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The Effect of Protectants and pH Changes on the Cellular Growth and Succinic Acid Yield of *Mannheimia succiniciproducens* LPK7

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The harmful effects of succinic acid and oxidative stress on cell growth were determined during batch fermentation with *Mannheimia succiniciproducens* LPK7, a powerful succinic acid-producing strain, and conditions were optimized to minimize these effects. In terms of toxicity, the cell concentration decreased as the concentration of succinic acid increased. By changing the pH from 6.5 to 7 during fermentation, the cell concentration increased by about 10%, and the level of succinic acid production was 6% higher than that of the control. In addition, by introducing protectants, the cell concentration increased by about 10%, and the level of succinic acid produced was increased by 3%.

Keywords: *Mannheimia succiniciproducens* LPK7, succinic acid, protectants (biotin, glutathione, trehalose), cellular growth, oxidative stress

Succinic acid is a well-known chemical composed of four carbons and two carboxyl groups. This compound is utilized as a food and drug additive and is an intermediate of several industrial products. The production of succinic acid is growing significantly as the market for biodegradable polymers increases.

Succinic acid has been produced by chemical processes based on petroleum feed stocks. However, the rise of oil prices and the increase in environmental concerns have forced the succinic acid industry to change the method of production from a chemical process to biological fermentation. The latter process offers some advantages over the former. First, the biological production of succinic acid is a carbon dioxide-consuming process [16]. Furthermore, fermentation is a less polluting process than petroleum-based processes.

Several bacteria produce large amounts of succinic acid, including *Actinobacillus succinogenes* [2, 5, 12, 17], *Anaerobiospirillum succiniciproducens* [4, 6, 13, 15], and *Mannheimia succiniciproducens* [3, 8, 9, 18]. *M. succiniciproducens* MBEL55E isolated from bovine rumen shows an outstanding ability to produce succinic acid [9].

A large amount of organic acids including succinic acid have been produced by *M. succiniciproducens* LPK7 during fermentation [10, 11]. Hydrogen ions derived from organic acids inside cells cause oxidative stress, inhibiting the normal production of succinic acid. Therefore, experiments to eliminate the effects of hydrogen ions and to strengthen cells by introducing protectants to the medium were performed.

The bacterial strain used in this study was *M. succiniciproducens* LPK7, provided by the Korea Advanced Institute of Science and Technology. It is a genetically engineered strain that was generated by the deletion of the *ldhA*, *pflB*, and *pta-ackA* genes from *M. succiniciproducens* MBEL55E to reduce by-product formation [7, 9].

Cell growth was measured in terms of optical density using a UV–Vis spectrophotometer (Shimadzu, Japan) at 610 nm. Succinic acid and glucose concentrations were determined by high-performance liquid chromatography (HPLC; UV 730D detector, RI 750F monitor; Younglin, Korea) with an ion-exchange column (Aminex HPX-87H, 300 mm×7.8 mm; Bio-Rad, USA). The mobile phase for organic acid analysis was 0.005 N H_2SO_4, whereas 0.01 N H_2SO_4 was used for glucose analysis at a flow rate of 0.6 ml/min.

Batch fermentation was performed in MMH3 medium (yeast extract 5 g/l, NaCl 1.0 g/l, $(NH_4)_2SO_4$ 1.0 g/l, K_2HPO_4 8.708 g/l, $CaCl_2{\cdot}2H_2O$ 0.02 g/l, and $MgCl_2{\cdot}6H_2O$ 0.2 g/l) with 18 g/l glucose. The 5-l fermentor (Biotron, Korea)

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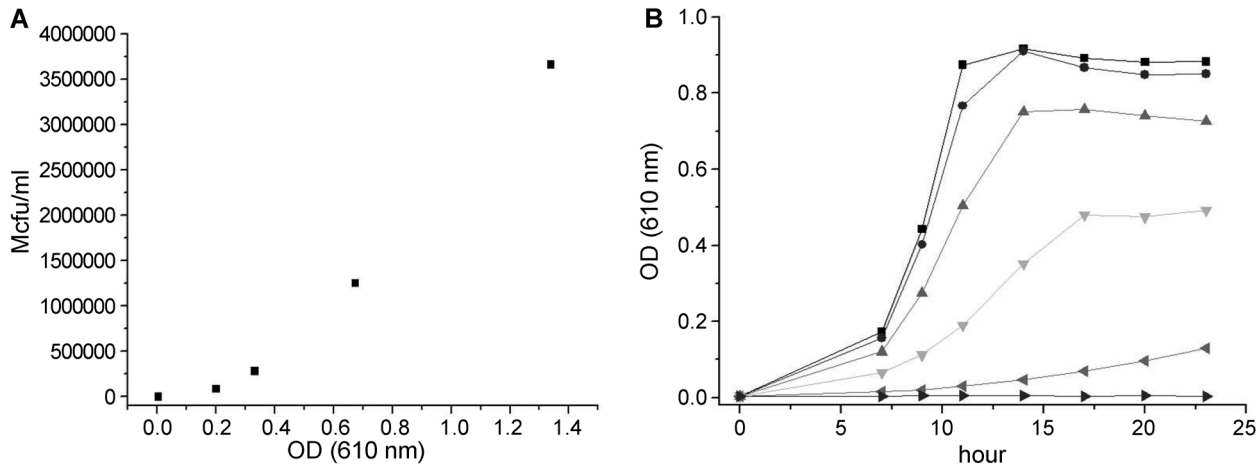

Fig. 1. The effects of succinic acid concentration on the cellular growth of *M. succiniciproducens*.
A. Viable cell count versus OD 610 nm profiles. **B**. Succinic acid toxicity test. ■, 0; ●, 0.1; ▲, 0.2; ▼, 0.3; ◀, 0.4; and ▶, 0.5% succinic acid.

containing 2 l of MMH3 medium with glucose was sterilized at 121°C for 15 min. During fermentation, the temperature, pH, and agitation speed were maintained at 39°C, 6.5, and 150 rpm, respectively. CO_2 gas was continuously flowed

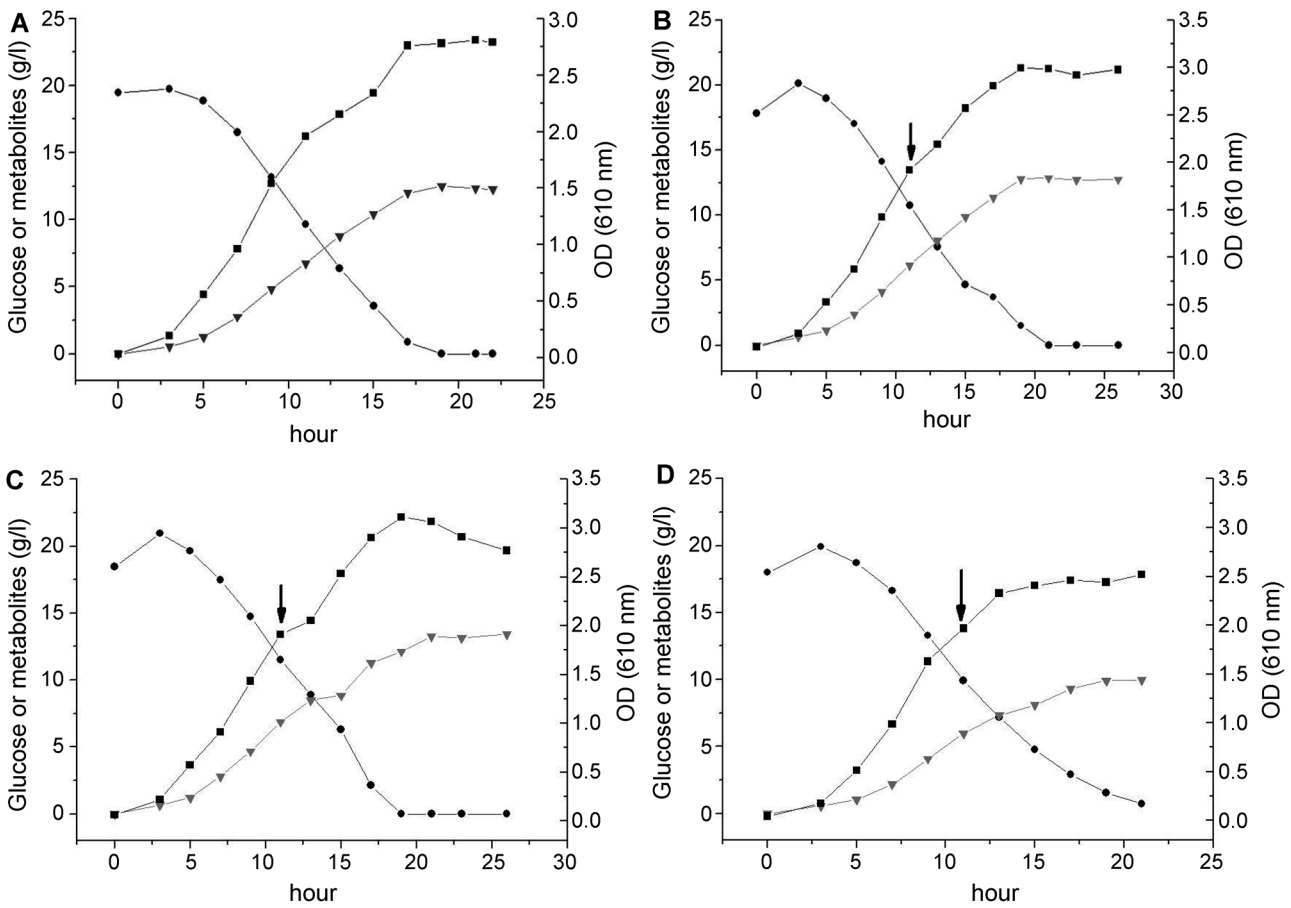

Fig. 2. The effects of pH change on the cellular growth of *M. succiniciproducens*.
The pH was changed, at the point indicated by the arrow, from 6.5 to (**A**) 6.5, (**B**) 6.75, (**C**) 7.0, and (**D**) 7.25. ●, Glucose; ■, cell growth; ▼, succinic acid production.

into the fermentor. Antifoam 204 (Sigma) was used to reduce foam. Cellular growth was measured and the biochemical composition of the medium was determined by HPLC.

For the succinic acid toxicity test, *M. succiniciproducens* LPK7 was grown in MH3 medium (NaCl 1.2 g/l, $(NH_4)_2SO_4$ 1.2 g/l, K_2HPO_4 10.44 g/l, yeast extract 3 g/l, $CaCl_2{\cdot}2H_2O$ 0.02 g/l, and $MgCl_2{\cdot}6H_2O$ 0.2 g/l) with 5 g/l glucose. After sterilization, CO_2 gas and 1 ml of precultured *M. succiniciproducens* LPK7 were introduced into each flask containing medium. The flasks were incubated at 39°C and monitored over 23 h. A cell viability test was also performed to verify the relationship between the measured optical density and the viable cell number. The number of viable cells was determined and expressed in terms of colony forming units.

The effects of changing the pH of batch fermentations as well as of adding cell protectants were examined. For the pH change experiments, the initial pH was maintained at 6.5 and changed to 6.75, 7.00, and 7.25 during the exponential growth phase. For the protectant experiments, separate solutions of 57.1 mg/l biotin, 1 g/l trehalose, and 24.6 mg/l glutathione were prepared. The protectants were introduced into the medium in each fermentor using a presterilized filter immediately before inoculation with the precultured medium [1, 14].

The succinic acid toxicity test consisted of two steps: a viable cell number test, and a flask culture test of cell growth in the presence of succinic acid at several concentrations: 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%. The growth rate of *M. succiniciproducens* LPK7 decreased as the concentration of succinic acid increased (Fig. 1). In addition, cell concentrations did not increase at all in medium containing 0.5% succinic acid.

Batch fermentations at higher pH levels were performed to determine the effects of pH changes on succinic acid fermentation. Fermentations performed in medium where the final pH was 6.75 and 7.00 showed improved cellular growth compared with the control (Fig. 2). However, at a final pH of 7.25, the cells did not grow as well as the control, and the succinic acid concentration was very low. In addition, the rate of glucose consumption at pH 7.25 was lower than that at pH 6.75 and 7.00. Although cell concentrations reached their highest levels at pH 7.00, they began to decrease after reaching the maximum (Fig. 2). This result may be due to the relatively high concentration of sodium ions introduced by added sodium hydroxide, which was used to control the pH at 7.00. The accumulation of organic acids in the medium might also induce this decrease in cell concentration. In terms of stable cellular growth, the optimum final pH was found to be 6.75. At this pH, the cell concentration was about 7% higher than the control. However, approximately 6% more succinic acid was produced at pH 7.00 than at pH 6.50. Thus, a pH of 7.00 is optimal for fermentation in terms of succinic acid yield.

Experiments to determine the effects of the cell protectants biotin, glutathione, and trehalose on succinic acid fermentation were performed. For the biotin-supplemented fermentation, the cellular growth rate and the maximum

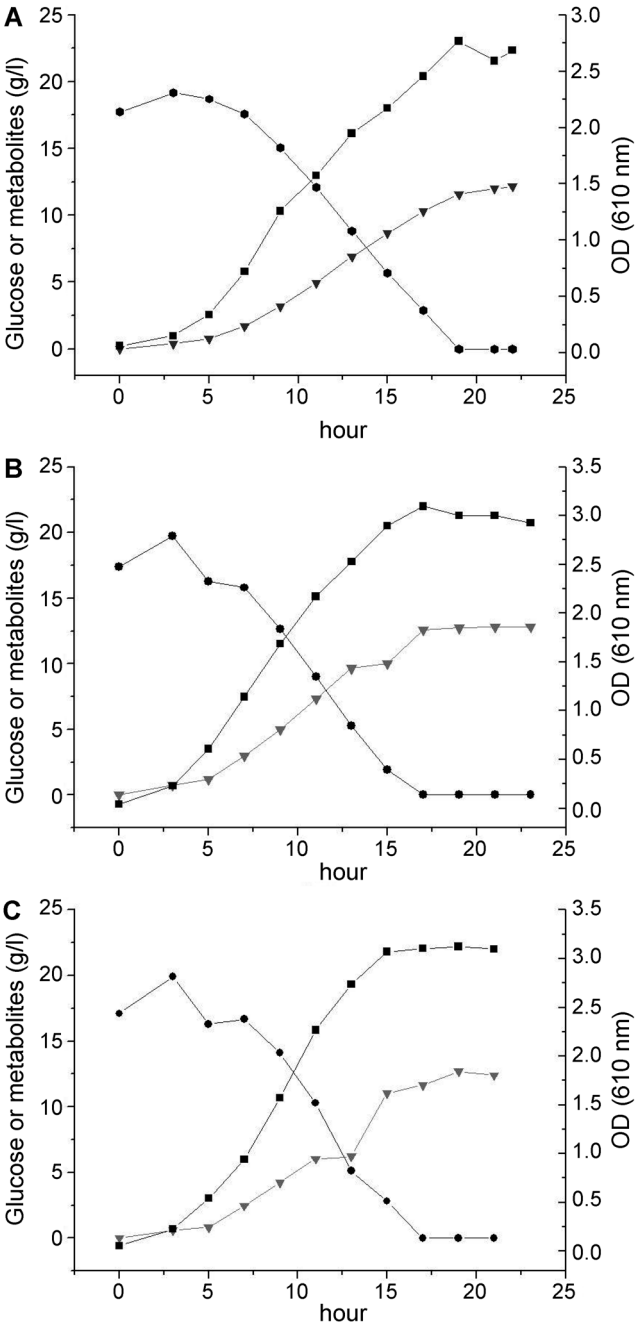

Fig. 3. The effects of protectants on the cellular growth of *M. succiniciproducens*.
Batch fermentations were performed to determine the effect of the protectants (**A**) biotin, (**B**) glutathione, and (**C**) trehalose on cell growth and succinic acid production. ●, Glucose; ■, cell growth; ▼, succinic acid.

cell concentration were both lower than those of the control (Fig. 2 and 3). Succinic acid production was also less than the control. Biotin, which is considered to be a mycobacterial supplement, seemed to be useless in the succinic acid fermentation carried out by *M. succiniciproducens* LPK7. However, when glutathione or trehalose was added, cells grew approximately 10% more than the control and produced more succinic acid than the control. Glutathione and trehalose may cause these effects by reducing oxidative stress due to the accumulation of hydrogen ions in the cells.

In conclusion, this study showed useful methods to increase the production of succinic acid, which could be applied to various industrial fermentations. The toxicity of succinic acid toward *M. succiniciproducens* LPK7 was verified. Cellular growth decreased as succinic acid concentrations in the medium increased. In addition, methods to improve succinic acid productivity were developed. By increasing the pH, cell growth was approximately 10% higher than in fermentations where the pH was kept constant at 6.5. Furthermore, by introducing protectants, the cell concentration was increased by about 10% and more succinic acid was produced compared with the control. These results were worthy of notice, because they showed an effective way to improve the production of succinic acid using microorganisms. Further research will involve if the methods developed in this study can be applied to larger scale fermentations.

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