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Cell Recycled Culture of Succinic Acid-Producing *Anaerobiospirillum succiniciproducens* Using an Internal Membrane Filtration System

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Cell recycled culture of succinic acid-producing *Anaerobiospirillum succiniciproducens* was anaerobically carried out using an internal membrane filter module in order to examine the physiological response of *A. succiniciproducens* to a high-cell-density environment. The optimal growth of *A. succiniciproducens* and its enhanced succinic acid productivity were observed under CO_2-rich conditions, established by adding $NaHCO_3$ and Na_2CO_3, in the cell recycled system. *A. succiniciproducens* grew up to 6.50 g-DCW/l, the highest cell concentration obtained so far, in cell recycled cultures. The cells did not change their morphology, which is known to be easily changed in unfavorable or stress environments. The maximum productivity of succinic acid was about 3.3 g/l/h, which is 3.3 times higher than those obtained in batch cultures. These results can serve as a guide for designing highly efficient cell recycled systems for succinic acid at a commercial level.

Keywords: *Anaerobiospirillum succiniciproducens*, succinic acid, membrane filtration, continuous culture

Succinic acid, a dicarboxylic acid, has been considered as one of biochemicals of commercial interest because it can be used for the manufacture of synthetic resins and biodegradable polymers and as an intermediate for the synthesis of various chemicals [22, 28]. Even though, to date, most succinic acids have been produced by chemical processes, biological productions mainly exploiting succinic acid-producing bacteria have attracted great interest as an alternative route for producing succinic acid [13]. In the view of metabolism and physiology, succinic acid has a dual role as an intermediate of the TCA cycle [12] and as fermentative end-products of several anaerobic and facultative microorganisms [4, 26]. The well-known succinic acid-producing anaerobic and facultative microorganisms are *Anaerobiospirillum succiniciproducens* [3], *Actinobacillus succinogenes* [5], and *Mannheimia succiniciproducens* [14]. Among them, the facultative anaerobe *M. succiniciproducens* MBEL 55E has been genome-scale analyzed [6] and its genome information is successfully used for *in silico* metabolic engineering [10].

Like *M. succiniciproducens* MBEL 55E, the obligate anaerobe *A. succiniciproducens* has been considered as one of the best succinic acid producers so far because of its higher metabolic capacity, such as high succinic acid yield on glucose or whey (lactose), and less by-product formation. Therefore, batch and fed-batch cultures of *A. succiniciproducens* have been intensively carried out to study environmental and physiological responses of the cells: for example, effects of CO_2-HCO_3^- levels [17], effect of medium compositions [18], and effects of carbon sources [16, 19]. Besides such bioreactor studies, phosphoenolpyruvate carboxykinase (PCK) of *A. succiniciproducens*, known as an important enzyme in the succinic acid formation pathway, was also studied biochemically and structurally [1, 7].

Although the previous knowledge on *A. succiniciproducens*, based solely of batch culture studies, is of value in better understanding succinic acid fermentation, it is still not sufficient and is limited to predict the behavior and fermentation performance of *A. succiniciproducens* under high-cell-density conditions. The high-cell-density cultivation is a preferred bioprocess for biocommodity chemical productions, such as of ethanol or lactic acid [2] from feedstock. In general, cell recycled systems using a membrane filtration have been regarded as one of the effective methods for growing cells to high density in a bioreactor [21]. Increasing the cell concentration in a membrane bioreactor correlates strongly with increasing the productivity of the product of interest, as demonstrated for ethanol production by *Saccharomyces cerevisiae* [20] and for the production of *Bacillus thuringiensis* spores [8]. Therefore, the cell recycled culture of *A. succiniciproducens* using a membrane bioreactor will provide more practical information on *A. succiniciproducens* in a high-cell-density condition, which is the more realistic and preferred bioprocess condition for succinic acid. An internal

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membrane reactor system was used for the high-cell-density culture of *A. succiniciproducens* and its growth behavior and fermentation performance were compared.

In this study, for the first time, we report the cell recycled culture of succinic acid-producing *A. succiniciproducens* and its physiological responses to a high-cell-density environment.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Anaerobiospirillum succiniciproducens (ATCC 29305) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were anaerobically grown in sealed anaerobic bottles containing 100 ml of minimal salts medium1 (AnS1) [16, 19] supplemented with 10 g/l of glucose, 2.5 g/l of yeast extract, and 2.5 g/l of polypeptone, and CO_2 as the gas phase. The AnS1 medium (per 1 l) contained 3 g of K_2HPO_4, 1 g of NaCl, 1 g of $(NH_4)_2SO_4$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgCl_2 \cdot 6H_2O$, and 1 g of Na_2CO_3. The media were heat-sterilized (15 min at 121°C) in anaerobic bottles with nitrogen headspace, and glucose, yeast extract, and polypeptone were aseptically filter-sterilized. The nitrogen headspace was replaced by CO_2 in an anaerobic chamber (Forma Scientific, U.S.A.) and then concentrated H_2SO_4 was added to the sterile medium in order to adjust the pH of the medium to 6.5±0.1. $Na_2S \cdot 9H_2O$, a reducing chemical, and resazurin, an oxygen indicator, were added into the media. After a reduced oxygen-free condition was established in the AnS1 medium, it was inoculated with 2.5 ml of a glycerol stock culture, purged with CO_2 gas, sealed with a rubber stopper and an aluminum cap, and anaerobically incubated at 39°C for 12–13 h.

Membrane Module and Experimental Setup

The membrane filter module used in this study consisted of 13 vertical cylindrical ceramic tubes with an inner diameter of 8 mm, an outer diameter of 11 mm, and a height of 80 mm (Fig. 1). The filter module was installed inside a 2.5-l jar fermentor (Korea Fermentor Co., Korea). The cylindrical ceramic tubes in the filter module have micropores to retain growing cells inside a reactor and let only cell-free culture medium penetrate. The continuous cell recycle bioreactor was operated by controlling the culture volume in the reactor constantly. Filtration fluxes (or filtrate flow rates) were regulated by adjusting pump speeds. Continuous cell recycled cultures of *A. succiniciproducens* were carried out at 39°C in the internal-membrane bioreactor containing 1.5 l of minimal salts medium2 (AnS2) [16, 19] supplemented with 20 g/l of glucose, 5 g/l of yeast extract, and 5 g/l of polypeptone. The AnS2 medium (per liter) contained 3 g of K_2HPO_4, 2 g of NaCl, 5 g of $(NH_4)_2SO_4$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.4 g of $MgCl_2 \cdot 6H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, and 5 g of Na_2CO_3. The pH was automatically controlled at 6.5 by feeding 10 N NaOH, NH_4OH, or Na_2CO_3 solution.

Feeding solutions of 30 l, basically the same as AnS2 medium containing 100 g/l glucose, were prepared as described above for AnS1 and AnS2 media and then aseptically purged with oxygen-free CO_2 gas for 24 h before uses in order to establish an oxygen-free condition inside the feeding solution. When the residual glucose concentration dropped below 10 g/l in a batch mode, the reduced feeding solution was added at calculated volumetric rates while cell-free culture broth was removed from the bioreactor through the filter module. Foaming was controlled by adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO, U.S.A.). The CO_2 gas sparging rate was controlled at 0.15 vvm unless otherwise stated, and the agitation speed was controlled from 200 to 800 rpm.

Analytical Methods

The concentrations of glucose, succinic acid, and acetic acid were measured by high-performance liquid chromatography (Hitachi L-3300 RI monitor, L-4200 UV-VIS detector, D2500 chromato-integrator; Tokyo, Japan) equipped with an ion-exchange column (Aminex HPX-87H, 300 mm×7.8 mm; Hercules, CA, U.S.A.) using 0.012 N H_2SO_4 as a mobile phase. Cell growth was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer (Ultrospec3000; Pharmacia Biotech, Sweden). Dry cell weight (DCW) was calculated from a standard curve relating the OD_{660} to dry cell weight (1 OD_{660}=0.33 g-DCW/l) [19]. All chemicals used were of reagent grade and were obtained from either Junsei Chemical Co. (Tokyo, Japan) or Sigma Chemical Co. Gases were scrubbed free of oxygen by passing through a gas purifier (P.J. Cobert Associates, Inc., St. Louis, MO, U.S.A.).

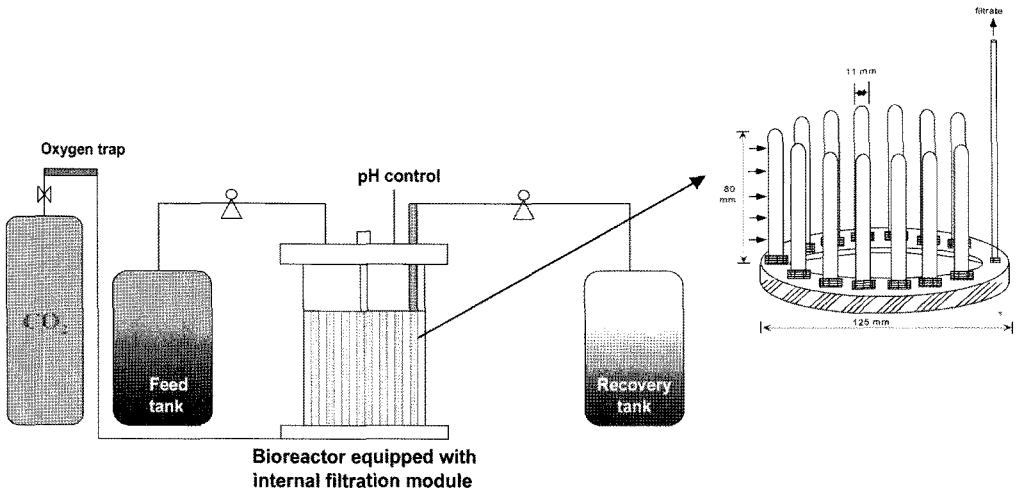

Fig. 1. Schematical description of an internal membrane module and experimental setup for cell recycled culture.

Results and Discussion

Effect of Agitation Speed on High-Cell-Density Culture of *A. succiniciproducens*

High-cell-density cultures of organic acid-producing microorganisms such as lactic acid bacteria have been intensively studied using fed-batch or membrane-based continuous modes [11]. Specifically, cell recycle systems using membrane modules have shown promising performances such as dramatically enhanced volumetric productivity [9, 27]. However, a membrane-fouling phenomenon is occasionally observed in membrane reactor systems and reduces the filtration performance of membranes [24]. To overcome or reduce the fouling problem, the agitation speed is often controlled and higher speeds reduce the accumulation of cells on the surface of a membrane, thus improving the membrane filtration flux. However, the shear stress generated by high agitation speeds may also affect the growth and physiology of shear-stress-sensitive microorganisms, in particular under unfavorable conditions such as high cell density.

We therefore studied the effects of agitation speeds (or shear stress) on the growth and succinic acid formation of *A. succiniciproducens* in a high-cell-density environment. After 10 h of a batch culture with an internal membrane module, a continuous mode was started at a constant dilution rate of 0.2 h^{-1}. The initial condition of the cell recycled culture was 400 rpm, 8.5 g/l of glucose, 12.5 g/l of succinic acid, 2.7 g/l of acetic acid, and 0.99 g-DCW/l. Agitation speeds of up to 400 rpm did not significantly influence cell growth, succinic/acetic acid formation, and glucose consumption (data not shown). Therefore, the agitation speed was stepwisely increased from 400 to 800 rpm at 5 h, and thereafter, the speed of 800 rpm was maintained to examine the physical response of *A. succiniciproducens* (Fig. 2).

The highest speed of agitation (800 rpm) and highest cell density environment (3.63 g-DCW/l) generated in the cell recycled culture did not cause morphological changes of *A. succiniciproducens* (data not shown), which is one of the indicators of the physiological/ metabolic state of the cells. All cells grown under the high shear stress condition showed rod and spiral shapes (active state), not a sphere shape (inactive state) [17]. Active rod and spiral cells grew gradually and reached 3.63 g-DCW/l in 15 h (Fig. 2), which is 3.5 times higher than those obtained in batch cultures (0.99 g-DCW/l). A ratio of succinic acid and acetic acid (S/A), another indicator for the physiological/metabolic state of the cells [19], was not significantly affected by these high stress environments. These results suggest that *A. succiniciproducens* resists well against unfavorable or harsh stressed environments, which is one of the suitable properties of industrially relevant microorganisms. Thus, the fixed agitation speed of 800 rpm was used for the following experiments, unless otherwise stated.

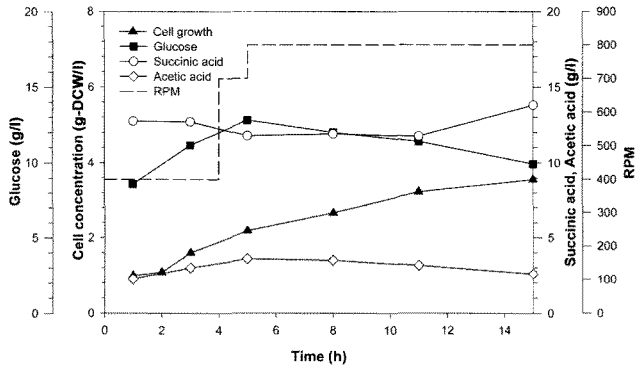

Fig. 2. Effect of agitation speed on cell growth and succinic acid production.

Effect of External CO_2 Supply

Our previous study [17] showed that external CO_2 supply was one of the critical factors influencing the balanced growth and succinic acid formation of *A. succiniciproducens* in batch cultures. The required CO_2 can be supplied into a bioreactor by either oxygen-free CO_2 gas or carbonate ion (CO_3^{2-}). Both work effectively as a CO_2 source for growth and succinic acid formation in batch cultures of *A. succiniciproducens*. Unlike batch cultures, however, a cell recycled culture maintains the high concentration of cell biomass as mentioned above, and thus, *A. succiniciproducens* may need more external CO_2 for its proper growth and succinic acid formation.

First, we controlled the level of CO_2 in the cell recycled culture by feeding of $NaHCO_3$ solution as an extra CO_2 source at a fixed volumetric flow rate of CO_2 gas (1.5 vvm). *A. succiniciproducens* was first grown up to 2.67 g-DCW/l without any extra feeding, thereafter, 1.2 M $NaHCO_3$ solution was fed at a rate of 0.55 ml/min (Fig. 3). *A. succiniciproducens* showed a tendency of growing faster after feeding of the $NaHCO_3$ solution and grew up to 4.79 g-DCW/L in 23 h.

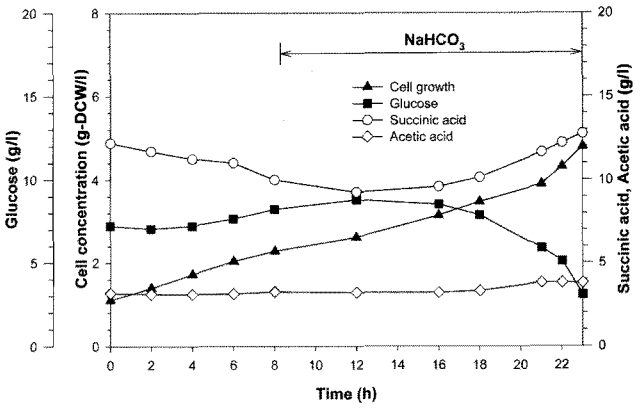

Fig. 3. Effect of constant feeding of $NaHCO_3$ on cell growth and succinic acid production.

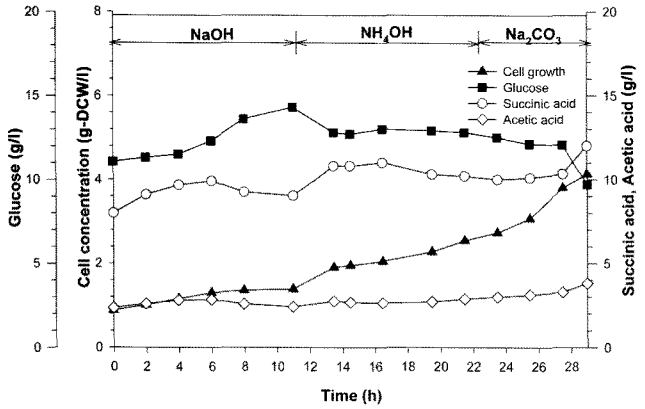

Fig. 4. Effect of pH control bases (NaOH, Na_2CO_3, and NH_4OH) on cell growth and succinic acid production.

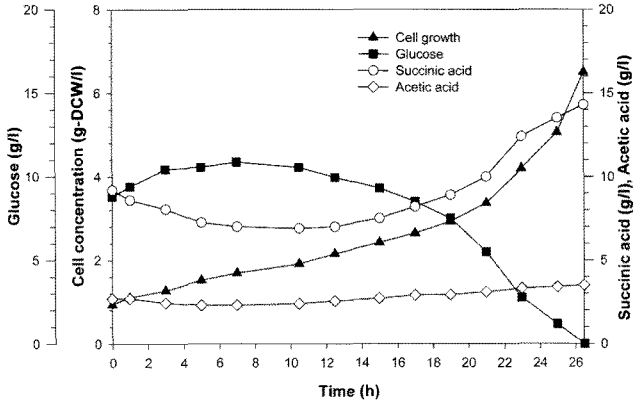

Fig. 5. Effect of $NaHCO_3$ and Na_2CO_3 on cell growth and succinic acid production.

This tendency suggests that feeding of $NaHCO_3$ can be an alternative for a CO_2 source in the cell recycled culture.

Next, we tried to choose a pH control base from two candidates, Na_2CO_3 and NH_4OH. In a practical point of view, some pH control bases have dual functions, pH controllers and nutritional sources. Microorganisms grown in a pH-controlled bioreactor take up some pH control bases as a nutrient source [11]. In a particular, NH_4OH and Na_2CO_3 can be consumed by microorganisms as a nitrogen source (NH_4^+) and as a CO_2 source (CO_3^{2-}), respectively. For this reason, we examined the effect of NH_4OH and Na_2CO_3 on cell growth and succinic acid formation under a high-cell-density condition. *A. succiniciproducens* was first grown using 10 N NaOH as a pH base, which was switched to 30% (w/v) NH_4OH, followed by 1.5 M Na_2CO_3 as shown in Fig. 4. The adding of Na_2CO_3 and NH_4OH as pH bases enhanced cell growth compared with NaOH feeding at a fixed flow rate of CO_2 gas (Fig. 4). Close performance comparison of Na_2CO_3 and NH_4OH as a pH controller showed that Na_2CO_3 was better when growth rate and succinic acid formation rate were considered.

Using $NaHCO_3$ and Na_2CO_3 in a Cell Recycled Culture

Finally, we carried out cell recycled culture with the fixed operating parameters described above: feeding of 1.2 M $NaHCO_3$ at a rate of 0.55 ml/min, a dilution rate of 0.23 h^{-1}, Na_2CO_3 as a pH control agent, and agitation speed of 800 rpm. *A. succiniciproducens* grew gradually until 19 h, and thereafter grew sharply and reached 6.50 g-DCW/l in 26 h, the highest cell concentration obtained so far (Fig. 5). All cells grown up to 6.50 g-DCW/l showed rod and spiral shapes (data not shown), indicating no morphological change of the cells, like observed at 3.63 g-DCW/l. The concentration of succinic acid (S) and acetic acid (A) were 14.3 g/l and 3.5 g/l, respectively, at the end of cell recycled culture and the ratio of S/A was 4.1:1, which are similar values obtained in batch cultures with wood hydrolysates [1], glucose [3], and whey (lactose) [2] as carbon sources. The productivity of succinic acid was 3.3 g/l/h and is higher than those obtained by batch (1.1 g/l/h) [23], fed-batch (1.35 g/l/h) [19], and continuous (2.1 g/l/h) cultures [25]. These results support that higher productivity of succinic acid and higher concentration of active cells can be achieved by cell recycled cultures, as demonstrated in this study.

In conclusion, we report for the first time that *A. succiniciproducens* could be grown to a relatively high density (6.50 g-DCW/l) by a cell recycled culture with an internal filter module and was able to resist harsh and stressful environmental conditions. Higher productivity of up to 3.3 g-succinic acid/l/h was obtained without causing any physiological/metabolic changes of the cells, as supported by no morphological changes and a constant S/A ratio. Even though the strategy described in this study is just the beginning of the effort towards development of an efficient bioprocess for succinic acid, this study can be used as a good guide for high-cell-density process for succinic acid production. To better understand the behavior of the cells, further study is necessary to achieve a higher cell density (over 6.50 g-DCW/l) as well as higher concentration and productivity of succinic acid.

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