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Optimization and Scale-Up of Succinic Acid Production by *Mannheimia succiniciproducens* LPK7

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The effects of culture conditions on succinic acid production and its possible scale-up have been studied. *Mannheimia succiniciproducens* LPK7, engineered for enhanced production of succinic acid and reduced by-product secretion, was used for the experiments. *Mannheimia succiniciproducens* LPK7 is a knock-out strain of wild type deficient in the *ldhA*, *pflB*, and *pta-ackA* genes, and is derived from *Mannheimia succiniciproducens* MBEL55E. Process optimization of factors including optimal temperature, pH, carbon source, and nitrogen source was performed to enhance the production of succinic acid in flasks. To observe scale-up effects, batch fermentation was carried out at various working volumes. At a working volume of 7.0 l, the final succinic acid concentration and yield were 15.4 g/l and 0.86 g/g. This result shows similar amount of succinic acid obtained in lab-scale fermentation, and it is possible to scale up to larger fermentors without major problems.

Keywords: *Mannheimia succiniciproducens* LPK7, scale-up, succinic acid, anaerobic fermentation

Succinic acid is a member of the C_4-dicarboxylic acid family and is used in many industries including the manufacturing of foods, pharmaceuticals, and cosmetics. Additionally, it is an intermediate for the production of 1,4-butanediol, tetrahydrofuran, and gammabutyrolactone [13, 15, 17]. Thus far, succinic acid has been produced by chemical processes based on petroleum materials. Recently, concerns have been raised about the fermentative production of succinic acid. Fermentative production of succinic acid has several advantages. First, it is an anaerobic process conducted by microbes, including rumen anaerobic bacteria such as

Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, and Mannheimia succiniciproducens [17]. CO_2 that contributes to the greenhouse effect is consumed in this process. This process can be integrated into the ethanol fermentation processes, which produce large amounts of CO_2. Additionally, fermentative production of succinic acid from renewable biomass is a green technology and is environment-friendly compared with chemical processes.

Many researchers are therefore studying processes for the production of succinic acid. Nutritional and physiological factors such as media composition, pH, and temperature of the cell culture are essential for operating fermentative processes. For optimal production of succinic acid, physiological and nutritional factors have to be studied; however, scale-up for industrial application production has rarely been studied. In the present investigation, the newly developed strain *Mannheimia succiniciproducens* LPK7 was used [11]. Process optimization was performed to maximize the production of succinic acid. In order to observe scale-up effects, different sizes of fermentation culture have been tested and secretion profiles of organic acid have been analyzed.

Mannheimia succiniciproducens LPK7 is deficient in *ldhA*, *pflB*, and *pta-ackA* genes and modified from *Mannheimia succiniciproducens* MBEL55E in order to produce more succinic acid with less by-product formation [11].

The effects of different environmental and nutritional parameters on the production of succinic acid were examined in flasks. Flask cultures were carried out in 500-ml flasks containing 270 ml of modified MH3 medium with 5 g/l glucose. Modified MH3 medium contained (per liter) NaCl 1.2 g, $(NH_4)_2SO_4$ 1.2 g, K_2HPO_4 10.44 g, yeast extract 3 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, and $MgCl_2 \cdot 6H_2O$ 0.2 g. The medium was sterilized at 121°C for 15 min. After sterilization, CO_2 gas was sparged into the flask. The medium was inoculated with 10% seed culture and incubated at 39°C. The effects of different temperature and pH on succinic

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Table 1. Effects of temperature and pH on succinic acid production in flasks.

	Initial pH	pH 6.0	pH 6.5	pH 7.0	pH 7.5
36°C	Cell density (g DCW/l)	0.156	0.377	0.529	0.619
	Succinic acid (g/l)	0.82	2.17	2.91	3.43
	Final pH	5.09	4.99	5.18	5.30
39°C	Cell density (g DCW/l)	0.110	0.433	0.542	0.579
	Succinic acid (g/l)	0.63	2.42	3.12	3.64
	Final pH	5.40	5.15	5.29	5.36
42°C	Cell density (g DCW/l)	0.037	0.146	0.275	0.029
	Succinic acid (g/l)	0.33	1.27	2.26	0.28
	Final pH	5.56	5.65	5.77	6.49
45°C	Cell density (g DCW/l)	0	0	0	0
	Succinic acid (g/l)	0	0	0	0
	Final pH	5.65	6.12	6.48	6.86

acid production were tested at various temperatures and pH values (36°C, 39°C, 42°C, and 45°C *vs.* pH 6.0, 6.5, 7.0, and 7.5). The initial pH of the medium was adjusted with 5 M NaOH. After these experiments, the effects of pH on succinic acid production were tested in a fermentor at 39°C because pH was not continuously controlled in the flasks. The fermentation medium used was the same as the flask culture medium. Experiments to determine the optimal carbon source and nitrogen source were performed at 39°C. Flask cultures to determine the optimal carbon source were performed using modified MH3 and 5 g/l of carbon sources. The carbon sources used were arabinose, glucose, lactose, maltose, sorbitol, and sucrose. For nitrogen source tests, instead of yeast extract, the same amount of beef extract, malt extract, peptone, tryptone, or urea was added in modified MH3 medium. Flasks were filled with this medium and 5 g/l glucose was added.

In order to obtain scale-up factors, batch fermentation experiments were performed at various working volumes using the optimal culture conditions obtained from the lab-scale tests. The working volumes were 0.5 l, 2.5 l, and 7.0 l in 1 l, 5 l, and 10 l vessels, respectively (manufactured by Biotron). The fermentation medium was MMH3 medium with 18 g/l of glucose. MMH3 medium contains (per liter) yeast extract 5 g, NaCl 1 g, K_2HPO_4 8.708 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, and $MgCl_2 \cdot 6H_2O$ 0.2 g. The medium was sterilized in the vessel at 121°C for 15 min. After sterilization, CO_2 gas was sparged into the vessel, and temperature and pH were adjusted to 39°C and pH 6.5 with 5 M NaOH. Agitation speed and pH were maintained at 150 rpm and pH 6.5. The effects of different agitation speeds on the production of succinic acid were tested at 75, 150, and 300 rpm, respectively.

The concentrations of products and carbon sources were analyzed using high-performance liquid chromatography (UV730D detector, RI750F monitor; Younglin, Korea) equipped with an ion-exchange column (Aminex HPX-87H, 300 mm×7.8 mm; Bio-Rad) using 0.005 N H_2SO_4 as a mobile phase at 55°C and a flow rate of 0.6 ml/min. Cell growth was monitored by measuring the absorbance at 610 nm using a UV-Vis spectrophotometer (Shimadzu). Cell concentration was defined as gram dry cell weight per liter (g DCW/l). An OD_{610} of 1.0 was equivalent to 0.6216 g DCW/l.

One of the strains used for high production of succinic acid is *Mannheimia succiniciproducens* [17]. *Mannheimia succiniciproducens* has been mutated to produce more succinic acid without by-products [11]. In order to obtain maximum succinic acid production, optimization of conditions was performed. The results are shown in Table 1, Table 2, Table 3, and Table 4.

Temperature and pH were important for cell growth and also for the production of succinic acid. As shown in Table 1 and Table 2, various temperatures and pH values were tested to determine the effects on cellular growth and succinic acid production. When choosing temperature and pH, other papers regarding *Mannheimia succiniciproducens* were considered [3, 11, 14]. At all pH ranges, the highest succinic acid concentration was obtained at 39°C. Although the dry cell weight and succinic acid concentration at 36°C were lower than that at 39°C, the differences between 36°C and 39°C were small. At 45°C, the organism failed to grow and no significant succinic acid production was observed. For pH tests, although the initial pH was adjusted, the pH was changed at the end-point. Because pH was not continuously controlled in flasks, the effects of pH on

Table 2. Effects of pH on succinic acid production at 39°C in a fermentor.

pH	6.0	6.5	7.0	7.5
Cell density (g/DCW l)	0.467	0.437	0.412	0.379
Succinic acid (g/l)	3.31	3.34	1.94	0.84

Table 3. Effects of carbon source on succinic acid production.

Carbon source	Arabinose	Glucose	Lactose	Maltose	Sorbitol	Sucrose
Cell density (g DCW/l)	0.116	0.575	0.554	0.467	0.079	1.021
Succinic acid (g/l)	0.76	2.85	2.44	2.81	0.38	2.59

succinic acid production were tested in a controlled-pH fermentor at 39°C. Succinic acid production was maximized at pH 6.5. However, the result at pH 6.0 was very similar to that at pH 6.5. *Mannheimia succiniciproducens* was originally isolated from the bovine rumen of Korean cows [5]. In general, healthy cows should have a rumen temperature of 38–42°C and a pH of 6 or 7. These temperature and pH values are similar to the optimal conditions obtained in this experiment for production of succinic acid.

Experiments to determine the optimal carbon and nitrogen sources for succinic acid production were performed at 39°C and pH 6.5. The cell yield was highest when sucrose was used as the carbon source (see Table 3.). However, the succinic acid concentration was lower than when glucose was used as carbon source. Thus, the optimal carbon source for production of succinic acid appears to be glucose. When yeast extract was used as a nitrogen source, both the dry cell weight and succinic acid concentration were higher than in any other case (see Table 4.). Yeast extract was found to be the best nitrogen source among tested nitrogen sources. Glucose is one of the most abundant carbohydrates used by most microbes, thus it is reasonable that *Mannheimia succiniciproducens* utilizes glucose more easily. Additionally, yeast extract contains large amounts of nitrogen, amino acids, and inorganic salts that are essential for cell growth, so yeast extract is an excellent substrate for cell growth and production of succinic acid.

This experiment was performed to observe scale-up effects for application in industrial processes. Optimal temperature and pH conditions as well as carbon and nitrogen sources determined by flask experiments were used for scale-up tests. Although 5 g/l glucose was used in flask culture, 18 g/l glucose was used during scale-up tests, as the amount of 5 g/l glucose converted to succinic acid by *Mannheimia succiniciproducens* was too low. When 36 g/l glucose was used to produce succinic acid, the glucose was not consumed by *Mannheimia succiniciproducens* completely and accumulated in the medium (data not shown). MMH3 medium is simpler than MH3 medium; thus experiments for scale-up used MMH3 medium and 18 g/l glucose. In

order to determine the scale-up capacity, fermentation was carried out at various working volumes (0.5, 2.5, and 7.0 l); all the fermentors were cylindrical shaped and height-to-diameter ratios were 1.48, 1.32, and 1.89, respectively. The volume-to-surface ratios were equivalent to 5.67, 7.50, and 12.00. Agitation speed was maintained at 150 rpm and ratios of fermentor diameter-to-impeller diameter were 1.69, 1.85, and 1.73. The fermentation results are shown in Fig. 1. When the working volume was increased from 0.5 l to 7.0 l, both cell concentration and succinic acid production were reduced by about 15% and 17%, respectively. When scale-up experiments were performed, parameters such as temperature and pH were difficult to maintain at the same value, especially at all locations within the fermentor. As the absolute quantity of carbon and nitrogen sources increased, the production of succinate, pyruvate, and endotoxins was increased. The surrounding conditions were thus harmful to cell growth. Therefore, when working volumes increased, cell concentration and succinic acid concentration were decreased. However, the cellular growth and organic acid secretion working volumes of 2.5 l and 7.0 l were similar. Under these conditions, the maximum succinic acid concentration was about 15 g/l at stationary phase. Owing to anaerobic fermentation during the production of succinic acid, it was not necessary to consider the oxygen transfer rate as an important factor for scale-up. Thus, the scale-up from 2.5 l to 7.0 l did not affect the production of succinic acid. However, when the working volume was greater than 7.0 l, fermentation for production of succinic acid showed slightly reduced cell concentration and succinic acid concentration.

Agitation speed was an important factor for culture optimization. In order to determine an optimal agitation speed at a working volume of 7.0 l, the agitation speed was changed from 75 to 300 rpm. The medium composition and culture conditions were the same as in previous experiments. As shown in Fig. 2, the greatest cell growth was achieved at 150 rpm. Although at 75 rpm, cell density and succinic acid concentration were lower than at 150 rpm, pyruvic acid production at 75 rpm was about 2-fold higher than at

Table 4. Effects of nitrogen source on succinic acid production.

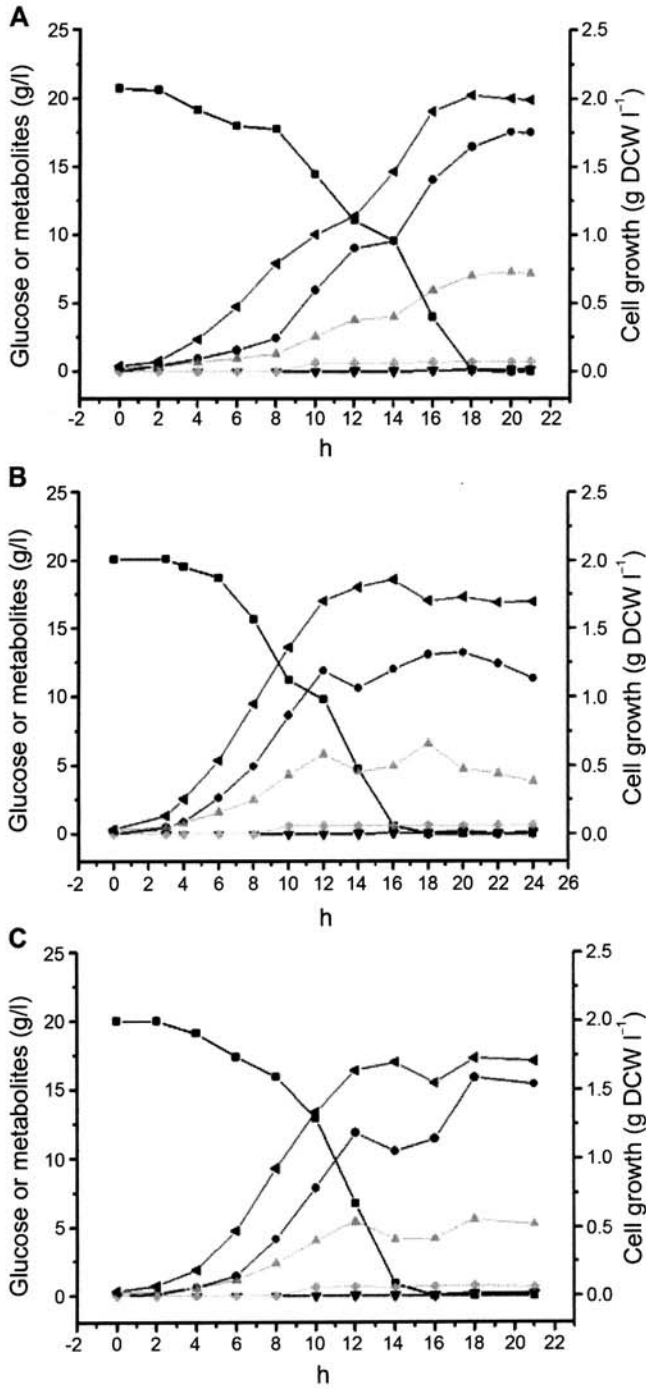| Nitrogen source	Beef extract	Malt extract	Peptone	Tryptone	Urea	Yeast extract
Cell density (g DCW/l)	0.268	0.059	0.150	0.209	0.050	0.564
Succinic acid (g/l)	1.74	0.02	0.93	0.73	0.02	3.57

Fig. 1. Batch fermentation profiles of *M. succiniciproducens* for the scale-up experiment, determined using various working volumes: (**A**) 0.5 l, (**B**) 2.5 l, (**C**) 7.0 l.
●, Succinic acid; ■, glucose; ▲, pyruvic acid; ▼, formic acid; ◆, acetic acid; ◀, cell growth.

Fig. 2. Effect of agitation speed on succinic acid production at a working volume of 7.0 l; (**A**) 75 rpm, (**B**) 150 rpm, (**C**) 300 rpm.
●, Succinic acid; ■, glucose; ▲, pyruvic acid; ▼, formic acid; ◆, acetic acid; ◀, cell growth.

150 rpm. At 300 rpm, succinic acid concentration was lower than in any other condition, and pyruvic acid production was higher. *Mannheimia succiniciproducens* cuts glucose in half, and then adds one carbon of CO_2 to a 3-carbon intermediate (pyruvic acid). Finally, succinic acid, a 4-carbon compound, is produced. When the agitation
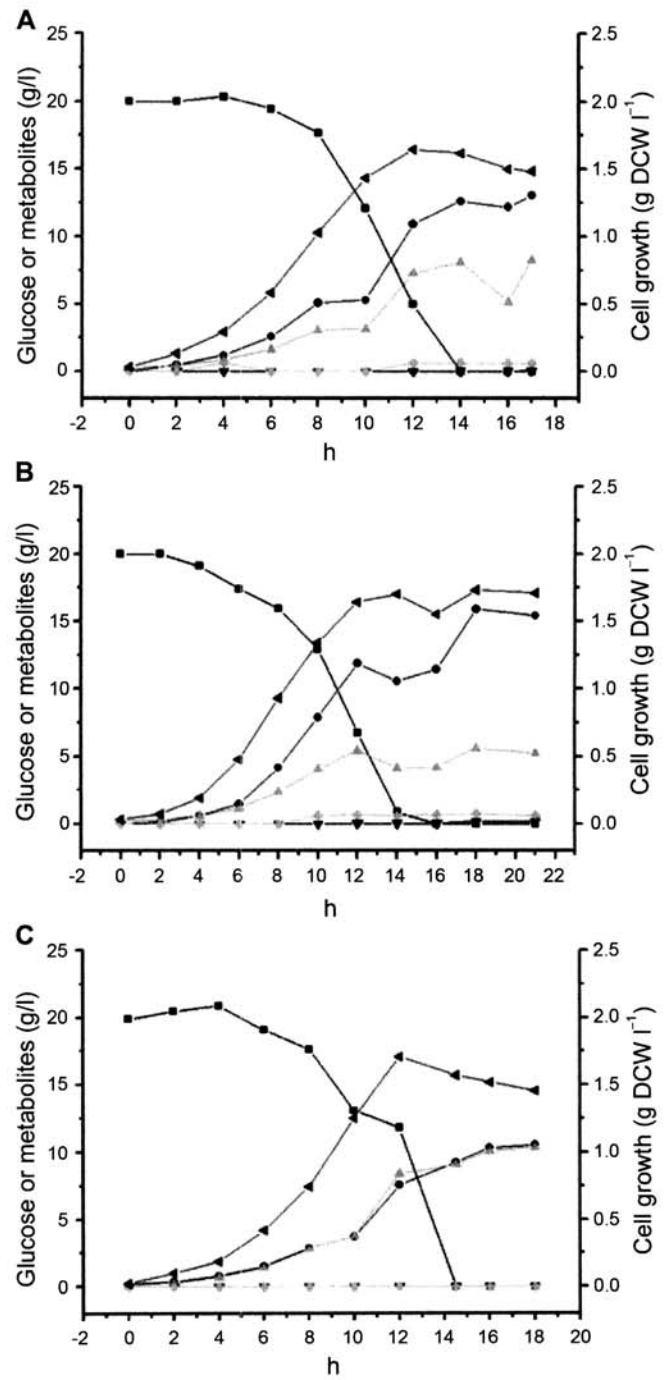
speed was slow, CO_2 was not sufficiently dissolved in the medium. The results were therefore similar to that at 75 rpm. On the other hand, when the agitation speed was fast, cells were broken because of shear stress. As a result, pyruvic acid from inside the cells was released into the broth. Considering the purification cost, the optimal agitation

speed of fermentation for production of succinic acid at a working volume of 7.0 l seems to be 150 rpm in this study.

In conclusion, the optimal conditions of temperature, pH, carbon source, and nitrogen source were 39°C, pH 6.5, glucose, and yeast extract, respectively. Fermentation for the production of succinic acid was performed under anaerobic conditions. The oxygen transfer rate, which is usually the main problem in scale-up processes, therefore does not need to be considered. Although cell concentration and succinic acid concentration were decreased slightly, the scale-up experiment from flask to fermentor showed the possibility of extended application to commercial production processes. When scale-up is performed, determination of the optimal agitation speed is very important. The optimal agitation speed for large-scale fermentation was 150 rpm in this study.

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