature on the long-term stability for semibatch production of fructo-oligosaccharides: (●), 50°C; (○) 55°C.

Table 2
Comparison of Typical Composition of Reaction Products with Semibatch and Continuous Processes

Sugars		Semibatch process,[a] %	Continuous process,[b] %
Mono- and disaccharides	F	1.0	1.0
	G	29.0	27.0
	GF	15.0	29.0
	F+G+GF	45.0	57.0
Fructo-oligosaccharides	GF_2	26.0	19.4
	GF_3	19.5	15.5
	GF_4	7.8	6.7
	GF_5	1.7	1.4
	$\sum_{n=2}^{5} GF_n$	55.0	43.0
Total		100.0	100.0

[a]Processing time = 20 h.
[b]Dilution rate = 0.05 h^{-1}.

CONCLUSIONS

By comparison with the free cell system, the final ratio of total fructo-oligosaccharides to total sugars was similar with the system of immobilized cells although GF_5 was formed besides GF_2, GF_3, and GF_4, as illustrated in Table 1. The difference in the composition of fructo-oligosaccharides may have resulted from the diffusional resistance associated with immobilized cells.

It appears that the semibatch process provided a means of steady production of fructo-oligosaccharides at 50°C over a period of 1200 h although the process was operated under unsteady-state conditions. Furthermore, the performance of the semibatch process was superior to the continuous process. The decrease in reactor performance with the continuous process was owing to prolonged exposure to high concentrations of glucose, which is considered to be a competitive fructosyltransferase inhibitor.

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REFERENCES

1. Cheetham, P. S. J., Imber, C. E., and Isherwood, J. (1982), *Nature* **299,** 628.
2. Cheetham, P. S. J., Garrett, C., and Clark, J. (1985), *Biotechnol. Bioeng.* **27,** 471.
3. Greenberg, N. A. and Mahoney, R. R. (1983), *Food Chem.* **10,** 195.
4. Prenosil, J. E., Stuker, E., and Bourne, J. R. (1987), *Biotechnol. Bioeng.* **30,** 1019.
5. Hidaka, H., Eida, T., Adachi, T., and Saitoh, Y. (1987), *Nippon Nogeikagaku Kaishi* **61,** 915.
6. Oku, T., Tokunaga T., and Hosoya, N. (1984), *J. Nutr.* **114,** 1574.
7. Gupta, A. K. and Bhatia, I. S. (1980), *Phytochemistry* **19,** 2557.
8. Gupta, A. K. and Bhatia, I. S. (1982), *Phytochemistry* **21,** 1249.
9. Torrey, S. (1983), *Enzyme Technology: Preparation, Purification, Stabilization, Immobilization,* Noyes Data Corporation, Park Ridge, NJ, p. 204.
10. Jung, K. H., Lim, J. Y., Yoo, S. J., Lee, J. H., and Yoo, M. Y. (1987), *Biotechnol. Lett.* **9,** 703.
11. Jung, K. H., Yun, J. W., Kang, K. R., Lim, J. Y., and Lee, J. H. (1989), *Enzyme Microb. Technol.,* **11,** 491.
12. Lee, J. H., Skotnicki, M. L., and Rogers, P. L. (1982), *Biotechnol. Lett.* **4,** 615.
13. Jain, V. K., Toran-Diaz, I., and Baratti, J. (1985), *Biotechnol. Bioeng.* **27,** 273.
14. Kasche, V. (1983), *Enzyme Microb. Technol.* **5,** 2.