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Continuous high cell density culture of *Anaerobiospirillum succiniciproducens* with membrane filtration for the production of succinic acid

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Abstract

An internal membrane bioreactor system was employed for continuous succinic acid production from glucose in order to prove its performance and practicality. Succinic acid-producing *Anaerobiospirillum succiniciproducens* required more CO_2 for the proper growth and succinic acid production in cell recycled continuous culture than in batch culture. The maximum productivity obtained in cell recycled continuous culture was about 3.3 g/L-h which was ca. 3.3 times higher than that obtained in batch culture.

Introduction

Succinic acid, a dicarboxylic acid, is recently being considered as an important chemical because it can be used for the manufacture of synthetic resins and biodegradable polymers and as an intermediate for the synthesis of various chemicals (1). To date, succinic acid has mostly been produced by chemical processes. However, fermentative production of succinic acid from renewable biomass by anaerobic bacteria has attracted great interest as an alternative to chemical production of succinic acid from petroleum feedstocks (2). An anaerobic bacterium *Anaerobiospirillum succiniciproducens* has been considered as one of the best succinic acid producers because it can produce a significant amount of succinic acid from glucose (3, 4). Therefore, several studies have been carried out on the succinic acid fermentation by *A. succiniciproducens*: effects of hydrogen supplementation and glucose concentration (3); optimization of medium composition for succinic acid production (4); fermentation of lactose and whey (5); batch fermentation of glycerol (6). Although batch and fed-batch cultures of *A. succiniciproducens* have been carried out in several research groups, cell recycled continuous culture has received little attention. It is well known that cell recycled system using membrane filtration has been found to be effective in maintaining high cell concentrations in bioreactors [7]. In addition, productivity of products generally increases with the cell concentration in bioreactor. Therefore, the aim of the present study was to establish the operational conditions for the higher cell density of *A. succiniciproducens* and for the continuous production of succinic acid by cell recycled continuous culture using membrane filter.

Materials and methods

Organism and growth conditions. *Anaerobiospirillum succiniciproducens* (ATCC 29305) was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in sealed anaerobic bottles containing 100 mL minimal salts medium1 (AnS1) 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 contained per liter: 3 g K_2HPO_4, 1 g NaCl, 1 g $(NH_4)_2SO_4$, 0.2 g

$CaCl_2 \cdot 2H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, and 1 g Na_2CO_3. The medium was heat sterilized (15 min at 121°C) in anaerobic bottle with nitrogen headspace. Concentrated H_2SO_4 was added to the sterile medium in order to adjust the pH to 6.5. The nitrogen headspace was replaced by CO_2, and $Na_2S \cdot 9H_2O$ was added to a final concentration of 1 mg/L to establish strict anaerobic condition. After 15 min, the reduced medium was inoculated with 2.5 mL glycerol stock culture and incubated at 39 °C for 12-13 h.

Filter module and experimental set-up. The filter module used in this study consisted of 13 vertical cylindrical ceramic tubes with an inner diameter, an outer diameter, and height of 8, 11, and 80 mm, respectively. More details of the filter module are given elsewhere [7]. The filter module was installed inside a 2.5-L jar fermenter (Korea Fermentor Co., Korea). Continuous cell recycle cultures were carried out at 39 °C in the jar fermenter containing 1.5L minimal salts medium2 (AnS2) supplemented with 20 g/L of glucose, 5 g/L of yeast extract and 5 g/L polypeptone. The AnS2 medium contained per liter: 3 g K_2HPO_4, 2 g NaCl, 5 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, and 5 g Na_2CO_3. The pH was controlled at 6.5 using 1.5 M Na_2CO_3. Feeding solutions were purged with oxygen-free CO_2 gas for 24 h in order to establish anaerobic condition before uses. When the residual glucose concentration became 2 g/L in batch operations, the reduced feeding solution was added at various dilution rates while cell-free liquid was removed from the bioreactor through the filter module. Foaming was controlled by adding Antifoam 289 (Sigma Chemical Co. St. Louis, MO). CO_2 gas sparging rate and agitation speed were controlled at 0.25 vvm and 200 rpm, respectively. All chemicals used were of reagent grade and were obtained from either Junsei Chemical Co. (Tokyo, Japan) or Sigma Chemical Co. Gas was scrubbed free of oxygen by passing through a gas purifier (P.J. Cobert Associates, Inc., St. Louis, MO).

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 x 7.8 mm, Hercules, CA) 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).

Results and discussion

Effect of agitation speed. In general, membrane-fouling phenomenon is one of critical problems in the operation of membrane bioreactor. To overcome or reduce the membrane fouling during cell recycled continuous culture using membrane bioreactor, agitation speed is often increased in order to generate shear force, and then it reduces accumulation of cells on the surface of membrane. Although the shear force generated by the increasing agitation speed can reduce the membrane fouling, it may negatively affect some shear force-sensitive microorganisms. Therefore, we firstly examined the effect of agitation speed on the growth and succinic acid production of *Anaerobiospirillum succiniciproducens*. When agitation speed was stepwisely increased from 400 to 800 rpm during cell recycled continuous culture, there was no deformation of cell morphology at the agitation speed of 800 rpm (data not shown). Cell concentration gradually increased and reached an OD_{660} of 11. Although cell concentration

increased, glucose began to accumulate slightly over 700 rpm, and accordingly succinic acid concentration start to decreased (Fig. 1).

Effect of pH control bases. Our preliminary study showed that external CO_2 supply was one of the critical factors in the optimal growth and succinic acid production of *A. succiniciproducens* (3). The required CO_2 can be supplied by either oxygen-free CO_2 gas or carbonate ion such as CO_3^{2-}. Both the CO_2 sources are effective in the growth and succinic acid production in batch cultures of *A. succiniciproducens*. However, cell recycled continuous culture maintains higher cell density than batch culture does, and thus more CO_2 can be required in cell recycled continuous cultures rather than batch cultures. Therefore, the degree of requirement of CO_2 was secondly examined by adding of Na_2CO_3 (also CO_2 source), or NH_4OH as pH control bases in the condition of constant flow rate of CO_2 gas (Fig. 2). Adding of Na_2CO_3 was more effective in cell growth and succinic acid production than NH_4OH. Also, glucose consumption was enhanced when Na_2CO_3 was used as a pH control base. This means that *A. succiniciproducens* required CO_2 more in higher cell density condition.

Constant feeding of $NaHCO_3$. For the sufficient supplement of CO_2, we carried out constant feeding of $NaHCO_3$ during cell recycled continuous culture. When 1M $NaHCO_3$ was fed at 0.55 mL/min, cell concentration gradually increased and reached an OD_{660} of 20. Accordingly, glucose was completely consumed in 26h of continuous culture. The concentration and productivity of succinic acid were 14.2 g/L and 3.3 g/L-h, respectively, at the end of continuous culture (Fig. 3). The succinic acid productivity of 3.3 g/L-h was ca. 3.3 times higher than that obtained in batch cultivation.

In this study, we showed for the first time that *Anaerobiospirillum succiniciproducens* could be grown to a relatively high density by cell recycle culture, resulting in the production of succinic acid with high productivity. The strategy described in this study is just the beginning of the effort towards the efficient production of succinic acid. Optimization of operation condition will allow us to achieve higher cell density as well as higher succinic acid concentration and productivity.

Acknowledgment

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Figure 1. Effect of rpm on cell growth and succinic acid production

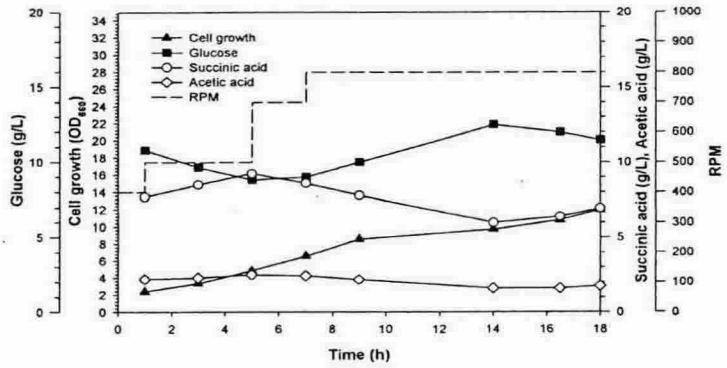

Figure 2. Effect of pH control bases on cell growth and succinic acid production

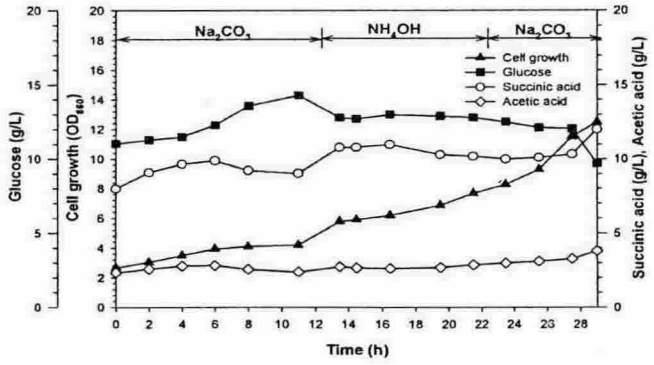

Figure 3. Effect of constant feeding of $NaHCO_3$ on cell growth and succinic acid production