Recovery of succinic acid produced by fermentation of a metabolically engineered *Mannheimia succiniciproducens* strain

Hyohak Song\(^a,b\), Yun Suk Huh\(^b\), Sang Yup Lee\(^a,b,c,d,*\), Won Hi Hong\(^b\), Yeon Ki Hong\(^e\)

\(^a\) Metabolic and Biomolecular Engineering National Research Laboratory, Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), 335 Gwahangno, Yuseong-gu, Daejeon 305-701, Republic of Korea

\(^b\) Department of Chemical and Biomolecular Engineering (BK21 Program), Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), 335 Gwahangno, Yuseong-gu, Daejeon 305-701, Republic of Korea

\(^c\) Department of BioSystems, BioProcess Engineering Research Center and Bioinformatics Research Center, Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), 335 Gwahangno, Yuseong-gu, Daejeon 305-701, Republic of Korea

\(^d\) Center for Systems and Synthetic Biotechnology, Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), 335 Gwahangno, Yuseong-gu, Daejeon 305-701, Republic of Korea

\(^e\) Department of Chemical and Biological Engineering, Chungju National University, 123 Geomdan-ri, Iryu-myeon, Chungju, Chungbuk 380-702, Republic of Korea

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**Abstract**

There have recently been much advances in the production of succinic acid, an important four-carbon dicarboxylic acid for many industrial applications, by fermentation of several natural and engineered bacterial strains. *Mannheimia succiniciproducens* MBEL55E isolated from bovine rumen is able to produce succinic acid with high efficiency, but also produces acetic, formic and lactic acids just like other anaerobic succinic acid producers. We recently reported the development of an engineered *M. succiniciproducens* LPK7 strain which produces succinic acid as a major fermentation product while producing much reduced by-products. Having an improved succinic acid producer developed, it is equally important to develop a cost-effective downstream process for the recovery of succinic acid. In this paper, we report the development of a simpler and more efficient method for the recovery of succinic acid. For the recovery of succinic acid from the fermentation broth of LPK7 strain, a simple process composed of a single reactive extraction, vacuum distillation, and crystallization yielded highly purified succinic acid (greater than 99.5% purity, wt%) with a high yield of 67.05 wt%. When the same recovery process or even multiple reactive extraction steps were applied to the fermentation broth of MBEL55E, lower purity and yield of succinic acid were obtained. These results suggest that succinic acid can be purified in a cost-effective manner by using the fermentation broth of engineered LPK7 strain, showing the importance of integrating the strain development, fermentation and downstream process for optimizing the whole processes for succinic acid production.

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**Keywords:** *Mannheimia succiniciproducens*; Succinic acid; By-products; Metabolic engineering; Downstream process

**1. Introduction**

To date, most of commercially available succinic acid, a dicarboxylic acid having the molecular formula of \(\text{C}_4\text{H}_6\text{O}_4\), has been produced by chemical processes using petroleum as a starting material (Song and Lee, 2006; Zeikus et al., 1999). However, there has recently been much interest in the production of succinic acid from renewable resources by microbial fermentation because of the dramatic increase in petroleum price and ever increasing environmental concerns. Succinic acid is produced in many organisms as an intermediate of tricarboxylic acid (TCA) cycle and also as one of the fermentation end-products of anaerobic metabolism. Several bacteria, including *Actinobacillus succinogenes* (Guettler et al., 1999), *Anaerobiospirillum succiniciproducens* (Lee et al., 1999; Samuelov et al., 1991), and *Mannheimia succiniciproducens* (Lee et al.,
2002), have been found to produce much larger amounts of succinic acid than other microorganisms, while some by-products such as acetic acid, formic acid, lactic acid, and ethanol are also formed. It is widely accepted that the concomitant production of by-products limits in part the possibility of its fermentative production in industrial scale, since it reduces the succinic acid yield and makes the downstream process more complex and costly (Huh et al., 2004; Kim et al., 2004; Song and Lee, 2006).

The downstream processing typically accounts for over 50–80% of the total production cost in the classical fermentation-based processes (Daniel and Eyal, 1995; Williams, 2004). The downstream processes that have been developed for the recovery of succinic acid from fermentation broth consisted of simultaneous acidification and electrodialysis or ion-exchange chromatography, followed by crystallization, and thus, they are rather complex and costly (Berglund et al., 1991; Datta et al., 1992; Davison et al., 2004). In addition, these processes typically yield large amounts of solid and slurry wastes that need to be further treated and properly disposed, especially calcium and sodium sulfates which are generated and precipitated by adding acids during the acidification step (Glassner and Datta, 1992; von Friel and Schügerl, 1999).

Much effort has been exerted to tackle the aforementioned problem by the development of engineered strains and/or better fermentation strategies and downstream processes. Among these, supplementation of hydrogen gas (Lee et al., 1999), co-fermentation of glucose and glycerol (Lee et al., 2001), and the use of chemically mutagenized (Guettler et al., 1996) and recombinant strains (Hong and Lee, 2001; Lin et al., 2005) allowed reduced production of by-products to some extent, which improved the downstream processes as well. In another case, Davison et al. (2004) reported that sorption using a packed column of sorbent resins is an efficient method for in situ separation of succinic acid without generation of solid wastes. However, much improvement in recovery method is still needed to make the whole process economically feasible.

*M. succiniciproducens* MBEL55E, a gram-negative capnophilic bacterium, produces a substantial amount of succinic acid as a major fermentation product under anaerobic conditions. Like other succinic acid-producers, this strain also produces acetic, formic, and lactic acids as by-products (Lee et al., 2002). More recently, we reported the development of *M. succiniciproducens* LPK7 strain, in which the *ldhA* (lactate dehydrogenase), *pfb* (pyruvate formate lyase), and *pta-ackA* (phosphotransacetylase-acetate kinase) genes were inactivated (Lee et al., 2006) by metabolic engineering based on its complete genome sequence (Hong et al., 2004). The resulting LPK7 strain produced much less by-products compared with other succinic acid producers including the wild-type *M. succiniciproducens*.

In this paper, we report the development of a simpler, more efficient, and environmentally friendly downstream process for the recovery of succinic acid from the fermentation broth of the LPK7 strain. We also investigated in detail the effects of strain improvement on the efficiency of downstream processes by comparing the recovery results obtained using the fermentation broth of LPK7 and MBEL55E strains.

2. Materials and methods

2.1. Organism and growth conditions

All chemicals used in this study were of reagent grade and were purchased from either Sigma–Aldrich (St. Louis, MO) or Junsei Chemical (Tokyo, Japan) unless otherwise described. An industrial grade of CO2 (Special gas, Daejeon, Korea) was used. *M. succiniciproducens* MBEL55E (KCTC 0769BP; Korean Collection for Type Cultures, Daejeon, Korea) and LPK7 [*ldhA::Kn*, *pfb::*::Cm*, *pta-ackA::*::Sp*] (KCTC 10626BP) strains were used in this study. Seed cultures were prepared by growing cells at 39 °C in a scaled anaerobic flask containing 250 ml of MH4 medium with CO2 headspace. The MH4 medium contained per liter: 5 g yeast extract, 1 g NaCl, 0.2 g CaCl2·2H2O, 0.2 g MgCl2·6H2O, and 8.709 g K2HPO4. The medium was heat sterilized at 121 °C for 15 min in an anaerobic flask with CO2 as a gas phase. Glucose was separately sterilized and added to the medium to give a final concentration of ca. 100 mM, the actual concentration of which was measured again before the fermentation started. The initial pH of the sterilized medium was adjusted to 7.0 by adding 5N NaOH. Spectinomycin was added to a final concentration of 50 µg ml⁻¹ for the cultivation of LPK7 strain. The medium was inoculated with 2.5 ml of glycerol stock culture and incubated at 39 °C in an anaerobic chamber (ThermoForma, Marietta, OH) until the optical density at 600 nm (OD600) reached about 1.5–1.7.

Batch fermentation was conducted at 39 °C in a 6.6 l Bioflo 3000 fermentor (New Brunswick Scientific Co., Edison, NJ) containing 2.25 l of MH4 medium plus 100 mM glucose, into which seed culture (10%, v/v) was added. The pH was controlled at 6.5 ± 0.1 by the automatic addition of 28% (w/w) ammonia solution. The agitation speed was kept at 200 rpm. Foaming was controlled by adding Antifoam 289 (Sigma, St. Louis, MO). The fermentor was continuously flushed with CO2 gas, which was scrubbed free of oxygen by passing through an oxygen trap (Agilent, Waldbronn, Germany), at a flow rate of 0.25vvm during the entire period of the fermentation. A 0.2 µm HEPA filter (Milli-pore, Billerica, CA) was fitted in the inlet line of the fermentor to sterilize CO2 gas.

2.2. Downstream process for succinic acid recovery

Samples for the recovery of succinic acid were taken when glucose in fermentation broth was completely consumed. The fermentation broth was centrifuged for 10 min at 5590 × g and 4 °C to separate cells. To remove by-products and salts from the clear fermentation broth, reactive extraction was performed as follows. Organic solvent (10 ml of 1-octanol) containing 0.25 mol kg⁻¹ tri-n-octylamine (TOA) was mixed with 10 ml of fermentation broth in a 30 ml vial. Phase mixing was then carried out by stirring with a magnetic bar at 1000 rpm and 25 °C for 2 h. When needed, multiple reactive extractions were per-
formed by repeating the above reactive extraction up to three
cycles. For the clear phase separation after the reactive extrac-
tion, mixed phases were centrifuged at 2480 × g and 25°C
for 15 min. After performing the reactive extraction as a pri-
mary separation method for the removal of by-product acids
from the fermentation broth, samples in aqueous solution were
vacuum distilled at 0.7 atm and 75°C for 2 h to facilitate crys-
tallization and to eliminate residual volatile carboxylic acids
such as acetic, formic and lactic acids. Crystallization of su-
cinic acid was carried out at 4°C and pH 2.0 by adding HCl.
Crystallized succinic acid was well dried at 100°C for 5 h.
The purity, purification yield, and the mass ratio of succinic
acid to by-products were calculated as reported previously
(Huh et al., 2004). The purity and recovery yield of succinic
acid at each purification process were determined by HPLC
equipped with an ion-exchange column (Supelcogel C-610H,
300 mm × 7.8 mm, SUPELCO, USA) using 0.1 wt% H3PO4
solution as mobile phase. The average values, which were
obtained from three independent measurements using at least
two separate fermentation broth, were used in all calcu-
lations.

2.3. Analytical procedures
The concentrations of glucose and metabolites in fer-
mmentation broth, including acetic, formic, lactic, pyruvic
and succinic acids, were measured by high-performance liq-
uid chromatography (ProStar 210, Varian, Palo Alto, CA)
equipped with the MetaCarb 87H column (300 mm × 7.8 mm,
Varian), ProStar 320 UV–vis (Varian) and Shodex RI-71 detec-
tors (Tokyo, Japan). The column was isocratically eluted
with 0.01N H2SO4 at a flow rate of 0.6 ml min−1 and
60°C. Cell growth was monitored by measuring the OD600
using a spectrophotometer (Ultrospec3000, Amersham Biosciences, Uppsala, Sweden). Dry cell weight (DCW) was
calculated form the pre-determined standard curve relating the
OD600 to dry weight (1 OD600 = 0.451 g DCW l−1; Lee et al.,
2006).

3. Results
3.1. Fermentation of M. succiniciproducens

*M. succiniciproducens* MBEL55E (wild-type) and LPK7
(Δ*ldhA*, Δ*pflB*, and Δ*pta-ackA*) strains were cultivated in
the MH4 medium (Song et al., 2007) supplemented with
100 mM glucose as a carbon source at 39°C with con-
tinuous supply of CO2. At the end of the fermentation,
the concentrations of cell, acetic, formic, lactic and succinic
acids were 3.320 ± 0.019 g DCW l−1, 3.573 ± 0.003,
3.950 ± 0.137, 0.960 ± 0.002, and 8.815 ± 0.009 g l−1 (for
MBEL55E) and 1.615 ± 0.029 g DCW l−1, 0.372 ± 0.049, 0,
0, and 13.64 ± 0.362 g l−1 (for LPK7), respectively. Total carbon recovery values obtained with
the MBEL55E and LPK7 strains were similar (98.0 ± 1.95
and 97.5 ± 2.93%, respectively). MBEL55E and LPK7 strains com-
pletely consumed glucose in 7 and 15 h, respectively (Fig. 1).
As reported previously (Lee et al., 2006), the LPK7 strain
grew slowly due to the knock-out of the *ldhA*, *pflB*, and *pta-
ackA* genes. Also, the final cell concentration obtained with
MBEL55E strain was about twice higher than the LPK7 strain.
Lee et al. (2006) reasoned that less production of acetyl-CoA
due to the *pflB* gene knock-out and the loss of ATP production
due to the *pta-ackA* gene knock-out might retard the growth of
the LPK7 strain. However, the succinic acid concentration at
the end of fermentation was significantly higher for the LPK7
strain. Accordingly, the final succinic acid yield on glucose
obtained with the LPK7 strain was 1.58 times higher than that
obtained with the MBEL55E strain. Even though the overall
volumetric succinic acid productivity of the LPK7 strain was
slightly lower than that of MBEL55E strain due to the lower
growth rate, the specific succinic acid productivity of the former

| Table 1 | Comparison of the fermentation results between the wild-type *M. succiniciproducens* MBEL55E strain and a metabolically engineered *M. succiniciproducens* LPK7 strain
<table>
<thead>
<tr>
<th>MBEL55E</th>
<th>LPK7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial glucose (g l−1)</td>
<td>18.03 ± 0.097</td>
</tr>
<tr>
<td>Final biomassb (g DCW l−1) (carbon recovery%)c</td>
<td>3.320 ± 0.019 (21.66 ± 0.124)</td>
</tr>
<tr>
<td>Final acetic acid (g l−1) (carbon recovery%)c</td>
<td>3.573 ± 0.003 (19.81 ± 0.016)</td>
</tr>
<tr>
<td>Final formic acid (g l−1) (carbon recovery%)c</td>
<td>3.950 ± 0.137 (14.29 ± 0.495)</td>
</tr>
<tr>
<td>Final lactic acid (g l−1) (carbon recovery%)c</td>
<td>0.960 ± 0.002 (3.324 ± 0.011)</td>
</tr>
<tr>
<td>Final pyruvic acid (g l−1) (carbon recovery%)c</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Final succinic acid (g l−1) (carbon recovery%)c</td>
<td>8.815 ± 0.009 (37.29 ± 0.038)</td>
</tr>
<tr>
<td>Final succinic acid yieldd (g g−1)</td>
<td>0.489 ± 0.001</td>
</tr>
<tr>
<td>Overall volumetric succinic acid productivity (g l−1 h−1)</td>
<td>1.260 ± 0.009</td>
</tr>
<tr>
<td>Overall specific succinic acid productivity (g g DCW l−1 h−1)</td>
<td>0.741 ± 0.009</td>
</tr>
<tr>
<td>Total carbon recovery (%)</td>
<td>98.37 ± 0.781</td>
</tr>
</tbody>
</table>

a All data were obtained at the end of fermentation when glucose was completely consumed. Values are the means and standard deviations from two independent but fermentations.
b Carbon compound incorporated into the cell was calculated using the cell composition formula CH3OΔN02 (Mosey, 1983).
c Carbon compound in succinic acid was calculated as 3 mol carbon per succinic acid, considering CO2 fixation during succinic acid formation (Lee et al., 2006).
d Calculated as succinic acid produced per glucose consumed.
Time profiles of cell growth and metabolites production during the batch fermentation of (A) *M. succiniciproducens* MBEL55E and (B) *M. succiniciproducens* LPK7 strains in a complex MH4 medium supplemented with glucose. Symbols are biomass (■), glucose (●), succinic acid (●), acetic acid (♦), formic acid (△), lactic acid (□), and pyruvic acid (▽).

(1.160 ± 0.039 g g⁻¹ h⁻¹) was substantially higher than that of the MBEL55E strain (0.741 ± 0.009 g g⁻¹ h⁻¹).

As previously reported by Lee et al. (2002, 2006), the wild-type *M. succiniciproducens* MBEL55E produced relatively large amounts of acetic, formic, and lactic acids as by-products (Fig. 1A). On the other hand, only a small amount of acetic acid (0.372 ± 0.049 g l⁻¹, at the end of fermentation) was produced by the metabolically engineered *M. succiniciproducens* LPK7 strain (Fig. 1B). Furthermore, the LPK7 strain did not produce any formic and lactic acids during the entire period of fermentation. Although pyruvic acid was produced as another by-product by the LPK7 strain, the total amount of by-products including acetic and pyruvic acids was 5.192 ± 0.203 g l⁻¹ when 100 mM glucose was completely utilized. This value corresponds to about 60% of the total amounts of by-products produced by the MBEL55E strain.

The efficiency of the downstream process is strongly affected by the concentration of a targeted product as well as the number and characteristics of by-products present in the fermentation broth (Harrison et al., 2003), the LPK7 strain becomes advantageous in the recovery process; it not only produces one less by-product, but also produces as a by-product pyruvic acid having a pKa value of 2.49, which is relatively different from that of acetic (pKa of 4.75), formic (pKa of 3.84), lactic (pKa of 3.86), and succinic acid (pKa1 and pKa2 of 4.207 and 5.635, respectively), facilitating easier recovery of succinic acid.

### 3.2. Reactive extraction

In order to recover succinic acid from fermentation broth, a simple downstream process, consisting of reactive extraction, vacuum distillation, and crystallization, was developed in this study. As previously reported (Tung and King, 1990; Huh et al., 2004; Hong and Hong, 2000, 2005), the reactive extraction using TOA is an effective primary recovery method for the selective removal of carboxylic acid by-products. Fig. 2A shows the effects of the pH (ranging from 2.0 to 6.0) on the distribution coefficients of the acids produced by MBEL55E during the reactive extraction using TOA in 1-octanol at 25 °C. The distribution coefficient of component i (Di) is defined as follows

\[
D_i = \frac{C_{i,\text{org}}}{C_{i,\text{aq}}}
\]

where \(C_{i,\text{org}}\) and \(C_{i,\text{aq}}\) represent the equilibrium concentrations of component i in the organic phase and aqueous phase, respectively. At the extreme pH ranges (≥6.0 and ≤2.0), the changes in the distribution coefficients of all acids were not noticeable (data not shown). For all acids, the distribution coefficients...
increased monotonously as the pH decreased. In particular, the sharp rises in the values of the distribution coefficient for acetic, formic, lactic, and succinic acids were observed at the pH near their pK\textsubscript{a1} values, 4.75, 3.84, 3.86, and 4.207, respectively. Since TOA, as a tertiary amine extractant, extracts only the undissociated form of carboxylic acids, the increase in the concentrations of undissociated carboxylic acids below the pK\textsubscript{a1} values will enhance the extraction of acids.

As shown in Fig. 2A, the distribution coefficients for monocarboxylic acids, including acetic, formic, and lactic acids, were higher than that of a dicarboxylic acid, succinic acid, at all pH levels examined. Especially, the distribution coefficient for succinic acid showed a big drop between pK\textsubscript{a1} (4.207) and pK\textsubscript{a2} (5.635). The distribution coefficient for succinic acid did not change much at pH above 5.0, while those of acetic, formic, and lactic acids (mono-carboxylic acids) did. These behaviors are mainly attributed to the fact that the stronger acidity is, as represented by the pK\textsubscript{a} value, the more undissociated acid can be extracted using the same solvent system. This also suggests that the optimal condition for the selective removal of by-produced monocarboxylic acids, especially acetic, formic, and lactic acids, from the fermentation broth should be predetermined as shown in Fig. 2.

Fig. 2B shows the effects of pH on the distribution coefficients of acids produced by the LPK7 strain during the reactive extraction using TOA in 1-octanol at 25°C. As in the reactive extraction of the MBEL55E fermentation broth, the distribution coefficients for acetic and pyruvic acids increased with the decrease of pH. This observation is again due to that only the undissociated carboxylic acids can be extracted by TOA. In cases of monocarboxylic acids, the higher initial concentrations of the acids (formic acid > acetic acid > lactic acid) in the fermentation broth obtained with the MBEL55E strain made the greater distribution coefficients, which correspond to the concentrations of the undissociated carboxylic acids, except for the dramatic drop in the distribution coefficient for formic acid at pH between 5.0 and 6.0 (Fig. 2A). It was also observed for the fermentation broth of the LPK7 strain that the distribution coefficient was much higher for pyruvic acid than for acetic acid at all pH ranges (2.0–6.0) as the initial concentration of pyruvic acid (4.448 ± 0.105 g l\textsuperscript{-1}) was substantially higher compared with that of acetic acid (0.372 ± 0.049 g l\textsuperscript{-1}). As shown in Fig. 2, the distribution coefficient of succinic acid decreased to nearly 0 at pH higher than 5.0, while those of other acids were much higher, thus allowing selective extraction of other acids from the fermentation broth.

Based on the equilibrium distribution coefficients (Fig. 2), reactive extraction using TOA in 1-octanol was performed at pH 5.0. Acetic, formic, lactic, and pyruvic acids could be selectively removed from the fermentation broth by single batch reactive extraction. The purity of succinic acid and the ratio of succinic acid to by-products increased from 50.96 ± 0.089% to 55.70 ± 0.073% and 1.045 ± 0.029 to 1.250 ± 0.021 g g\textsuperscript{-1}, respectively, for the fermentation broth of MBEL55E. In the case of the fermentation broth of the LPK7 strain, the purity and ratio increased from 73.89 ± 0.093% to 77.48 ± 0.097% and 2.830 ± 0.019 to 3.440 ± 0.033 g g\textsuperscript{-1}, respectively (Table 2).

### 3.3. Vacuum distillation and crystallization of succinic acid

Vacuum distillation was applied in order to increase the succinic acid concentration and to remove the residual volatile organic acids from the succinic acid solution preliminarily purified by reactive extraction. This process allowed concentration of succinic acid from the fermentation broths produced by the MBEL55E and LPK7 strains by about 11.3-and 7.34-folds, respectively.

As the final recovery process, the crystallization was carried out using the concentrated succinic acid solution at low temperature (4°C) and low pH (2.0). HCl was added to selectively crystallize succinic acid based on the fact that the degree of solubility for carboxylic acids is dependent on both temperature and pH. No further increase in the purity of crystallized succinic acid was observed by lowering the pH and temperature below 2.0 and 4°C, respectively.

Table 2 summarizes the ratios of succinic acid to by-products, the purities of succinic acid, and the purification yields after single reactive extraction, vacuum distillation, and crystallization using the fermentation broths of the MBEL55E and LPK7 strains. The vacuum distillation and crystallization steps caused the dramatic drop in the purification yields of succinic acid from 99.17 ± 0.081% to 66.44 ± 0.054% (for MBEL55E broth) and from 95.79 ± 0.077% to 67.05 ± 0.036% (for LPK7 broth). In return of sacrificing the purification yield, we were able to obtain highly purified succinic acid crystals. Also, the ratios of succinic acid to by-products jumped from

### Table 2

<table>
<thead>
<tr>
<th>Strains</th>
<th>Fermentation broth\textsuperscript{b}</th>
<th>Reactive extraction</th>
<th>Crystallization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio (g g\textsuperscript{-1})\textsuperscript{c}</td>
<td>Purity (%)\textsuperscript{d}</td>
<td>Ratio (g g\textsuperscript{-1})</td>
</tr>
<tr>
<td>MBEL55E</td>
<td>1.045 ± 0.029</td>
<td>50.96 ± 0.089</td>
<td>1.250 ± 0.021</td>
</tr>
<tr>
<td>LPK7</td>
<td>2.830 ± 0.019</td>
<td>73.89 ± 0.093</td>
<td>3.440 ± 0.033</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values are the means and standard deviations three independent measurements using at least two separate fermentation broths.

\textsuperscript{b} All data were obtained at the end of fermentation when glucose was completely consumed.

\textsuperscript{c} The ratio of succinic acid (g) to the total amounts of by-products (g).

\textsuperscript{d} The weight percentage of succinic acid to total dry weight of the sample.

\textsuperscript{e} The weight percentage of succinic acid to the initial amount of succinic acid.
1.25 ± 0.021 to 53.70 ± 0.051 g g⁻¹ (for MBEL55E broth) and from 3.44 ± 0.033 to 222.08 ± 0.08 g g⁻¹ (for LPK7 broth). In particular, the final purity of succinic acid obtained after crystallization using the fermentation broth of the LPK7 strain increased from 73.89 ± 0.093% to 99.55 ± 0.017%, while that obtained using the fermentation broth of the MBEL55E strain was 98.17 ± 0.010%.

3.4. Multiple reactive extractions of MBEL55E fermentation broth

With an aim to further increase the final purity of succinic acid for the fermentation broth of the MBEL55E strain, multiple reactive extractions were carried out up to three times (Table 3). As expected, the gradual increase in the ratio of succinic acid to by-products was observed: 1.257 ± 0.021 g g⁻¹ after one extraction, 1.477 ± 0.0234 g g⁻¹ after two extractions, and 1.644 ± 0.0221 g g⁻¹ after three extractions (Fig. 3A). Also, the purity of succinic acid increased as the number of extraction increased: 55.70 ± 0.102% (first), 59.63 ± 0.095% (second), and 62.18 ± 0.107% (third). This led to the subsequent increase in the final purity of succinic acid, 98.17 ± 0.010% (first), 98.97 ± 0.184% (second), and 99.16 ± 0.196% (third) after vacuum distillation and crystallization. However, the ratio of succinic acid to by-products and the purity of succinic acid after the multiple reactive extractions using the fermentation broth of the MBEL55E strain were still lower than those obtained by single reactive extraction of the fermentation broth of the LPK7 strain (Fig. 3B). These results suggest that reduced production of by-products during the fermentation is important to achieve efficient recovery of succinic acid to a high purity, which can be generalized to state that the upstream optimization (e.g., strain development) is very important in improving the overall process performance.

4. Discussion

Although much effort has been devoted to bring many commodity chemicals produced by microbial fermentation into commercialization, only a few of them such as citric acid, lactic acid and ethanol are currently available mainly due to the economic feasibility. In particular, traditional downstream processes for the recovery of carboxylic acids from fermentation broth are very costly and rather complex for commercial viability. Moreover, these processes yield large amounts of solid and slurry wastes, like gypsum, to be further treated and disposed (Datta et al., 1992; von Frielang and Schügerl, 1999), leading to generation of environmental problems. For the purification of succinic acid, electrodialysis has been a widely used separation process, in which ionized compounds are separated from non-ionized compounds by ion-exchange membrane. However, the current efficiency is rather low, because the electrodialysis cannot selectively remove acetate and formate from succinate. Major drawbacks of this process include the high-energy consumption and the difficulty of scaling-up. Reactive extraction method reported here is environmentally friendly and more effective than any other purification processes. This advantage became more evident when the LPK7 strain was employed. Thus, it is important to optimize the whole bioprocess from the upstream strain development to downstream recovery processes to make the fermentative succinic acid production industrially attractive.

A capnophilic rumen bacterium *M. succiniciproducens* MBEL55E is able to convert more than 37% carbon equivalent of glucose to succinic acid during its anaerobic fermentation. The
second highest fraction of carbon was recovered in the form of cell mass (22%), and the rest was found in acetic (20%), formic (14%), and lactic (5%) acids. Formation of by-products not only reduces the yield and productivity of succinic acid, but also increases the recovery costs. Thus, it is widely accepted that minimizing or eliminating the formation of by-products during fermentation is crucial for maximizing the yield of succinic acid as well as for enhancing the efficiency of downstream processes.

Lee et al. (2006) developed the LPK7 strain by knocking out the ldhA, pflB, and pta-ackA genes, which resulted in reduced formation of acetic acid and no production of formic and lactic acids. A small amount of acetic acid is still produced by several alternative pathways including those involved in amino acid metabolism (Lee et al., 2006). The LPK7 strain showed some retarded cell growth due to the reduced formation of ATP and acetyl-CoA by knocking-out key enzymes forming them. This observation is consistent with the previous reports showing that cells which were partially restricted in the synthesis of acetyl-CoA by inactivating the related genes showed slower growth compared with their parent strains (Kakuda et al., 1994; Chang et al., 1999; Yang et al., 1999a,b; Contiero et al., 2000). Acetyl-CoA is mainly derived from pyruvate by means of pyruvate dehydrogenase complex and/or pyruvate formate-lyase, and is further converted to acetate, ethanol, or biomass via fatty acids dehydrogenase complex and/or pyruvate formate-lyase, and is CoA is mainly derived from pyruvate by means of pyruvate metabolism (Lee et al., 2006). The LPK7 strain showed much enhanced succinic acid production. The specific succinic acid productivity, which represents the capacity of cells to produce succinic acid per hour, of the LPK7 strain was much higher than that of the wild-type MBEL55E strain due to the imbalance in the reducing power. This phenomenon is similarly observed in metabolically engineered Escherichia coli NZN111 (pfl and ldh genes inactivated) and AFP111 (pfl, ldh, and ptsG genes inactivated) strains under anaerobic conditions (Venuri et al., 2002; Lin et al., 2005). Nonetheless, we thought that it is still more important to reduce the number and total amounts of by-products. As expected, the LPK7 strain showed much enhanced succinic acid production. The specific succinic acid productivity, which represents the capacity of cells to produce succinic acid per hour of the LPK7 strain was as high as 2.97 g l⁻¹ h⁻¹, which is higher than that obtainable with MBEL55E (Lee et al., 2006). The succinic acid yield obtained with MBEL55E strain was 0.747 mol succinic acid/mol glucose. On the other hand, the succinic acid yield obtained with the LPK7 strain was as high as 1.18 mol succinic acid/mol glucose, which is 69% of theoretical succinic acid yield.

The most notable finding in this study was that the reduced by-products formation by strain improvement through rational metabolic engineering allowed efficient recovery of succinic acid with a high purity and yield by simple downstream processes. Single reactive extraction was good enough to recover substantial amount of succinic acid from the fermentation broth also containing acetic and pyruvic acids. Vacuum distillation and crystallization following the reactive extraction yielded highly pure succinic acid. Apparently, more succinic acid with less by-products in the fermentation broth of LPK7 allowed recovery of succinic acid with a purity higher than 99.5%. This is in contrast with the fermentation broth of MBEL55E, which showed the final succinic acid purity of 98.2% by the same recovery processes, which could be increased to 99.2% after three reactive extractions. It should be noted that one reactive extraction process costs ca. 10% of the total succinic acid production cost, and thus, employing three reactive extraction steps is not economically desirable. On the other hand, it may be possible to consider countercurrent extraction instead of multiple extractions.

It can be seen from Table 2 that the purification yield after crystallization is apparently low. This is because we performed only single crystallization step as a proof-of-concept. In the actual process, one can recycle the residual mother liquor for repeated crystallization, which will obviously increase the purification yield.

In conclusion, we reported the development of a simple downstream process composed of single reactive extraction, vacuum distillation and crystallization for the economical recovery of succinic acid. This was possible by employing the M. succiniciproducens LPK7 strain which produces a large amount of succinic acid without much by-products. Thus, these results clearly show how strain improvement through metabolic engineering contributes to increasing the purity and yield of a desired product, and consequently reducing the costs of downstream processing by employing simple recovery steps.

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References


