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Succinic Acid Production by Continuous Fermentation Process Using *Mannheimia succiniciproducens* LPK7

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To achieve a higher succinic acid productivity and evaluate the industrial applicability, this study used *Mannheimia succiniciproducens* LPK7 (knock-out: *ldh*A, *pfl*B, *pta-ack*A), which was recently designed to enhance the productivity of succinic acid and reduce by-product secretion. Anaerobic continuous fermentation of *Mannheimia succiniciproducens* LPK7 was carried out at different glucose feed concentrations and dilution rates. After extensive fermentation experiments, a succinic acid yield and productivity of 0.38 mol/mol and 1.77 g/l/h, respectively, were achieved with a glucose feed concentration of 18.0 g/l and 0.2 h^{-1} dilution rate. A similar amount of succinic acid production was also produced in batch culture experiments. Therefore, these optimal conditions can be industrially applied for the continuous production of succinic acid. To examine the quantitative balance of the metabolism, a flux distribution analysis was also performed using the metabolic network model of glycolysis and the pentose phosphate pathway.

Keywords: *Mannheimia succiniciproducens* LPK7, continuous fermentation, succinic acid, flux distribution analysis

Succinic acid is a valuable 4-carbon intermediate that is useful in the production of 1,4-butanediol, tetrahydrofuran, and gammabutyrolactone [1] and applied in the food, pharmaceutical, and cosmetic industries [15, 19]. To date, succinic acid has been commercially produced by a substitution reaction of malic acid in chemical processes that depend on petroleum.

However, the fermentative production of succinic acid from a renewable biomass has attracted recent interest [6], as it has several advantages over chemical processes, including a reduced production cost [7, 11, 14], plus it does not contribute to the accumulation of CO_2 in the environment, making fermentatively produced succinic acid a "green" chemical [5, 16].

Some anaerobic bacteria, such as *Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes*, have already been extensively studied because of their ability to produce a relatively large amount of succinic acid [18]. However, unknown genomic information remains a barrier for metabolic engineering to enhance productivity and prevent by-product formation [8]. Recently, a Gram-negative capnophilic bacterium, *Mannheimia succiniciproducens* MBEL55E, was isolated from Korean cow rumens by Lee *et al.* [9, 11], and shown to be able to produce a large amount of succinic acid as a major fermentation product under anaerobic conditions in the presence of CO_2 [13]. In addition, *Mannheimia succiniciproducens* LPK7 was developed based on a metabolic network analysis and genetic modifications to reduce the by-products and increase the productivity of succinic acid [8].

Accordingly, for the purposes of industrial application, this study investigated the continuous cultivation of *Mannheimia succiniciproducens* LPK7. Various continuous culture experiments were conducted to determine the optimal operating conditions for producing more succinic acid and less by-product secretion. In addition, a metabolic flux analysis was performed to confirm the validity of the succinic acid measurements and by-product and flux distributions.

The strain used in this study was *Mannheimia succiniciproducens* LPK7, which is a knock-out strain deficient in the *ldh*A, *pfl*B, and *pta-ack*A genes from *Mannheimia succiniciproducens* MBEL55E to produce succinic acid with less by-product formation [13]. The cells were grown in sealed anaerobic bottles containing 20 ml of an MH3 medium and 5 g/l of glucose under a CO_2 atmosphere. The MH3 medium for the seed culture contained (per liter): polypeptone 2.5 g, yeast extract 2.5 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 1.0 g, K_2HPO_4 9.676 g, $CaCl_2{\cdot}2H_2O$ 0.2 g, and

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Table 1. Comparison of fermentation performances according to concentration of glucose fed to a continuous culture of LPK7 in steady-state.

Glucose feed concentration	9.0 g/l				18.0 g/l		
Dilution rate (h^{-1})	0.1	0.15	0.2	0.3	0.1	0.2	0.3
Succinate concentration (g/l)	12.89	9.94	7.83	5.21	10.72	8.87	3.51
Yield (mol/mol)	1.09	0.84	0.66	0.44	0.45	0.38	0.15
Productivity (g/l/h)	1.29	1.49	1.57	1.56	1.07	1.77	1.05

$MgCl_2{\cdot}6H_2O$ 0.2 g. The medium was heat sterilized for 15 min at 121°C. The anaerobic bottles were inoculated with 1 ml of a glycerol stock culture and incubated at 39°C.

For the flask cultures, exponentially growing cells were inoculated into sealed anaerobic bottles containing 230 ml of the MH3 medium, whereas the batch cultures were carried out in a 5 l fermentor (manufactured by Biotron) containing 2.5 l of an MMH3 medium and 18 g/l of glucose. The MMH3 medium contained (per liter): yeast extract 5 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 1.0 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 fermentation was carried out at a temperature of 39°C, with any foaming controlled by the addition of Antifoam 204 (from Sigma), and the culture pH was maintained at 6.5 by the addition of a sterile solution of 5 N NaOH. CO_2 gas was sparged throughout the fermentation at a flow rate of 0.5 l/min and the agitation speed controlled at 180 rpm. The continuous culture was also carried out under the same conditions as the batch cultures, using the MMH3 medium containing 9 g/l, and 18 g/l of glucose, respectively, which was fed into the fermentor using a peristatic pump (from Cole-Parmer).

The product concentrations and carbon sources were analyzed using high-performance liquid chromatography (UV730D detector, RI750F monitor; Younglin, Korea) and an ion-exchange column (Aminex HPX-87H, 300 mm× 7.8 mm; Bio-Rad) with 0.005 N H_2SO_4 as the mobile phase at 55°C and a flow rate of 0.6 ml/min. The cell growth was monitored by measuring the absorbance at 610 nm using a UV-Vis spectrophotometer (Shimadzu). The succinic acid yield was defined as the moles of succinic acid produced to the moles of glucose consumed.

Glycolysis and the pentose phosphate pathway were investigated using a metabolic flux analysis, where glycolysis is the sequence of reactions that converts glucose into pyruvic acid, and the pentose phosphate pathway is a cytosolic process that generates NADPH and the synthesis of pentose sugars. The biomass was calculated based on the composition of *Escherichia coli*, including polysaccharide synthesis, murein synthesis, glycerol synthesis, nucleotide synthesis, aromatic amino acid synthesis, synthesis 1 (cysteine, glycine, and serine), synthesis 2 (alanine, leucine, isoleucine, and valine), and synthesis 3 (chorimate) [2, 3]. The metabolites measured included glucose as the substrate and several organic acids (pyruvate, succinate, and acetate) as the secreted products. The flux distributions were calculated by considering the stoichiometric relationships of the metabolic reactions, whereas the intracellular fluxes were estimated based on the assumption of steady-state cellular growth. The possible uptake of other carbon sources

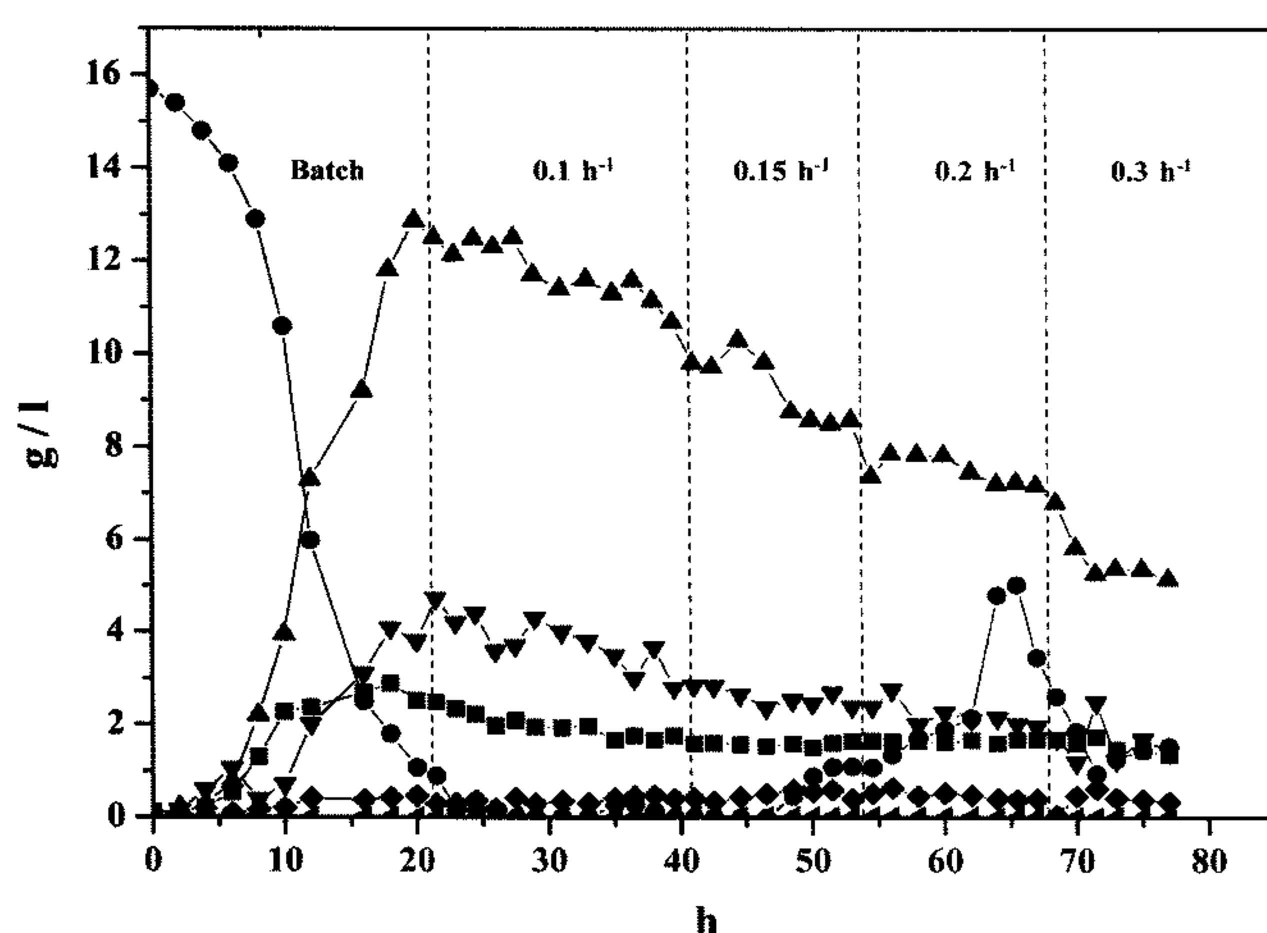

Fig. 1. Continuous fermentation profiles of *M. succiniciproducens* LPK7 at 50 mM glucose feeding concentration.

●, Glucose; ■, Cell concentration (OD_{610}); ▲, succinic acid; ▼, pyruvic acid; ◆, acetic acid; ◄, lactic acid.

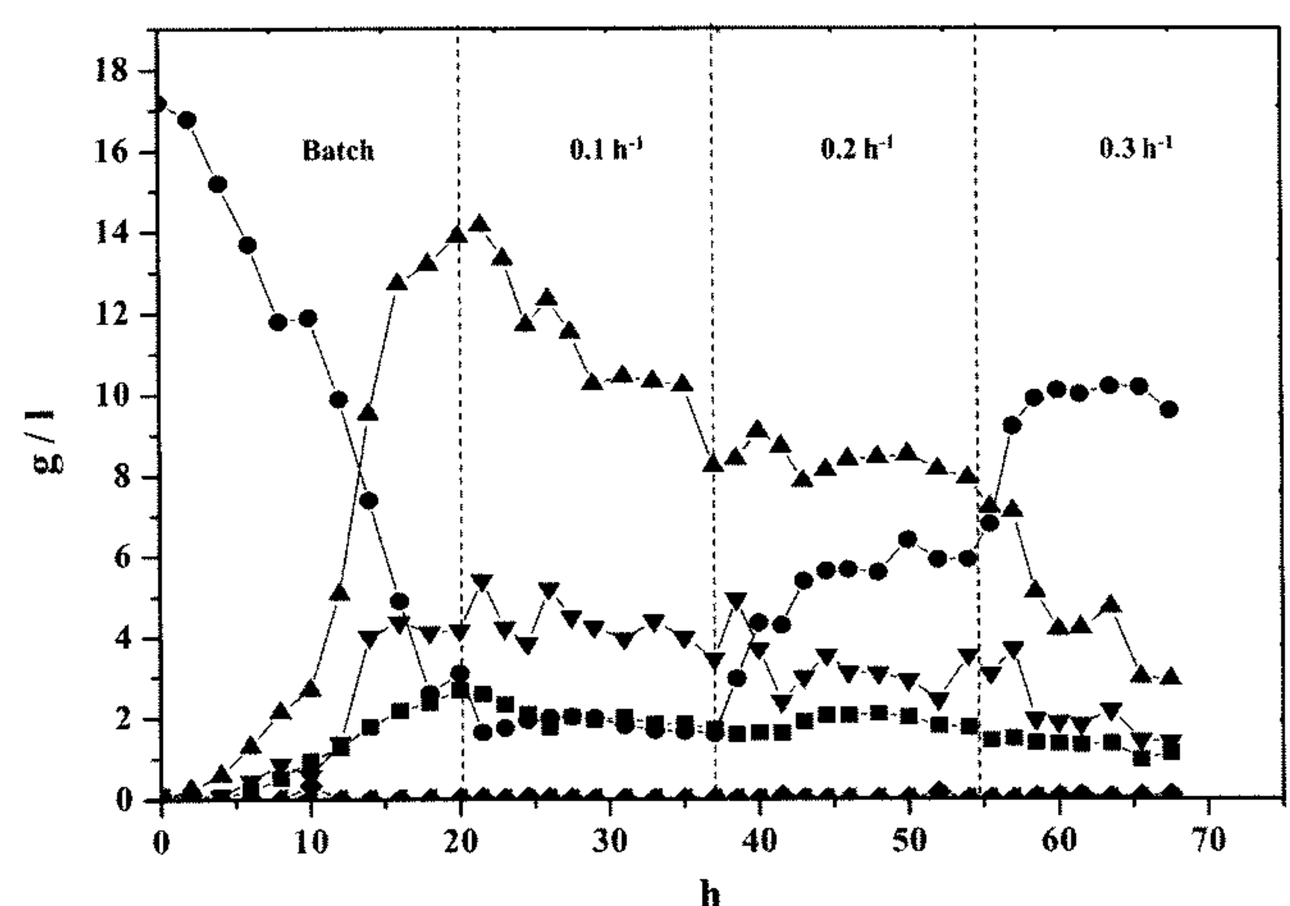

Fig. 2. Continuous fermentation profiles of *M. succiniciproducens* LPK7 at 100 mM glucose feeding concentration.

●, Glucose; ■, Cell concentration (OD_{610}); ▲, succinic acid; ▼, pyruvic acid; ◆, acetic acid; ◄, lactic acid.

Table 2. Comparison of flux distribution according to concentration of glucose fed to a continuous culture of LPK7 in steady-state.

Glucose feed concentration	Dilution rate (h^{-1})	Glucose flux	Glycolysis flux	PPP[b] flux	Biosynthesis flux (Gly[c])	Biosynthesis flux (PPP)	Secretion flux	Glycolysis flux ratio[a]	PPP flux ratio[a]	Biosynthesis flux (Gly) ratio[a]	Biosynthesis flux (PPP) ratio[a]	Secretion flux ratio[a]
9.0 g/l	0.1	0.0179	0.0053	0.0126	0.00179	0.00123	0.0111	29.6	70.4	10.0	6.84	62.0
	0.15	0.0314	0.0094	0.0221	0.00639	0.00227	0.0190	29.9	70.4	20.4	7.20	60.5
	0.2	0.0366	0.0108	0.0256	0.00831	0.00265	0.0205	29.5	70.0	22.7	7.25	56.0
	0.3	0.0370	0.0110	0.0259	0.0146	0.00361	0.0181	29.7	70.0	39.4	9.74	48.9
18.0 g/l	0.1	0.0177	0.0053	0.0124	0.00420	0.00604	0.0113	29.9	70.1	23.7	34.1	63.8
	0.2	0.0218	0.0085	0.0199	0.00715	0.00245	0.0155	39.0	91.3	32.8	11.3	71.1
	0.3	0.0365	0.0108	0.0255	0.0185	0.00245	0.0161	29.6	69.9	50.8	6.72	44.1

[a]Product flux/glucose flux ratio.
[b]Pentose phosphate pathway.
[c]Gly colysis.

was also considered, owing to the use of yeast extract as the nitrogen source, which includes some carbon content.

To determine the flux distributions and optimal conditions, continuous fermentation was performed at various dilution rates and glucose feed concentrations. The results are shown in Table 1, Fig. 1, and Fig. 2. With a glucose feed concentration of 9.0 g/l, the cell density remained at almost 2.0 g DCW/l. When using a dilution rate below 0.1 h^{-1}, the

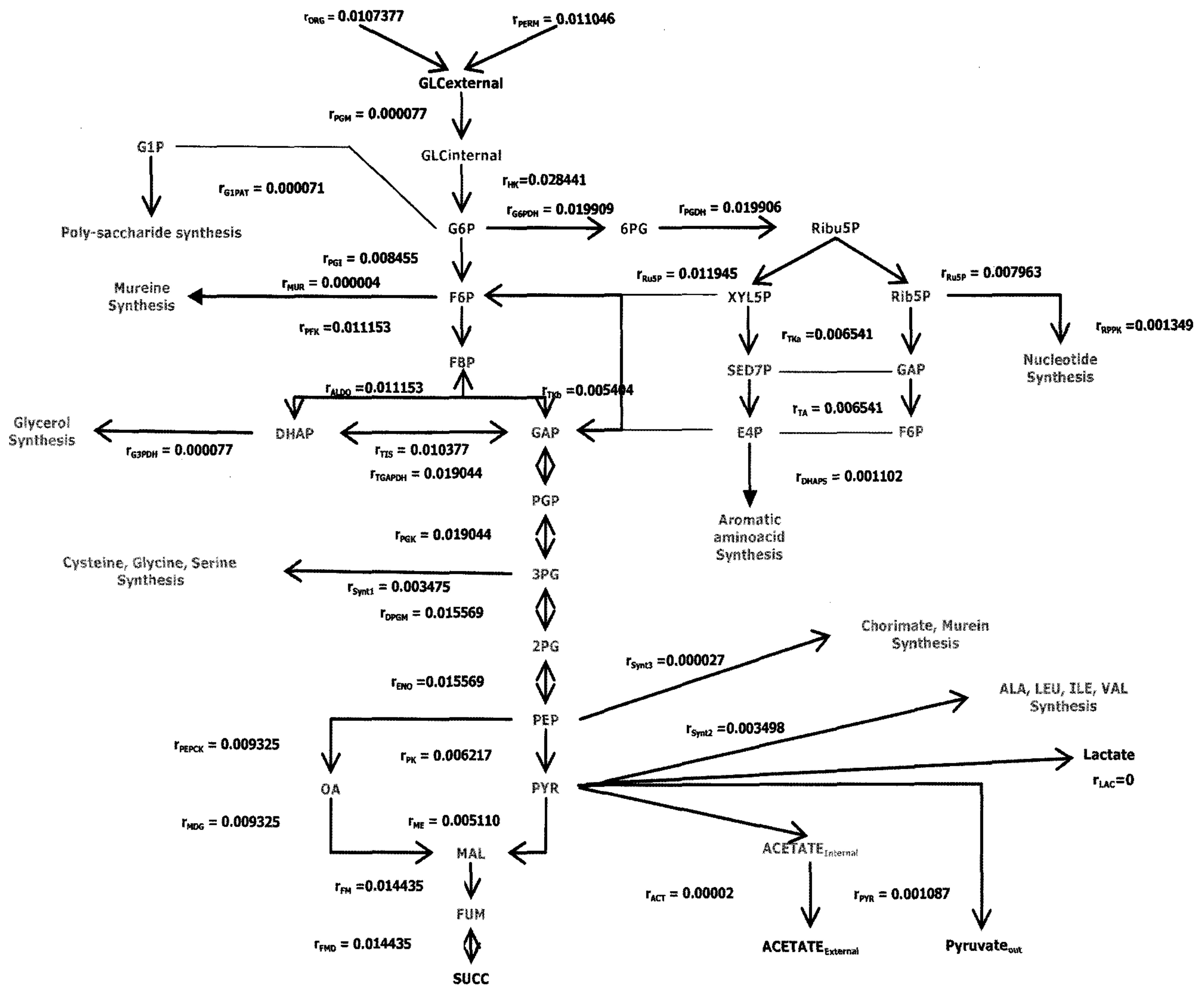

Fig. 3. Metabolic flux (g/h) distribution at a feeding glucose concentration of 18.0 g/l and 0.2 h^{-1} dilution rate.

glucose was completely consumed. However, at a dilution rate greater than 0.1 h^{-1}, glucose was accumulated in the medium and the succinic acid concentration and yield decreased, yet the productivity increased when increasing the dilution rate. With a glucose feed concentration of 18.0 g/l, the cell density also remained at 2.0 g DCW/l, and the results showed similar trends to those with a glucose feed concentration of 9.0 g/l. However, when using a dilution rate of 0.3 h^{-1}, the succinic acid concentration, yield, and productivity all decreased, as the dilution rate was greater than the maximum specific growth rate. When the glucose feed concentration was increased from 9.0 g/l to 18.0 g/l, the succinic acid yield decreased and glucose started to accumulate. Thus, the accumulated glucose seemed to act as an inhibitor, resulting in a lower succinic acid yield. In this experiment, the maximum succinic acid productivity was 1.77 g/l/h with a glucose feed concentration of 18.0 g/l and a 0.2 h^{-1} dilution rate. For the batch fermentation, the succinic acid concentration, productivity, and yield were about 15.0 g/l, 0.63 g/l/h, and 0.97 mol/mol, respectively. In previous batch fermentation results [13], the succinic acid concentration and yield were 13.4 g/l and 0.97 mol/mol, respectively. As seen in Table 1, the succinic acid concentration and yield from the continuous fermentation were analogous to the batch fermentation results, although the productivity of the continuous fermentation was much higher than that of the batch fermentation.

To determine the flux distributions, a metabolic flux analysis was performed, and the results are shown in Table 2. The glucose uptake rate, glycolysis flux, and pentose phosphate pathway flux all increased when increasing the dilution rate, although the ratio of glycolysis flux to glucose flux and the ratio of pentose phosphate pathway flux to glucose flux remained constant at about 30% and 70%, respectively, under the cultivation conditions. The biosynthesis fluxes in glycolysis and the pentose phosphate pathway increased when the dilution rate was increased, plus the ratio of biosynthesis flux to glucose flux increased with an increase in the dilution rate. As this experiment was carried out from a very low dilution rate and to a high dilution rate (similar to maximum specific growth rate), the cell growth rate also increased when increasing the dilution rate. Moreover, the metabolite secretion fluxes increased when the dilution rate was increased. However, the ratio of by-product secretion fluxes to glucose flux decreased when the dilution rate was increased, indicating that more carbon sources were consumed for biosynthesis rather than metabolite secretion when increasing the dilution rate.

In conclusion, this study showed a maximum succinic acid productivity of 1.77 g/l/h, where the optimal succinic acid production conditions were a glucose feed concentration of 18.0 g/l and a 0.2 h^{-1} dilution rate. Furthermore, since the continuous process was shown to produce a similar amount of succinic acid to the batch culture experiment, the optimal conditions can be applied to the industrial production of succinic acid.

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