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Ethanol production from starch by immobilized *Aspergillus awamori* and *Saccharomyces pastorianus* using cellulose carriers

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A simultaneous saccharification and fermentation (SSF) process was investigated to produce ethanol using two kinds of cellulose carriers that were respectively suitable for immobilization of *Aspergillus awamori* and *Saccharomyces pastorianus*. The maximum ethanol concentration attained by the batch operation was 25.5 g l^{-1}. Under suitable conditions, both cellulose carriers with immobilized cells could be reused efficiently for three cycles. The total amount of ethanol production was 66.0 g (per 1 l working volume) after the repeated operation. Ethanol productivity mainly depends on a saccharification process. There is a limit in durability in the repeated batch operation, and it is important to maintain high activity of the fungus in order to produce ethanol efficiently. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 52–57.

Keywords: simultaneous saccharification and fermentation; fungus; production of ethanol, cellulose carrier; *Aspergillus awamori*; *Saccharomyces pastorianus*; repeated batch operation

Introduction

In recent years, simultaneous saccharification and fermentation (SSF) processes have been widely investigated. Many researchers have approached the production of ethanol or organic acids from various substrates by SSF. Sugary substrates such as starch [3,4,23,24] and cellulose [5,17] were generally used for this purpose. In addition, poplar hardwood [14], wheat straw [16] and waste newspaper [19] were used for ethanol production in a unique case. SSF is a useful method for fermentation from polysaccharide as a carbon source, although the presence of end-product inhibition for enzyme activity has been reported [6,22]. Anuradha *et al* [1] concluded that SSF shows promise as a better alternative to the conventional process to obtain a high yield of lactic acid. Furthermore, Krishna *et al* [11] reported that better ethanol yields were obtained with SSF compared with the traditional saccharification and subsequent fermentation.

SSF processes are classified into two groups: (1) a combination of enzymatic saccharification and microbial fermentation. Krishna *et al* [10] studied ethanol production from sugar cane leaves using cellulase and *Saccharomyces cerevisiae*. Kim *et al* [8] used cellulase, β-glucosidase and *Brettanomyces custersii* in ethanol production from oak wood; and (2) using microorganisms in all stages. Suresh *et al* [20] reported ethanol production from rice using *Aspergillus niger* and *S. cerevisiae*.

Immobilization of the enzyme or microorganism is also available in SSF processes as well as other bioprocesses. Krishnan *et al* [12] used κ-carrageenan as a carrier for immobilized glucoamylase and *Zymomonas mobilis* in ethanol production from corn starch. *A. awamori* and *Z. mobilis* immobilized on calcium alginate gel were used for ethanol production from starch [13,21]. The entrapment method using gel beads is strongly susceptible to shear force, and substrate supply into the beads is likely to be insufficient; these gel beads are considered less effective carriers for large-scale production. A chemical and physical adsorption method for immobilization of the enzyme or microorganism has advantages over the entrapment method. The adsorption method is significantly simpler in terms of handling and does not require special apparatus for immobilization. Especially, porous carriers [7] have a number of superior features when used as support materials for the adsorption method, which overcome limitations of the entrapment method using gel beads. For example, phenol was degraded by immobilized *Pseudomonas putida* and *Cryptococcus elinovii* using the adsorption method [15]. Thus, the SSF process with immobilization is useful for performing multistage reactions or cultures with repeated batch operations. However, there are only few reports of the SSF process with immobilization using the adsorption method.

In the present work, the SSF process for ethanol production from starch was studied using immobilized *A. awamori* and *S. pastorianus* in mixed culture. In the experiment, two kinds of porous cellulose carriers, which are inexpensive and biodegradable, were used for immobilization. Since the ethanol productivity depends on the saccharification process in SSF, the optimal conditions with respect to oxygen supply for the fungus in saccharification were investigated first. Based on these optimal conditions for the fungus, conditions for the yeast with regard to the immobilization and fermentation process were investigated. We also estimated the feasibility of SSF with repeated batch operation.

Materials and methods

Microorganisms

The microorganisms employed were *A. awamori* IFO 4033 and *S. pastorianus* IFO 0751. *A. awamori* was used for saccharification as a biocatalyst that has glucoamylase activity, and *S. pastorianus* was used for the ethanol fermentation process. The stock culture for *A. awamori* was maintained on potato dextrose agar slants containing 4 g potato infusion, 20 g glucose and 15 g agar per liter. The stock

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culture for *S. pastorianus* was maintained on slants containing 10 g glucose, 5 g bactopeptone, 3 g malt extract, 3 g yeast extract and 15 g agar per liter.

Carriers

Cellulose foam carrier (FC) for fungus: A cellulose foam carrier (FC) having a 3.0-mm cubic shape was used for immobilization of *A. awamori*. FC was provided by Biomaterial (Fukui, Japan). This carrier has a sponge-like structure and a mean pore diameter of 1.26 mm. FC was suited for immobilization of the fungus [2]. Figure 1A shows a scanning electron micrograph of FC.

Cellulose bead carrier (YC) for yeast: A cellulose bead carrier (YC) of 3.5-mm diameter was used for immobilization of *S. pastorianus*. YC was provided by Rengo (Osaka, Japan). This carrier has numerous small surface pores (mean diameter of 7 μm) as well as considerably larger pores (diameter of 100–200 μm) radiating from the center. YC was suited for immobilization of the yeast [18]. Figure 1B shows a scanning electron micrograph of YC.

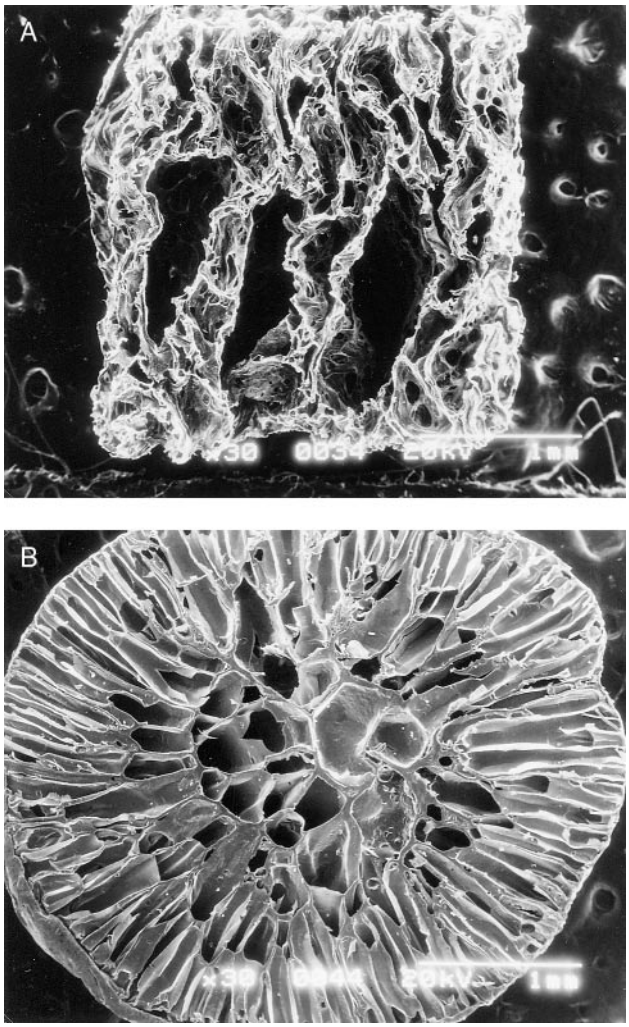

Figure 1 (A) Scanning electron micrograph of cellulose foam carrier (FC). (B) Scanning electron micrograph of cellulose bead carrier (YC).

Media

The preculture medium used for growth of *S. pastorianus* contained 20 g glucose, 5 g bactopeptone, 3 g malt extract and 3 g yeast extract per liter. The medium for immobilization of *A. awamori* and *S. pastorianus* contained 40 g glucose, 5 g polypeptone, 2 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$ and 1 g KH_2PO_4 per liter. The SSF medium for ethanol production contained 100 g starch, 5 g polypeptone, 2 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$ and 1 g KH_2PO_4 per liter. The initial pH of SSF medium was adjusted to 6.0, but the pH values of the others were not adjusted. The concentrations of polypeptone and yeast extract in SSF medium were varied by changes in the experimental conditions. Glucose was used instead of starch to verify the ethanol-fermenting ability of *S. pastorianus* alone.

Preparation of immobilized carrier

Immobilization for fungus: A spore suspension of *A. awamori* (10^7 spores) was inoculated in 200 ml of the immobilization medium with 20 ml of FC and then incubated in a 500-ml Erlenmeyer flask on a rotary shaker at 100 rpm and 30°C for 3 days. After the immobilization step, FC immobilized with *A. awamori* was transferred into SSF medium.

Immobilization for yeast: *S. pastorianus* was grown in the preculture medium on a rotary shaker at 150 rpm and 30°C for 1 day. To immobilize *S. pastorianus* on YC, 5 ml of the preculture medium in which *S. pastorianus* had grown was inoculated in 200 ml of the immobilization medium with 20 ml of YC. It was incubated in a 500-ml Erlenmeyer flask on a rotary shaker at 100 rpm and 30°C for 3 days. During the 3 days, the immobilization medium was exchanged five times at 12-h intervals. After the immobilization step, YC immobilized with *S. pastorianus* was transferred into SSF medium.

Saccharification and fermentation using immobilized fungus and yeast

For saccharification, 200 ml of the production medium (SSF medium) and 20 ml of FC immobilized with *A. awamori* were incubated in a 500-ml Erlenmeyer flask on a rotary shaker at 50, 100 and 150 rpm and 30°C for 8 days. For ethanol fermentation, 200 ml of the production medium containing 100 g glucose and 20 ml of YC immobilized with *S. pastorianus* were incubated in a 500-ml Erlenmeyer flask on a rotary shaker at 100 rpm and 30°C for 1 day.

SSF and SSF with repeated batch operation

SSF was carried out using a mixture of FC immobilized with *A. awamori* and YC immobilized with *S. pastorianus*. A 200-ml portion of SSF medium and 40 ml of the mixture of immobilized microorganisms were incubated in a 500-ml Erlenmeyer flask on a rotary shaker at 100 rpm and 30°C for 8 days. SSF with repeated batch operation was carried out as follows: at the end of the batch operation, the spent medium was removed using sterilized gauze, and the same volume of fresh SSF medium was added to the flask. The SSF process was carried out for four cycles at 4-day intervals.

Assays

Biomass expressed as a single culture or mixed culture of fungus and yeast was measured as the dry weight of the cells. Immobilized cells with the carriers were separated from the medium using a Tyler seven-mesh sieve, and washed with distilled water. They were dried at 105°C for 3 days in order to weigh the biomass. For free cells in the medium, the medium separated from immobilized cells was filtered through paper (pore size of 1 μm). The precipitate was then measured using the same procedure as for immobilized cells.

The glucose concentration was determined enzymatically using the Glucose C-II Test (Wako, Osaka, Japan). The ethanol concentration was analyzed by gas chromatography, which was performed with a flame ionization detector and a glass column (3×200 mm) of Shimalite (PEG 6000) using 2-methyl-1-propanol as an internal standard. The starch concentration was measured as follows [9]. The filtered medium containing starch was diluted 500 times with distilled water. One milliliter of the diluted medium was added to 5 ml of sulfuric acid solution containing 10 mg anthrone in a glass tube. The tube was then immersed in an iced water bath for 5 min and transferred into boiling water for 11 min. It was then placed in an iced water bath for 5 min and then held at room temperature. After 60 min, the absorbance at 620 nm was measured.

The yield was calculated by the ratio of ethanol produced to glucose or starch consumed.

Results and discussion

Saccharification by immobilized fungus

Immobilization of *A. awamori* was carried out using FC. After immobilization, the amount of immobilized *A. awamori* reached 26.6 g l^{-1} carrier. Figure 2A shows a scanning electron micrograph of *A. awamori* immobilized on FC; *A. awamori* was immobilized perfectly as if twisted around FC.

The effect of the speed of a rotary shaker on saccharification was also investigated. Saccharification of starch by immobilized *A. awamori* was carried out using several rotating speeds (Figure 3). In all cases, the starch concentrations were approximately 20 g l^{-1} after 8 days of saccharification. Saccharification rates were accelerated with the rotating speed at 100 and 150 rpm compared with that at 50 rpm. This could be due to insufficient oxygen supply into the production medium at 50 rpm. At rotating speeds of 100 and 150 rpm, 60–65% of the starch was saccharified after 4 days. On the other hand, the glucose concentrations produced were not in proportion to the decrease in the starch concentrations, and they were low under the 100 and 150 rpm conditions. This shows that the glucose produced is consumed efficiently due to aerobic metabolism of *A. awamori* under higher oxygen conditions. Furthermore, *A. awamori* grew during saccharification (Table 1). The amount of immobilized *A. awamori* reached 50.4 g l^{-1} carrier at 150 rpm after saccharification. At the low rotating speed (50 rpm), it showed sparse growth and did not maintain the saccharification activity for a long period (data not shown). Although the amount of free cells increased with an increase in the rotating speed, it was low compared with the amount of immobilized cells. From these results, it is considered that the rotating speed of 100 rpm, which provides a lower oxygen supply than 150 rpm, is suitable with regard to SSF because anaerobic culture is required for ethanol production. Therefore, subsequent experiments were performed at 100 rpm.

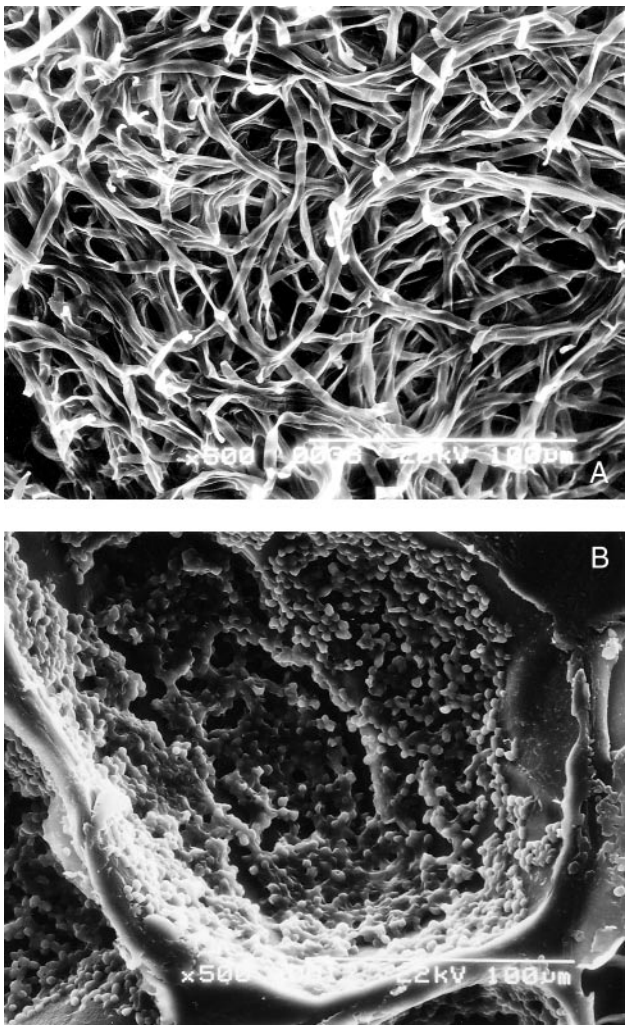

Figure 2 (A) Scanning electron micrograph of *A. awamori* immobilized on FC. (B) Scanning electron micrograph of *S. pastorianus* immobilized on YC.

Approximately 20% of the starch was not hydrolyzed by glucoamylase from *A. awamori*. This suggests that *A. awamori* cannot produce isoamylase, which catalyzes hydrolysis of the α-1,6-glucoside bond in starch.

Ethanol production from glucose by immobilized yeast

Immobilization of *S. pastorianus* was carried out using YC. After immobilization, the amount of immobilized *S. pastorianus* reached 37.6 g l^{-1} carrier. Figure 2B shows a scanning electron micrograph of *S. pastorianus* immobilized on YC, mainly within the large pores inside YC.

Next, the ability of immobilized *S. pastorianus* to carry out the ethanol fermentation was evaluated using the production medium with glucose. Immobilization and fermentation were carried out under optimum conditions determined in previous section. Figure 4 shows the time course of ethanol production from glucose by immobilized *S. pastorianus*. After 16 h, glucose was completely consumed and the ethanol concentration became 44.5 g l^{-1},

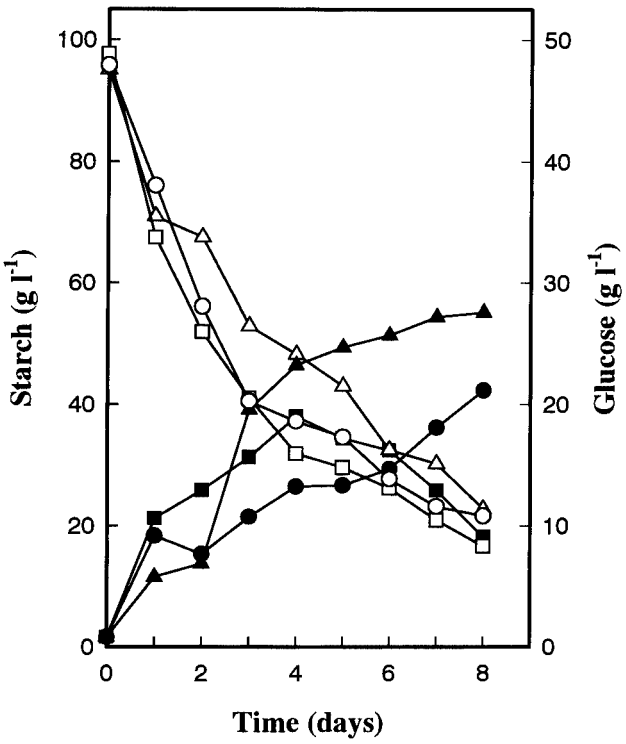

Figure 3 Time courses of saccharification from starch at different rotating speeds. Starch: (△) 50 rpm, (○) 100 rpm, (□) 150 rpm. Glucose: (▲) 50 rpm, (●) 100 rpm, (■) 150 rpm.

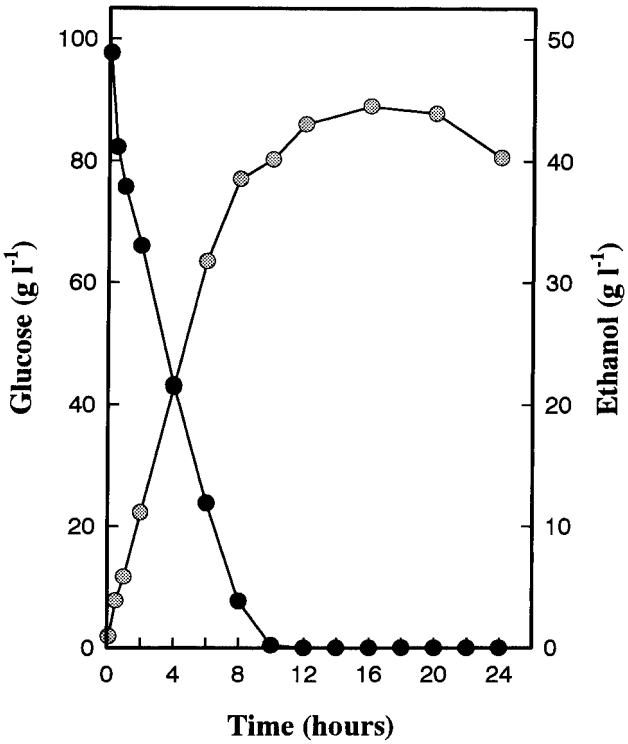

Figure 4 Time course of ethanol fermentation from glucose. Symbols: (●) glucose, (dotted circle) ethanol.

corresponding to a yield of 45.6% based on the glucose consumed. It was confirmed that the conditions for saccharification of starch were suitable for ethanol production and that YC was a useful carrier for *S. pastorianus*. These results suggest that the SSF process depends on the saccharification of starch because the fermentation rate is substantially higher compared with the saccharification rate of starch.

SSF using a mixture of immobilized fungus and yeast

The SSF process was carried out using a mixture of FC immobilized with *A. awamori* and YC immobilized with *S. pastorianus*. In these experiments, the mixtures of 20 ml of each carrier immobilized with cells were used (this mixing ratio is expressed as FC/YC=1/1 hereafter). Figure 5 shows the time course of SSF in FC/YC=1/1. The glucose produced from starch was used for fermentation promptly and was rarely observed during SSF. The amount of ethanol produced reached a peak, 25.5 g l^{-1}, after 5 days and then decreased slightly. At the end of SSF, the ethanol concentration was 23.5 g l^{-1}. This decrease is due to the consumption of ethanol by metabolism of the fungus or yeast at the end of starch saccharification. The yield of ethanol with regard to the consumed starch was 44.4%, which was slightly lower than

Table 1 Amounts of immobilized and free cells for different rotating speeds on saccharification by *A. awamori*

Rotating speed (rpm)	Immobilized cells (g l^{-1} carrier)	Free cells (g l^{-1})
After immobilization	26.6	–
50	28.8	1.2
100	38.2	3.8
150	50.4	11.4

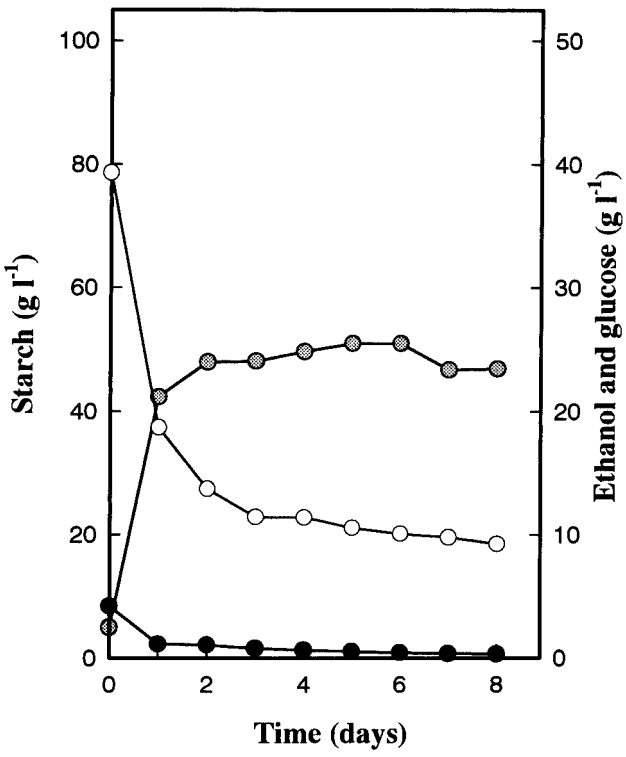

Figure 5 Time course of SSF from starch in FC/YC = 1/1. Symbols: (○) starch, (●) glucose, (dotted circle) ethanol.

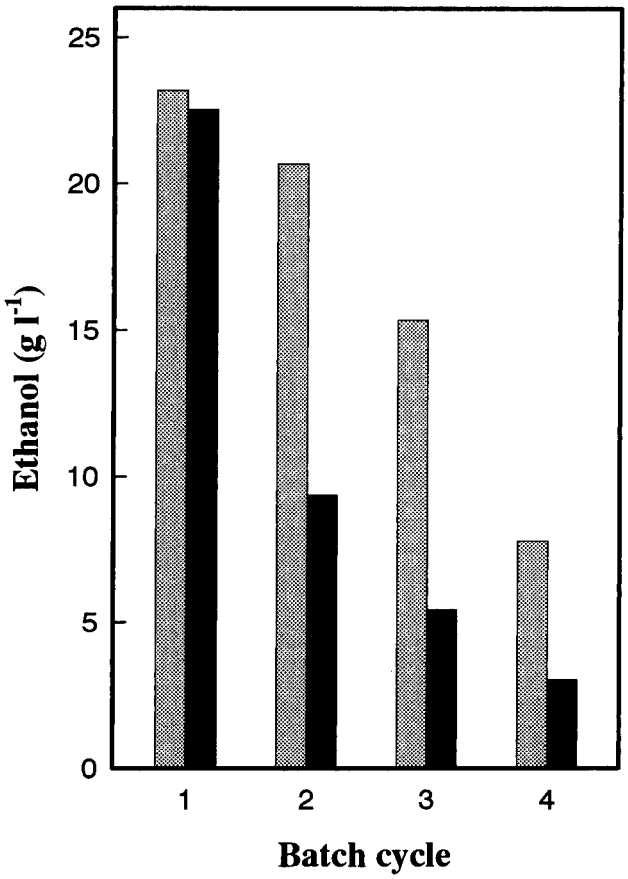

Figure 6 Ethanol production under repeated batch operation at different concentrations of polypeptone and yeast extract. Symbols: (dotted square) SSF medium, (■) modified SSF medium.

45.6% obtained from the glucose medium. Furthermore, the starch saccharification seemed to be saturated at 5–6 days with 18.5 g l^{-1} of starch remaining in the SSF process. This reason is explained in *Saccharification by immobilized fungus* section.

SSF with repeated batch operation

Effect of medium components on ethanol production: The effects of the nitrogen source in SSF medium and modified SSF medium on ethanol production were investigated under repeated batch operation using the mixing ratio of carriers with FC/YC=1/1. Modified SSF medium contained 1.0 g polypeptone and 0.4 g yeast extract per liter, which is 1/5 of the amounts in SSF medium. Figure 6 shows that the ethanol concentrations in both SSF medium and modified SSF medium were around 23 g l^{-1} at the first cycle. After the second cycle, the ethanol concentration in SSF medium gradually decreased, but that in modified SSF medium rapidly decreased and became less than 10 g l^{-1}. Thus, the nitrogen source in modified SSF medium was insufficient to produce enzymes for the starch hydrolysis such as glucoamylase. Therefore, the subsequent experiments were performed using SSF medium.

Effect of mixing ratio of immobilized fungus and yeast on ethanol production: The effects of the mixing ratio of the carriers, FC immobilized with *A. awamori* and YC immobilized with *S. pastorianus*, on ethanol production in the SSF process were investigated. Three ratios of carriers immobilized with cells were used for SSF in repeated batch operation. The profiles of starch saccharification and ethanol production were similar in the three SSF modes at the first cycle (Figure 7). The starch and ethanol concentrations at the end of the first cycle were in the range of 18.9–23.3 and 22.2–23.9 g l^{-1}, respectively. However, starch saccharification and ethanol production under each condition after

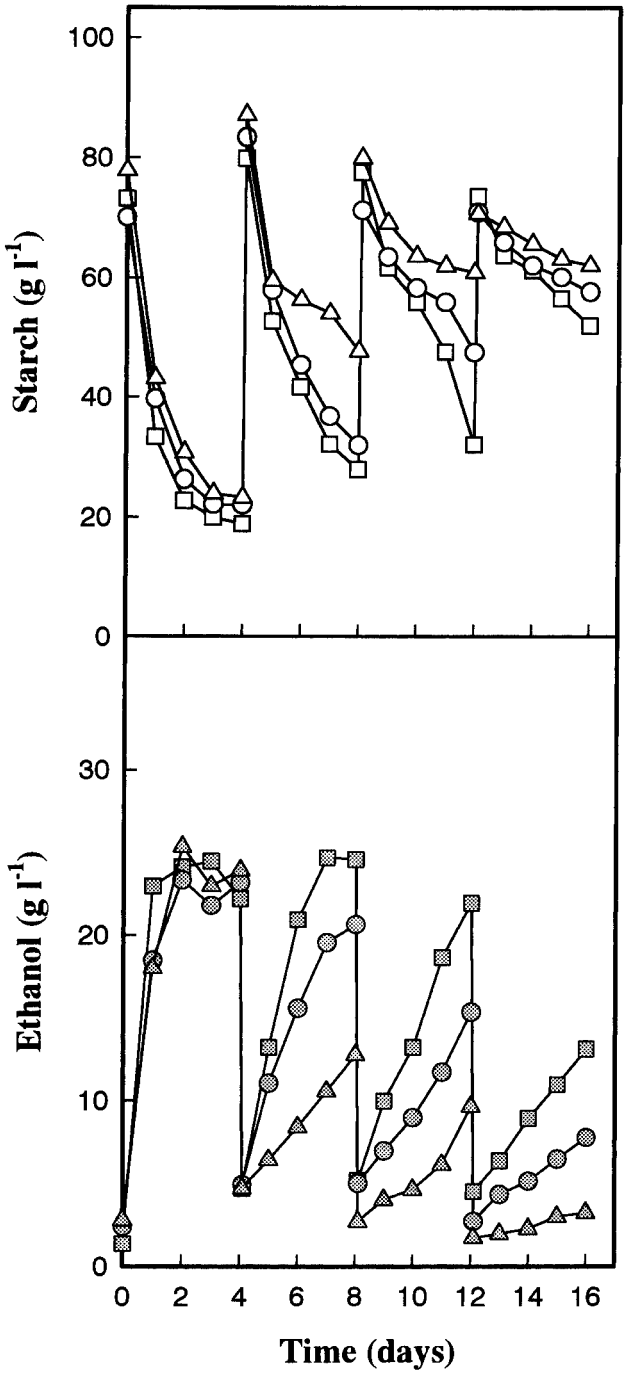

Figure 7 Time courses of SSF process in repeated batch operation at different mixing ratios of immobilized cells. Starch: (□) FC/YC = 3/1, (○) FC/YC = 1/1, (△) FC/YC = 1/3. Ethanol: (dotted square) FC/YC = 3/1, (dotted circle) FC/YC = 1/1, (dotted triangle) FC/YC = 1/3.

Table 2 Amounts of immobilized and free cells at different mixing ratios of immobilized cells during repeated batch operation

Mixing ratio (FC/YC)	Immobilized cells (g l^{-1} carrier)		Free cells at the end of each cycle (g l^{-1})			
	After immobilization	End of fourth cycle	First	Second	Third	Fourth
3/1	29.4	125.8	1.2	1.6	1.3	1.0
1/1	32.1	155.8	1.7	1.7	1.2	1.0
1/3	34.9	161.8	2.1	1.1	0.7	0.6

the second cycle showed different profiles. For example, the ethanol productivities in SSF with FC/YC=3/1 were maintained at a high level until the third cycle, and the ethanol concentrations were more than 20 g l^{-1}. The SSF process with FC/YC=3/1 showed the highest ethanol productivity. The total amount of ethanol produced was 66.0 g (per liter working volume), and the average yield with regard to starch consumed was 38.1% for four cycles. However, ethanol productivity at the fourth cycle decreased somewhat, and the ethanol concentration became 13.1 g l^{-1} due to less starch hydrolysis. On the other hand, ethanol production in SSF with FC/YC=1/3 and 1/1 decreased gradually after the second cycle, and the ethanol concentrations were 3.2 and 7.8 g l^{-1} at the fourth cycle. After the fourth cycle, ethanol fermentation was performed using glucose instead of starch in SSF medium. As the ethanol productivity was similar to that described in the *Ethanol production from glucose by immobilized yeast* section, it was recognized that immobilized *S. pastorianus* still had sufficient ethanol production activity. These results suggest that the saccharification by immobilized *A. awamori* is the dominant factor in the SSF process for repeated batch operation. The saccharification activity of immobilized *A. awamori* will not be stable for a long period. Genetic work may be needed to maintain the high saccharification activity of immobilized *A. awamori*.

Table 2 shows the cells immobilized within the carriers and the cells which leaked out and grew outside the carriers at each batch cycle. In all cases, cells in the carriers increased during the SSF process with repeated batch operation, but the free cells decreased slightly.

In conclusion, we successfully carried out ethanol production by the SSF process using immobilized cells, which were prepared by the adsorption method. This result will be applicable to other simultaneous fermentation processes by mixed microorganisms.

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