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Continuous Production of Succinic Acid Using an External Membrane Cell Recycle System

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Succinic acid was produced by continuous fermentation of *Actinobacillus succinogenes* sp. 130Z in an external membrane cell recycle reactor to improve viable cell concentration and productivity. Using this system, cell concentration increased to 16.4 g/l at the dilution rate 0.2 h^{-1}, up to 3 times higher than that of batch culture, and the volumetric productivity of succinic acid increased up to 6.63 g/l/h at the dilution rate 0.5 h^{-1}, 5 times higher than that of batch fermentation. However, in the continuous culture using a high dilution rate, operational problems including severe membrane fouling and contamination by lactic acid producer were observed. Another succinic acid producer, *Mannheimia succiniciproducens* MBEL55E, was also utilized in this system, and the cell concentration and productivity of succinic acid at the dilution rate of 0.3 h^{-1} were found to be above 3 and 2.3 times higher, respectively, compared with those obtained at the dilution rate of 0.1 h^{-1}. These observations give a deep insight into the process design for a continuous succinic acid production by microorganisms.

Keywords: External membrane cell recycle system, succinic acid, *Actinobacillus succinogenes* sp. 130Z, *Mannheimia succiniciproducens* MBEL55E

Succinic acid is a common metabolite formed by plants, animals, and several microorganisms. It is a C_4 dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle (TCA) and also as one of the fermentative end products of energy metabolism [2]. Many obligate or facultative anaerobic microorganisms produce succinic acid as the major end-product. Although the industrial potential for the succinic acid fermentations was recognized as early as 1980 [18], recent interest has been focused on the biological production of succinic acid as a green technology that can replace the petrochemical process with a biobased, renewable, and environmentally reasonable process [19].

To date, succinic acid has been produced commercially by chemical processes. In order to replace the chemical processes with a biological process, many approaches have been studied to reduce the fermentation, separation, and purification costs. Fermentative production of succinic acid from renewable biomass by anaerobic bacteria has become of great interest [5, 7] owing to the negligible cost of substrates. Isolation of a new bacterium has been studied to find a novel succinic acid producer that can produce succinate with higher yield and productivity [3, 4, 10, 14]. Continuous cultures of several succinate producers have been studied to increase volumetric productivities [9, 11]. For *A. succinogenes* sp. 130Z, several studies mainly focused on its physiological study using flask and batch cultures [3, 15]. No studies on the production of succinic acid in a continuous fermentation mode with a high cell density have been reported yet.

The high cell density culture has been recognized as a preferred bioprocess for many biocommodity chemical productions, such as lactic acid or ethanol [1]. To develop high cell density culture, the cell recycled system has been extensively studied owing to its dramatically enhanced volumetric productivity [6, 17]. For succinic acid production, an internal membrane cell recycled system was recently reported using *Anaerobiospirillum succiniciproducens*; however, the cells were grown only to 6.5 g-DCW/l [12]. Therefore, it is required to increase the cell concentration by further cell recycling into the bioreactor.

In this study, for the first time, we carried out continuous succinic acid fermentation using an external membrane cell recycle bioreactor by *A. succinogenes* sp. 130Z and *M. succiniciproducens* MBEL55E. The experimental setup is shown in Fig. 1, where a hollow fiber membrane filtration

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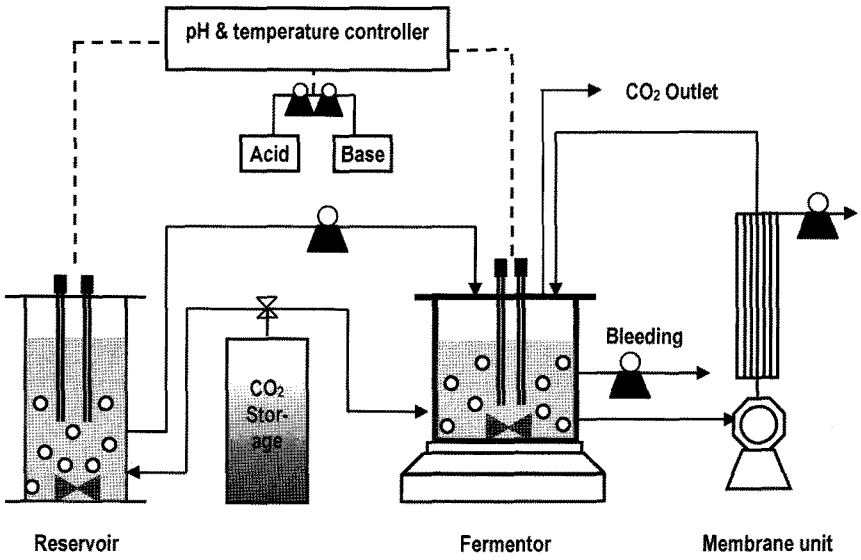

Fig. 1. Schematic diagram of the continuous culture system for succinic acid production with an external membrane cell recycle bioreactor by *Actinobacillus succinogenes* sp. 130Z.

unit (molecular weight cutoff (MWCO) 300K, 0.1 m^2 filtration area; Millipore Corporation, Bedford, MA, U.S.A.) was coupled with a 2.5-l jar fermenter. A diaphragm pump (Model P-07090-40; Cole-Parmer) was used for cell recycle at a flow rate ca. 600 ml/min.

In the experiment with *A. succinogenes*, feeding solution containing 60 g/l glucose and 20 g/l yeast extract was used to achieve a high cell density and productivity of succinic acid at various dilution rates and bleeding rates. As a control experiment, batch fermentation using the same concentration of glucose and yeast extract was performed, and cells were grown to 5.5 g/l after 12 h incubation (Supplementary Fig. 1). To increase viable cell density for higher productivity, continuous fermentation using an external membrane cell recycled system was studied at several dilution rates from 0.2 h^{-1} to 0.5 h^{-1}. As shown in Fig. 2, at the dilution rate of 0.2 h^{-1}, cell concentration was increased up to 16 g/l with similar product yield compared with that of batch culture. At the dilution rate of 0.4 h^{-1}, cell density was held to 13 g/l, and there was little difference compared with batch culture in product yield and ratio. Previous study proved that external CO_2 supply was one of the critical factors to determine growth and product yield in succinic acid fermentation [8]. In batch fermentation, the CO_2 can be supplied into the bioreactor by either oxygen-free CO_2 gas or carbonate ion in the medium. Because cell recycled culture maintained higher cell density compared with that of the batch mode, more CO_2 concentration was presumed to be required for its facile growth and succinic acid formation. Therefore, the CO_2 concentration dissolved in culture broth was monitored during the continuous fermentation by *A. succinogenes*. However as shown in Fig. 2, the dissolved CO_2 concentration did not decrease as culture time went on, so that CO_2 concentration was enough to support cell growth and product formation even in a higher cell density environment from the current cell recycled

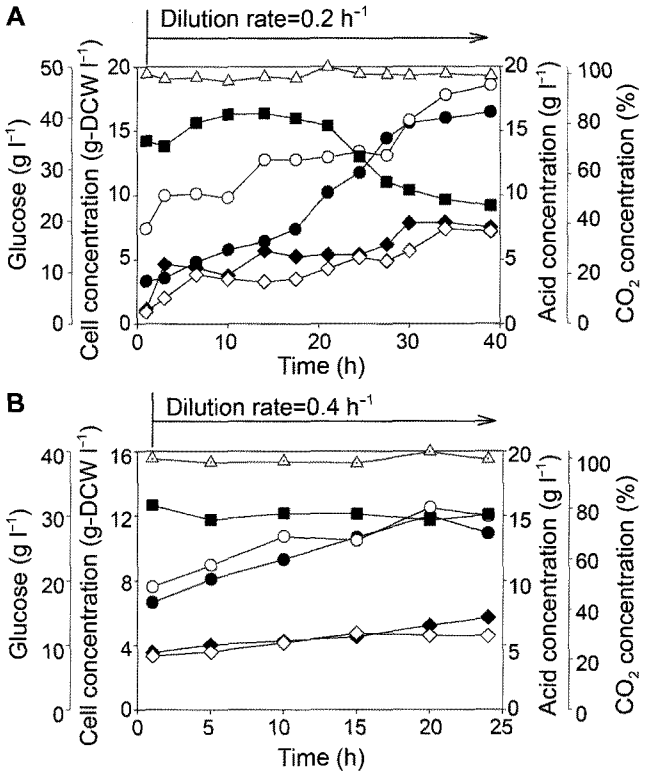

Fig. 2. Continuous culture by *Actinobacillus succinogenes* using an external membrane cell recycle system.
Symbols are the cell concentration (●), glucose (■), succinic acid (○), acetic acid (◆), formic acid (◇), and CO_2 concentration in the culture broth (△). **A.** At dilution rate 0.2 h^{-1}; **B.** At dilution rate 0.4 h^{-1}.

Table 1. Characteristics of the membrane cell recycle culture for succinic acid production by *Actinobacillus succinogenes* sp. 130Z at various dilution and bleeding rates.

Dilution rate (h^{-1})	Batch	0.2	0.3	0.4	0.5
Bleeding rate (h^{-1})		0.01	0.01	0.02	0.02
Cell density (g/l)	5.5	16.4	13.5	13.0	13.1
Succinic acid productivity (g/l/h)	1.33	3.71	4.50	6.25	6.63
Succinic acid yield (g/g)	0.573	0.562	0.549	0.588	0.504
Acetic acid yield (g/g)	0.223	0.225	0.217	0.212	0.252
Formic acid yield (g/g)	0.215	0.176	0.197	0.188	0.205

system.

The present experimental data dealing with the external membrane cell recycle system are arranged in Table 1. When the dilution rates were increased from 0.2 to 0.5 h^{-1}, the maximum succinic acid productivity increased from 3.71 to 6.63 g/l/h, and the succinic acid yield versus glucose amount was in a range of 50.4–58.8%. The highest productivity of succinic acid (6.63 g/l/h) obtained at a dilution rate of 0.5 h^{-1} was about 5 times higher than that of batch fermentation. End-product yield and distribution did not significantly change as dilution rates were varied, although at a dilution rate of 0.5 h^{-1}, the succinic acid yield decreased slightly and acetic and formic acids yields marginally increased. Cell densities were higher than 13 g/l, and the highest was 16.4 g/l obtained in the dilution rate of 0.2 h^{-1}, which was 3 times higher than that in batch culture (Supplementary Fig. 1). This shows that it can be one of the best processes to develop high cell density culture and productivity for succinic acid.

In the continuous cultures of *A. succinogenes* sp. 130Z with an external membrane cell recycle bioreactor, cell concentration increased several fold, but the membrane fouling was too severe to maintain long-term fermentation over 50 h. Additionally, the present experimental condition of high concentration of glucose (60 g/l) and low pH about 6.8 was suitable for growing lactic acid bacteria that are superior to compete against the original cells owing to their high tolerance for increased acidity from the organic acid produced [16], and thus unexpected lactic acid bacteria contamination was occasionally observed during the late fermentation stage. Different cell morphologies were observed between the initial batch stage and the late continuous fermentation stage, presumably due to the contamination of lactic acid producer (microscopic figure not shown). Fig. 3 shows an example of the contamination results at the dilution rate of 0.5 h^{-1}. In this case, succinic acid concentration reached its highest value of 15 g/l at around 25 h. As culture time went on, cell density increased sharply, but succinic acid concentration did not increase anymore and both acetic acid and formic acid concentrations decreased as well, and only lactic acid started to rapidly produce. Because the cultural condition of succinic acid was similar to that of lactic acid producers like *Lactobacillus* and *Leuconostoc*, which are common contaminant bacteria in many biological processes including ethanol fermentation [13], it is required to use a steam-sterilizable membrane such as ceramic membrane and to make a strictly closed culture condition to prevent contamination from the outside.

To prove the applicability of this system into other succinic acid producers, continuous fermentations by *M. succiniciproducens* MBEL55E were performed under various dilution rates with total cell recycling. For the stable continuous operation, a modified growth medium containing 20 g/l of glucose was used. The dilution rate was consecutively increased from 0.1 to 0.3 h^{-1} when the steady-state was achieved, confirming no change in the cell concentration.

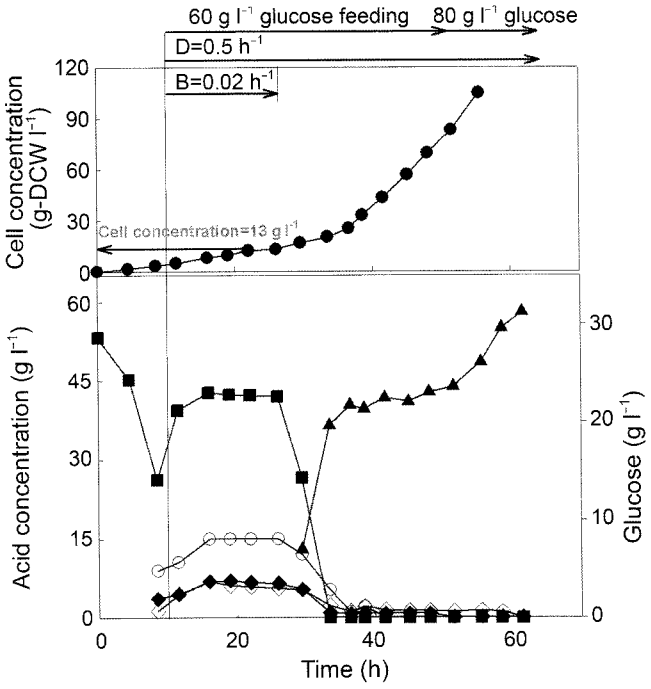

Fig. 3. Contamination by lactic-acid-producing microorganism during continuous culture by *Actinobacillus succinogenes* using an external membrane cell recycle system.
Cell concentration (●), glucose (■), succinic acid (○), acetic acid (◆), formic acid (◇), lactic acid (▲). D is dilution rate (h^{-1}) and B is bleeding rate (h^{-1}).

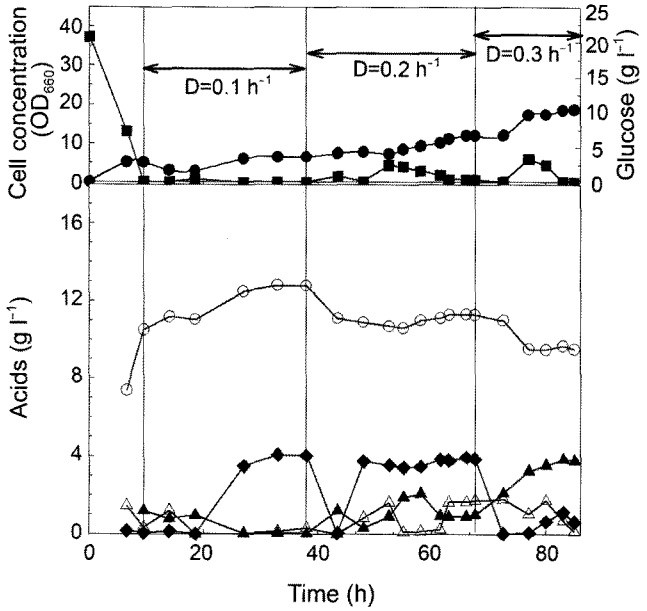

Fig. 4. Continuous culture by *Mannheimia succiniciproducens* MBEL55E using an external membrane cell recycle system.
Symbols are OD_{660} (●), glucose (■), succinic acid (○), acetic acid (◆), formic acid (◇), lactic acid (▲), and D is dilution rate (h^{-1}).

The representative time profiles of biomass, glucose, and acids in the fermentor are shown in Fig. 4. The glucose concentration remained less than 5.0 g/l throughout the fermentation. The data show that relatively constant levels of cell concentration, and succinic and acetic acids were well established at each dilution rate. This enables a high level of certainty on the reactor stability. Although the cell concentration often oscillated before setting to a constant level, it increased proportionally in response to a higher dilution rate. This is thought to be because the increased rate of cell-free effluent removal and cell recycling into the reactor through the membrane filtration unit accelerated the cell accumulation in the reactor. However, the gradual increase in lactic acid was observed at the cost of succinic and acetic acids as the cell concentration increased. This might be an indication that the increase of glucose feeding rate has an effect on the metabolic pathways of *M. succiniciproducens*, and can be elucidated by comparing the yields of succinic and lactic acids at each dilution rate. As shown in Fig. 4, the succinic acid concentration declined from 12.8 to 9.5 g/l as the dilution rate increased from 0.1 to 0.3 h^{-1}. Moreover, the dramatic decrease from 64% (0.1 h^{-1}) to 48% (0.3 h^{-1}) in the yield of succinic acid occurred. On the other hand, the steady-state lactic acid yield was observed to rise sharply up to 19% at the dilution rate of 0.3 h^{-1}. Nearly no lactic acid was observed during the period of steady-state at the dilution rate of 0.1 h^{-1}. This strongly implies that the higher glucose feeding rate triggered increase of the carbon flux responsible for lactate formation.

M. succiniciproducens has been known to be capable of forming succinic acid from glucose *via* two main intermediates, oxaloacetate and pyruvate. Both of these are converted from phosphoenolpyruvate (PEP) by using PEP carboxykinase and pyruvate kinase and then further successively converted to malate, formate, and succinate [4, 10]. In the course of the conversion from PEP and pyruvate to oxaloacetate and malate, the fixation of CO_2 is required to form succinate, respectively. However, the pyruvate produced in *M. succiniciproducens* is also converted to lactate, formate, ethanol, and acetate as end- products. This pathway does not demand CO_2 assimilation. Considering the constant CO_2 supplementation and agitation rates, the CO_2-limited condition as a result of the increased glucose feeding rate at a high dilution rate might result in the decrease of the succinic acid production whilst this could contribute to the enhanced production of lactic and acetic acids.

In conclusion, this study was the first approach to apply an external membrane cell recycle system for continuous fermentative succinic acid production in which several-fold higher cell concentration and volumetric productivity of succinic acid were observed compared with those of batch culture. Continuous culture using an external membrane cell recycle bioreactor may be proposed as a candidate for the practical production of succinic acid because of its capability of achieving high cell density and high succinic acid productivity. A further study is required to solve operating problems including metabolic flux change and membrane fouling.

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