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Continuous Production of Succinic Acid by a Fumarate-Reducing Bacterium Immobilized in a Hollow-Fiber Bioreactor

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Abstract

Enterococcus faecalis RKY1, a fumarate-reducing bacterium, was immobilized in an asymmetric hollow-fiber bioreactor (HFBR) for the continuous production of succinic acid. The cells were inoculated into the shell side of the HFBR, which was operated in transverse mode. Since the pH values in the HFBR declined during continuous operation to about 5.7, it was necessary to change the feed pH from 7.0 to 8.0 after 24 h of operation in order to enhance production of succinic acid. During continuous operation with a medium containing fumarate and glycerol, the productivity of succinate was 3.0–10.9 g/(L·h) with an initial concentration of 30 g/L of fumarate, 4.9–14.9 g/(L·h) with 50 g/L of fumarate, and 7.2–17.1 g/(L·h) with 80 g/L of fumarate for dilution rates between 0.1 and 0.4 h^{-1}. The maximum productivity of succinate obtained by the HFBR (17.1 g of succinate/[L·h]) was 1.7 times higher than that of the batch bioconversions (9.9 g of succinate/[L·h]) with 80 g/L of fumarate. Furthermore, the long-term stability of the HFBR was demonstrated with a continuously efficient production of succinate for more than 15 d (360 h).

Index Entries: Succinic acid; fumaric acid; *Enterococcus faecalis* RKY1; hollow-fiber bioreactor; immobilization.

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Introduction

Succinic acid ($HOOCCH_2CH_2COOH$) is a dicarboxylic acid produced as a metabolic intermediate in anaerobic microorganisms *(1,2)*. It has recently gained attention as an important chemical because it can be used for the manufacturing of potential chemicals such as 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone and for applications in agriculture, food, medicine, plastics, cosmetics, textiles, plating, and waste-gas scrubbing *(3,4)*. Currently, succinic acid is being produced commercially through chemical synthesis. However, biological production of succinic acid by microorganisms has attracted much interest as an alternative to the chemical production of succinic acid from petroleum feedstocks *(5,6)*.

Some anaerobic and facultative bacteria produce succinic acid as a fermentation product, but few species can produce it as a major product with a high yield *(7–9)*. Although *Anaerobiospirillum succiniciproducens* is well known as a good succinic acid producer *(3,5)*, the fermentation processes including this strict anaerobe are more difficult to handle than those including the facultative anaerobes. Some facultative anaerobes for succinic acid production, such as *Escherichia coli (10,11)*, *Actinobacillus succinogenes (12)*, and *Enterococcus faecalis* RKY1 *(13,14)*, have previously been reported by various researchers. Among these, *E. faecalis* RKY1 is able to produce succinic acid with a high yield if cultured anaerobically with glycerol as a hydrogen donor and fumaric acid as a hydrogen acceptor *(1,13–16)*.

Immobilization of whole microbial cells using a hollow-fiber bioreactor (HFBR) has many advantages over conventional batch operations. The major advantages of the HFBR are a large surface-to-volume ratio, easy separation of cells from the product stream, continuous removal of inhibitory wastes, no culture washout, decreased contamination risks, and higher volumetric productivities *(17)*. This potential immobilization technique has been applied to the production of versatile chemicals such as lactic acid *(18)*, acetic acid *(19)*, citric acid *(20)*, and ethanol *(21,22)*. To our knowledge, however, the direct operation mode of the HFBR systems was typically used by many research groups, but the transverse operation mode was rarely adopted. Moreover, the HFBR systems have not yet been applied to produce succinic acid. Accordingly, we investigated the production of succinic acid by *E. faecalis* RKY1 immobilized in an HFBR. In this article, we report that the HFBR system, as an efficient immobilization method, provides benefits in the continuous production of succinic acid, since a much higher productivity of succinic acid and long-term operation of the HFBR are possible.

Materials and Methods

Microorganism and Medium

E. faecalis RKY1, a facultative bacterium, was used in all the experiments reported here. The medium for cell growth contained 10 g/L of

glycerol, 22 g/L of fumaric acid, 20 g/L of Na_2CO_3, 10 g/L of yeast extract, 10 g/L K_2HPO_4, 1 g/L of NaCl, 0.05 g/L of $MgCl_2 \cdot 6H_2O$, and 0.01 g/L of $FeSO_4 \cdot 7H_2O$. The medium for batch and continuous experiments contained 20 g/L of glycerol, 15 g/L of yeast extract, 10 g/L of K_2HPO_4, 1 g/L of NaCl, 0.05 g/L of $MgCl_2 \cdot 6H_2O$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, and various concentrations of fumaric acid and Na_2CO_3 according to the experimental conditions. The storage stocks of the bacterium were preserved in serum bottles containing 50% (v/v) glycerol at –20°C until used. Glycerol, fumaric acid, Na_2CO_3, and K_2HPO_4 were from Yakuri (Osaka, Japan), yeast extract was from Difco (Detroit, MI), and all other chemicals were reagent grade.

Batch Culture

The seed broth was transferred to new medium every 12 h for 2 d. To prepare an inoculum for the bioconversion experiments, 0.6 mL of the seed cultures was inoculated into the growth medium (40 mL) in 50-mL serum bottles, followed by incubation at 38°C for 6 h on a shaking incubator (KMC-8480S; Vision, Taejon, Korea) at 200 rpm. The batch bioconversions were performed in a 2.5-L fermentor (KF-2.5L; Korea Fermenter, Incheon, Korea) with a 1-L working volume. All experiments were conducted at 38°C, and the culture pH was maintained by the automatic addition of 4 *N* Na_2CO_3. Before inoculation, nitrogen gas was passed through the fermentor vessel for 30 min to ensure anaerobic conditions.

Hollow-Fiber Bioreactor

A schematic diagram of the HFBR system used in these experiments is shown in Fig. 1. The HFBR (GUF-Lab; Sambo Globe, Ansan, Korea) consisted of a cylindrical polyvinyl chloride module (600 mm length and 25.4 mm id) containing 200 hollow fibers. The membranes used were asymmetric polysulfone hollow fibers (0.9 mm id × 1.6 mm od), with a molecular weight cutoff of 20 kDa (Fig. 2). The side ports on the shell side permitted access to the HFBR for inoculation or medium feeding and gas or cell bleeding. Prior to inoculation, the HFBR was sterilized with 0.1 *N* sodium hydroxide and 200 ppm of sodium hypochlorite overnight, followed by washing with sterile water until the pH inside the HFBR was 7.0. A later exponential phase inoculum (12 mL) was then introduced through the shell-side port, and the production medium was delivered to the HFBR through the same port using a peristaltic pump (MP-3N; Eyela, Tokyo, Japan) at various dilution rates. Since the HFBR was operated in the transverse mode, the medium was pumped into the shell side and passed across the membranes into the lumen side, from which products were collected. The culture temperature was maintained at 38°C by circulation of thermocontrolled water through the outer jacket of the HFBR.

Analytical Methods

The concentrations of succinic and fumaric acid were quantified by high-performance liquid chromatography (Waters 510 UV detector;

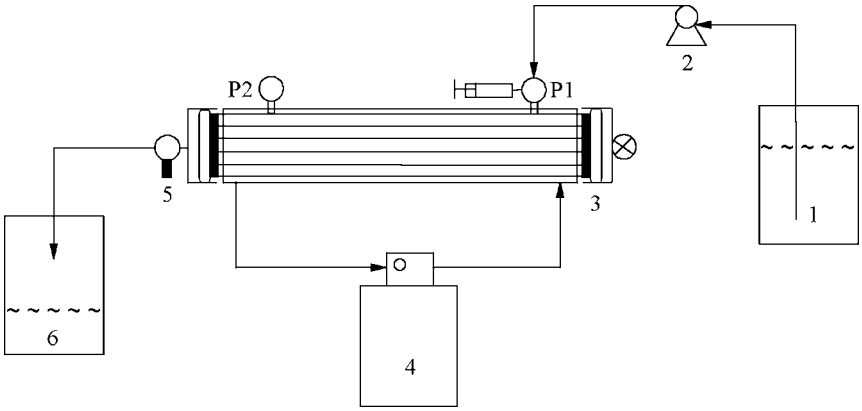

Fig. 1. Experimental setup for continuous production of succinic acid using an HFBR with transverse operation: 1, medium reservoir; 2, peristaltic pump; 3, HFBR; 4, circulator; 5, sampling port; 6, product reservoir; P1, inoculum or medium feeding port; P2, gas- or cell-bleeding port. The HFBR was maintained at constant temperature through the circulation of thermocontrolled water.

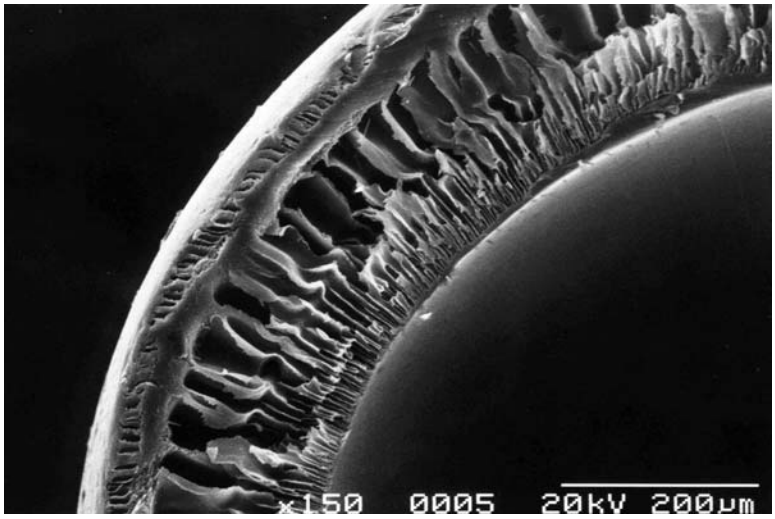

Fig. 2. Scanning electron micrograph of a cross-section of the hollow fiber used. Fiber (1 cm length) was fixed, dehydrated, critical point dried, gold coated, and then examined with an S-2400 (Hitachi, Tokyo, Japan) electron microscope.

Milford, MA) equipped with an ion-exclusion column (Aminex HPX-87H; Bio-Rad, Hercules, CA) using 5 m*M* H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min. The amounts of succinic and fumaric acids were represented as disodium succinate and disodium fumarate, respectively. Cell growth was monitored by measuring the optical density (OD) at 660 nm using a spectrophotometer (UV-160A; Shimadzu, Tokyo, Japan). The dry cell weight was determined using a calibration curve relating the OD at 660 nm to dry weight.

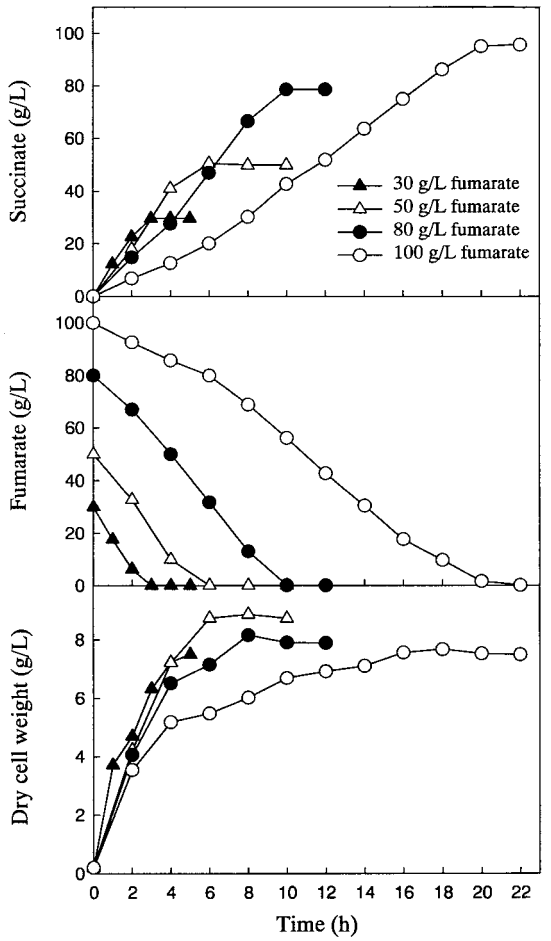

Fig. 3. Bioconversion of fumarate to succinate in batch culture by *E. faecalis* RKY1 with various fumarate concentrations.

Results and Discussion

Batch Bioconversion

Figure 3 shows the experimental data obtained from batch bioconversions with four different fumarate concentrations. Bioconversions were completed between 3 and 22 h, depending on the initial fumarate concentrations. The final succinate concentrations produced were 29.7, 49.8, 78.6, and 95.6 g/L when the media contained 30, 50, 80, and 100 g/L of fumarate, respectively. With a fumarate concentration of 100 g/L, the time required for succinate production (i.e., complete fumarate bioconversion) was more than seven times as long compared with 30 g/L of fumarate. The succinate productivities were 4.3–9.9 g/(L·h), depending on the initial fumarate concentrations, while the maximum cell mass (8.9 g/L), obtained with 50 g/L of fumarate, was much higher than that of the other fumarate concentrations.

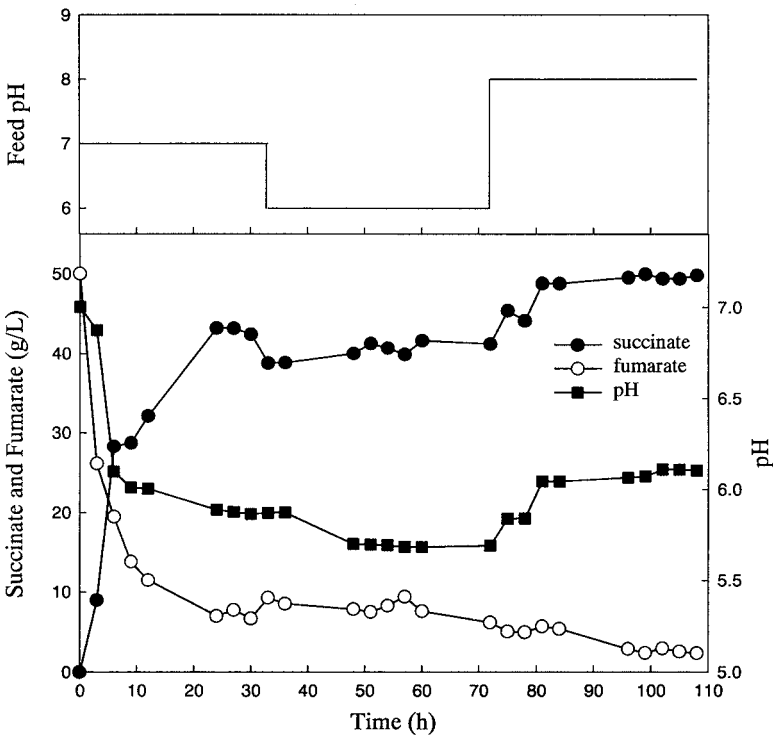

Fig. 4. Effect of feed pH on succinate production, fumarate consumption, and pH change at outlet port using *E. faecalis* RKY1 immobilized in HFBR (dilution rate of 0.1 h^{-1}; 50 g/L of fumarate).

Hollow-Fiber Bioreactor

E. faecalis RKY1 cells were inoculated into the HFBR, and then fresh medium was pumped into the HFBR through the shell side inlet port. During operation of the HFBR, *E. faecalis* RKY1 cells grew more densely near the medium inlet than the outlet. This could be accounted for since the cells near the medium inlet port were in constant contact with fresh medium. In addition, the medium's inlet pH was more favorable than that of the outlet, resulting in a greater bioconversion near the inlet port.

To investigate the characteristics of succinic acid production using the HFBR, the effects of the medium's pH were studied. Figure 4 shows the profiles of succinate production, fumarate consumption, and pH change at the outlet port during HFBR operation when using different medium pHs with a dilution rate of 0.1 h^{-1} and an initial fumarate concentration of 50 g/L. During the first 32 h of operation with the medium at pH 7.0, the maximum succinate concentration achieved was about 43.2 g/L, but the effluent pH decreased to about 5.7. After 32 h of operation, the pH of the medium being pumped into the HFBR was changed to 6.0. During this stage of operation, the maximum succinate concentration decreased slightly to about 41.6 g/L, and the effluent pH also decreased to about 5.6.

When the medium was changed again after 72 h of HFBR operation to a feed having a pH of 8.0, the succinate concentration reached a maximum value of 49.8 g/L and the effluent pH increased to about 6.1. Therefore, it was obvious that although the medium at pH 7.0, the optimum pH for batch bioconversions reported by Ryu et al. *(13)*, was continuously fed to the HFBR, the pH values within the HFBR were somewhat lower with an effluent pH below 6.0, which negatively affected cellular activity and hindered succinic acid production. By changing the medium's pH to 8.0, the succinic acid production was enhanced, because the pH values within the HFBR during operation were favorable for cellular activity and acid production. Thus, in further experiments, the medium at pH 7.0 was supplied to the HFBR for 24 h followed by a shift to the medium at pH 8.0 for the remainder of the experiment. One of the advantages of whole-cell immobilization in the HFBR is the high density achieved by the immobilized cells. A dry cell weight of about 80 g/L (data not shown) was obtained based on the total reactor volume, which corresponded to nine times the dry cell weight achieved with the batch culturing. Similar results were reported by VickRoy et al. *(18)*, who showed a 53-fold increase in cell mass over a batch system of *Lactobacillus delbrueckii*, and by Mehaia and Cheryan *(21)*, who obtained a 30-fold increase in cell mass with *Saccharomyces cerevisiae* in their respective HFBR systems.

Performance of the HFBR at various temperatures is shown in Fig. 5. The HFBR was operated for 129 h with variations in the operating temperature and an initial fumarate concentration of 50 g/L. After the HFBR was operated for 51 h at 38°C, the temperature was switched to 30°C until the 96 h of operation, followed by a second switch to 46°C until the end of the experiment. Succinate concentrations at the various temperatures reached a steady value of 45.7, 49.5, and 48.7 g/L, respectively. While the optimum temperature for HFBR operation and acid production was found to be 38°C, the overall performance was not significantly affected by operating at temperatures between 30 and 46°C.

To further enhance succinic acid productivity, we attempted to continuously produce succinic acid using the HFBR with various fumarate concentrations (30, 50, and 80 g/L) and dilution rates (0.1, 0.2, and 0.4 h^{-1}). The dilution rates presented in this work are defined as the flow rate (mL/h) divided by the total reactor volume (300 mL), which includes the fiber volume (240 mL) and the shell-side volume (60 mL), accessible to *E. faecalis* RKY1 cells between the araldite blocks. With an inlet fumarate concentration of 30 g/L, productivities of 3.0, 5.7, and 10.9 g/(L·h) were obtained with dilution rates of 0.1, 0.2, and 0.4 h^{-1}, respectively (Fig. 6). Therefore, as the dilution rates increased, the productivities also increased, but yields (g of succinate/g of fumarate) decreased from 0.99 to 0.91 g/g.

When a feed concentration of 50 g/L of fumarate was used (Fig. 7), productivities increased to between 4.9 and 14.9 g/(L·h) for dilution rates between 0.1 and 0.4 h^{-1}. Fumarate was completely consumed when the dilution rate was 0.1 h^{-1}. However, the steady-state concentrations of suc-

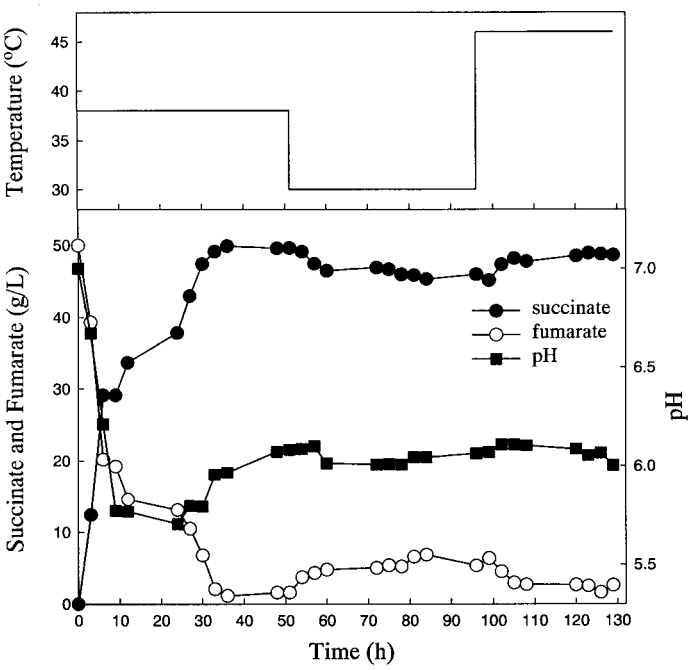

Fig. 5. Effect of temperature on succinate production, fumarate consumption, and pH change at outlet port using *E. faecalis* RKY1 immobilized in HFBR (dilution rate of 0.1 h^{-1}; 50 g/L of fumarate).

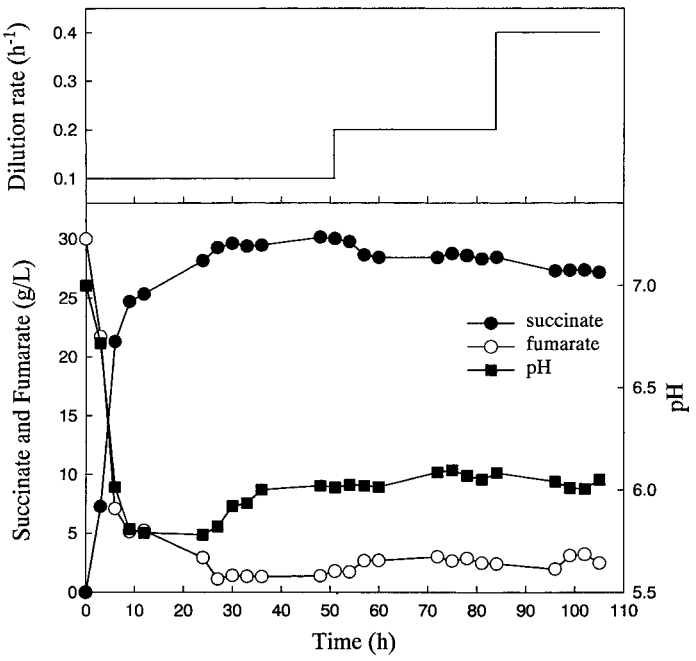

Fig. 6. Effect of dilution rate on succinate production, fumarate consumption, and pH change at outlet port using *E. faecalis* RKY1 immobilized in HFBR (30 g/L of fumarate).

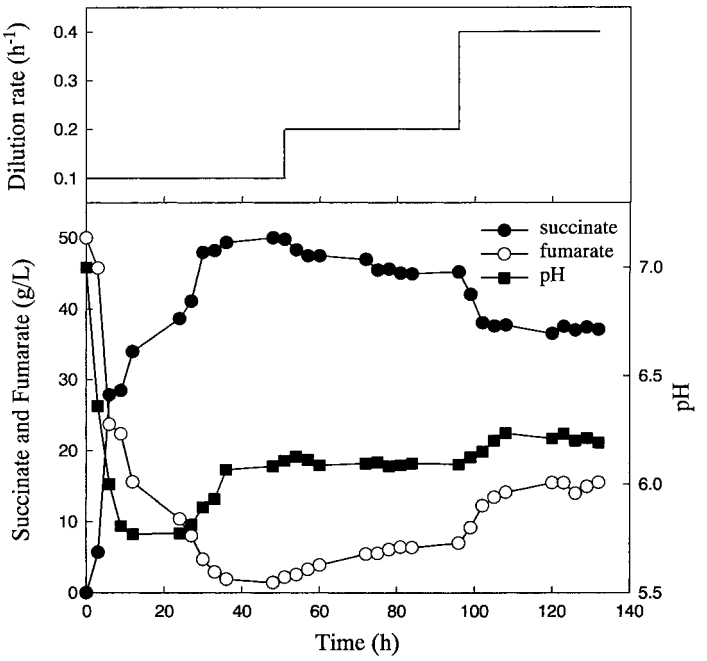

Fig. 7. Effect of dilution rate on succinate production, fumarate consumption, and pH change at outlet port using *E. faecalis* RKY1 immobilized in HFBR (50 g/L of fumarate).

cinate decreased from 49.3 to 37.2 g/L with an increase in the dilution rate from 0.1 to 0.4 h^{-1}.

When a feed concentration of 80 g/L of fumarate was used (Fig. 8), the productivities of succinate ranged between 7.2 and 17.1 g/(L·h). With a low dilution rate (0.1 h^{-1}), the succinate yield was about 0.90 g/g, while the maximum productivity of succinate, 17.1 g/(L·h), was achieved with a dilution rate of 0.4 h^{-1} yielding only 0.54 g/g.

Although fumarate was completely utilized at the lowest dilution rate (0.1 h^{-1}) when 30 and 50 g/L of fumarate in the feed were used, the productivities were lower than when the dilution rate was 0.2 and 0.4 h^{-1}, at which fumarate was not fully consumed. In general, the volumetric productivity of succinate increased with an increase in the dilution rate. However, the productivity of 17.1 g/(L·h) is 1.7–4.0 times higher than those of the batch bioconversions shown in Fig. 3; 14.3 times the batch fermentation using *A. succiniciproducens* ATCC 53488, as reported by Nghiem et al. *(5)*; 8.6 times the batch bioconversion using a recombinant *E. coli*, as reported by Wang et al. *(10)*; and 5.7 times continuous fermentation using *A. succiniciproducens* ATCC 29305, as reported by Samuelov et al. *(23)*, respectively.

Figure 9 shows the long-term stability of HFBR operation when the feed containing 50 g/L of fumarate was used and the dilution rate was set at 0.2 h^{-1}. Succinate production using the HFBR was consistent over 15 d (360 h) of operation. Under the steady-state conditions, succinate concen-

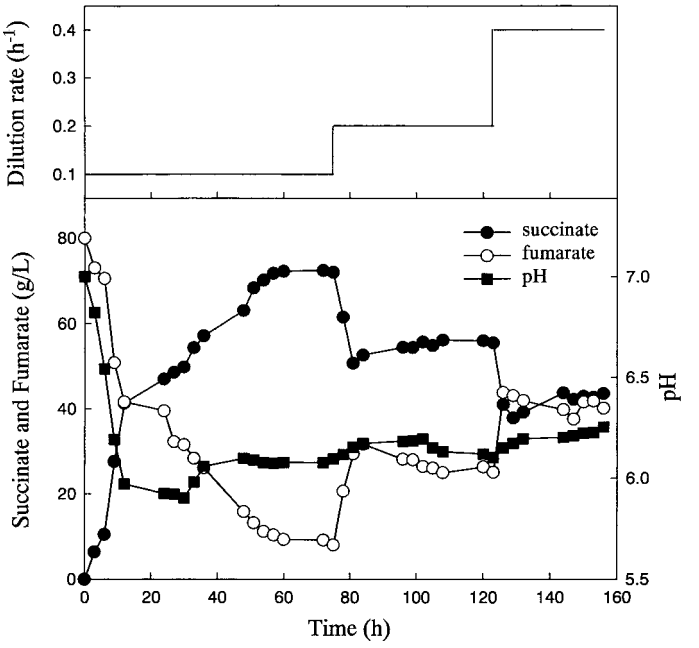

Fig. 8. Effect of dilution rate on succinate production, fumarate consumption, and pH change at outlet port using *E. faecalis* RKY1 immobilized in HFBR (80 g/L of fumarate).

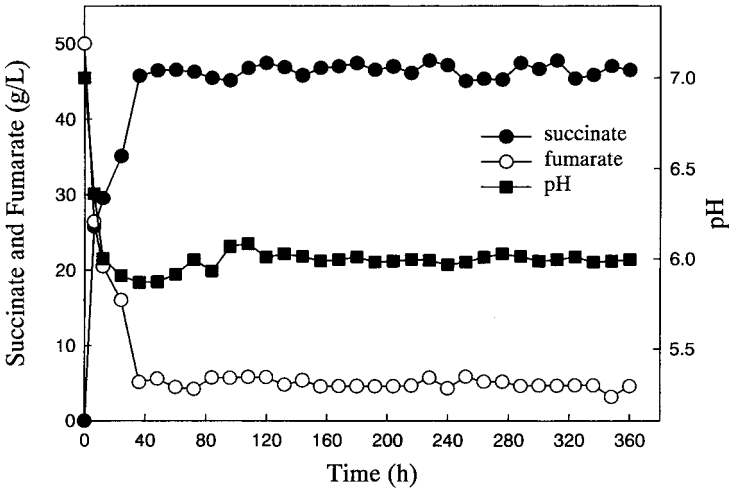

Fig. 9. Long-term stability of HFBR in transverse operation (dilution rate of 0.2 h^{-1}; 50 g/L of fumarate).

tration and productivity were 46.2 g/L and 9.2 g/(L·h), respectively. As fresh medium was continuously fed into the HFBR, excessive cell growth in the shell side was observed. In general, excessive cell growth in the HFBR causes operational problems, such as membrane clogging, during long-term operation *(17)*. Inloes et al. *(24)* reported that nitrogen-deficient

medium was used to control cell growth, and Mehaia and Cheryan *(21,22)* reported that they bled out some of the cells in the HFBR through the shell-side port to prevent excessive cell growth. Therefore, in our study it was necessary to bleed intermittently some of the cells through the shell-side port during any long-term experiment.

Conclusions

The continuous production of succinic acid using *E. faecalis* RKY1 cells immobilized in an HFBR was investigated in order to study the possibilities of enhancing productivity. Medium was fed into the shell side of the HFBR with a peristaltic pump, and the products were collected through the lumen-side outlet. For efficient HFBR performance, it was necessary to shift the medium's pH from 7.0 to 8.0 after 24 h of operation. Volumetric productivities during batch bioconversions with variations in the fumarate concentrations varied between 4.3 and 9.3 g/(L·h) at the end of bioconversion. During HFBR operation, as the dilution rate increased, productivity also increased but yield decreased. The maximum productivity of succinate obtained was 17.1 g/(L·h) using 80 g/L of fumarate in the feed at a dilution rate of 0.4 h^{-1}, which corresponded to a 1.7-fold increase over the maximum value seen in the batch bioconversions. In addition, the performance of the HFBR was maintained for 15 d (360 h) with efficient and consistent production of succinate.

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