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Bioconversion of Fumaric Acid to Succinic Acid by Recombinant *E. coli*

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ABSTRACT

Succinic acid was produced efficiently from fumaric acid by a recombinant *E. coli* strain DH5α/pGC1002 containing multicopy fumarate reductase genes. The effects of initial fumaric acid and glucose concentration on the production of succinic acid were investigated. Succinic acid reached 41 to over 60 g/L in 48.5 h starting with 50 to 64 g/L fumaric acid. Significant substrate inhibition was observed at initial fumaric acid concentration of 90 g/L. L-Malic acid became the major fermentation product under these conditions. Provision of glucose (5–30 g/L) to the fermentation medium stimulated the initial succinic acid production rate over two folds.

Index Entries: Recombinant *E. coli*; fumarate reductase; succinic acid; fumaric acid.

INTRODUCTION

Succinic acid is an intermediate of cellular metabolism. It has wide applications in agriculture, food, medicine, cosmetics, and polymer synthesis *(1)*. It is currently produced by chemical processes. Production of succinic acid through fermentation represents an alternative synthesis route via the utilization of renewable feedstocks. A strict anaerobe *Anaerobiospirillum succiniciproducens* has been employed to ferment glucose to a mixture of succinic acid and acetic acid *(2,3)*. Metabolic engineering methods have been used to create recombinant *E. coli* for enhanced succinic acid production from glucose by overexpression of the enzyme phosphoenolpyruvate carboxylase *(4)*. In addition to glucose, fumaric acid has been used as a substrate for the synthesis of succinic acid by recombinant *E. coli* strains with amplified fumarate reductase *(5)*. Although *A. succiniciproducens* is a good succinic acid producer, the operation of fermentation pro-

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cesses employing this strict anaerobe is not as easy as that employing the facultative organism *E. coli*. Since fumaric acid has been produced very efficiently from glucose at a weight yield of 85% *(6)*, we further explore bioconversion of fumaric acid to succinic acid as an alternative production method.

Fumarate reductase of *E. coli* is a complex flavoprotein enzyme bound to the cytoplasmic membrane *(7)*. It is composed of four nonidentical polypeptides A, B, C, and D. The 66-kDa A subunit contains a covalently bound flavin adenine dinucleotide prosthetic group. The 27-kDa B subunit contains catalytic iron-sulfur centers. The 15-kDa C and the 13-kDa D subunits bind the catalytic AB subunits to the inner surface of the cytoplasmic membrane. Fumarate reductase catalyzes the reduction of fumarate to succinate and is a key enzyme under anaerobic conditions when fumarate is the terminal electron acceptor *(8)*. It is structurally similar to, but functionally distinct from, the tricarboxylic-acid-cycle enzyme succinate dehydrogenase. The two enzymes catalyze the interconversion of fumarate and succinate with different substrate affinities and reaction rates *(9)*. The expression of fumarate reductase gene is induced, whereas that of the succinate dehydrogenase gene is repressed under anaerobic conditions *(8)*. The four genes *(frdABCD)* that code for the corresponding four fumarate reductase subunits have been cloned on a plasmid pGC1002 *(10)*. Total reductase activity was measured to be 31.2 U (μmol succinate oxidized/min at 38°C per 50 mg of cell protein) in a pGC1002-containing *E. coli* strain, whereas that of the host strain alone was only 8.1 U *(10)*.

Recombinant *E. coli* strains with amplified fumarate reductase activity have been shown to produce succinate from fumarate at significantly higher rates and yields than wild-type strain (molar yield ratio of 125 vs 17.6 in 4 d) *(5)*. Previous research mainly focused on the effect of cell concentration on the bioconversion of fumarate to succinate and the analysis of enzyme subunits produced. A detailed investigation of the effects of various levels of initial glucose concentration and initial fumaric acid concentration on succinic acid production have not been reported. In this study, a recombinant *E. coli* strain containing a multicopy plasmid (pGC1002) carrying the fumarate reductase genes *(frdABCD)* has been employed to convert fumaric acid to succinic acid. The effects of initial substrate and glucose concentrations on the bioconversion process have been examined.

MATERIALS AND METHODS

Strain and Plasmid

The laboratory *E. coli* strain DH5α was employed as the host strain in the study. Plasmid pGC1002 *(10)* is a pBR322-based multicopy vector containing the ampicillin-resistance gene and the fumarate reductase genes *frdABCD* coding for all four subunits of the enzyme complex.

E. coli Transformation

The plasmid pGC1002 was introduced into the host strain DH5α. The resulting recombinant *E. coli* strain was denoted as DH5α/pGC1002. Transformation of *E. coli* was performed as described by Sambrook et al. *(11)*.

Media and Cultivation

LB/amp medium for cell growth contains tryptone (10 g/L, Difco, Detroit, MI), yeast extract (5 g/L, Difco), NaCl (10 g/L), and ampicillin (50 mg/L). Fermentation medium contains $K_2HPO_4{\cdot}3H_2O$ (1.3 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (0.03 g/L), casamino acid (0.5 g/L, Difco), peptone (0.6 g/L), yeast extract (0.3 g/L), and various concentrations of glucose and fumaric acid neutralized with sodium hydroxide to pH 7.1–7.2.

Batch cultures for cell growth were conducted in 2-L Erlenmeyer flasks containing 500 mL LB/amp medium and were incubated in an incubator shaker at 37°C and 250 rpm. Batch cultures for bioconversion were conducted under microaerobic conditions in 50-mL Erlenmeyer flasks sealed with rubber plugs containing 25 mL fermentation medium and were incubated in an incubator shaker at 37°C and 80 rpm.

Analytical Methods

Fumaric acid, succinic acid, L-malic acid, acetic acid, glucose, and other organic acids were determined and quantified by HPLC with an Intelligent Pump (Hitachi, L-6200A), an Intelligent Auto Sampler (Hitachi, AS-4000, Tokyo, Japan), a Bio-Rad (Hercules, CA) Aminex HPX-87H ion-exclusion column (300 × 7.8 mm), a refractive index detector (Hitachi, L-3350 RI), and a Chromato-Integrator (Hitachi, D-2500). The column temperature was maintained at 60°C and the column was eluted with 5 m*M* sulfuric acid at a flow rate of 0.8 mL/min.

RESULTS AND DISCUSSION

The recombinant *E. coli* strain DH5α/pGC1002 was grown overnight to stationary phase in LB/amp medium. Cells were precipitated by centrifugation. An equal amount of cell mass (202.5 mg dry weight) was inoculated into each fermentation flask. Samples were taken at different time points during the fermentation and were analyzed by HPLC.

Figure 1 shows the profiles of the fermentation with initial fumaric acid and glucose concentrations of 50.8 and 23.7 g/L, respectively. The production of succinic acid was rapid; the volumetric production rate was 4.38 g/L/h for the first 6 h. The final succinic acid concentration reached 47.5 g/L at 32 h. The weight yield of succinic acid based on the amount of fumaric acid consumed was 0.93. The consumption of fumaric acid was

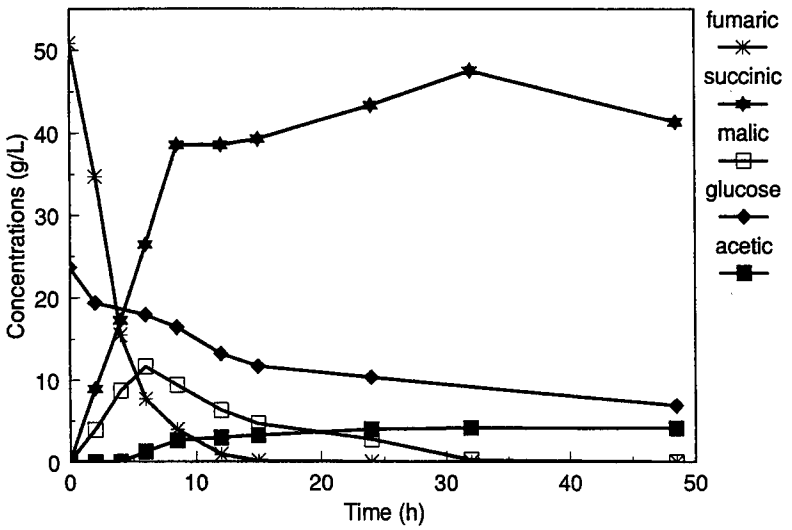

Fig. 1. General profiles of succinic acid fermentation from fumaric acid.

also fast with only 7.7 and 3.9 g/L of fumaric acid left at 6 and 8.5 h, respectively. Glucose was gradually consumed to 10.3 g/L at 24 h and 6.9 g/L at 48.5 h. L-Malic acid concentration first increased from zero to 11.7 g/L at 6 h and then gradually decreased to zero at 32 h. It is very likely that part of fumaric acid was initially converted to L-malic acid by a hydration reaction catalyzed by the enzyme fumarase and then L-malic acid was converted back to fumaric acid when the concentration of fumaric acid dropped to a very low level after 6 h. Acetic acid was also produced as a low-concentration byproduct in the bioconversion process. It was produced slowly after 4 h of incubation and its concentration finally accumulated to a level of about 4.1 g/L in the fermentation broth. The fermentation results are summarized in Table 1.

Effect of Initial Fumaric Acid Concentration

The effect of the initial fumaric acid concentration on the production of succinic acid by the recombinant *E. coli* strain DH5α/pGC1002 was investigated. The concentrations of initial glucose level were fixed at around 21–23 g/L. Four initial fumaric acid levels were examined: 50, 57, 64, and 90 g/L.

As shown in Fig. 2, the production of succinic acid was very rapid with initial fumaric acid concentrations of 50 to 64 g/L. At initial fumaric acid concentration of 90 g/L, however, the production of succinic acid was much slower. The initial volumetric production rates were 4.38, 4.32, 3.01, and 0.24 g/L/h in 6 h for initial fumaric acid concentrations of 50, 57, 64, and 90 g/L, respectively. Final succinic acid concentrations reached 41 to over 60 g/L for those fermentations started with 50 to 64 g/L fumaric acid.

Table 1
Summary of Fermentation Results Obtained at 32 h

Succinic acid concentration (g/L)	47.5
Succinic acid yield (g/g fumaric acid consumed)	93%
Specific productivity of succinic acid (g/g biomass•h)	0.183
Volumetric productivity of succinic acid (g/L•h)	1.48
Succinic acid : acetic acid (g/g)	11.4

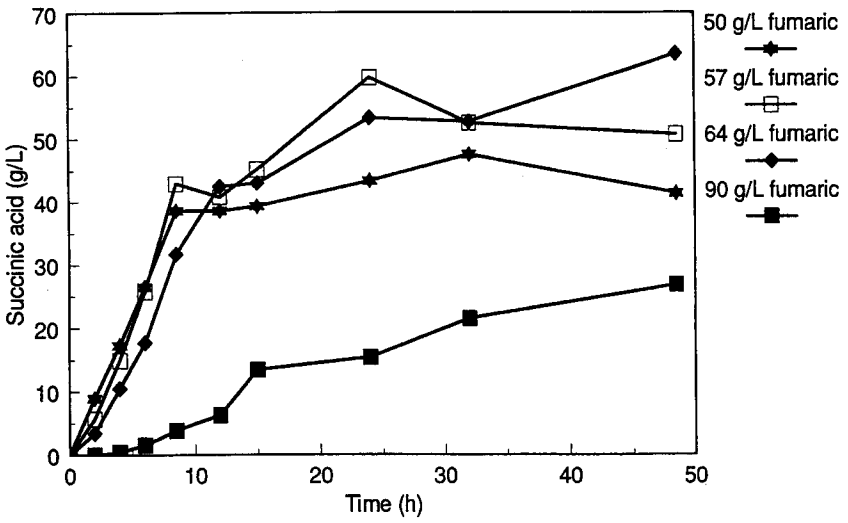

Fig. 2. Succinic acid production with various initial fumaric acid concentrations. The initial concentrations of glucose were approx 20 g/L.

When 90 g/L fumaric acid was used, substantial substrate inhibition effect was observed and final succinic acid concentration could only reach 26.8 g/L at 48.5 h.

As evident from Fig. 3A, the consumption rate of fumaric acid at initial concentration of 90 g/L was much slower than those at lower initial fumaric acid concentrations. Glucose profiles also indicated significant difference between the fermentation started with 90 g/L fumaric acid and those started with lower fumaric acid levels (Fig. 3B). L-Malic acid profiles were generally similar to that in Fig. 1 with one exception. For the fermentation with initial fumaric acid concentration of 90 g/L, L-malic acid continued to accumulate and became the major product at a final concentration of 49.7 g/L (Fig. 4A). Acetic acid finally accumulated to 1.4 g/L for the fermentation with 90 g/L initial fumaric acid, and to 4.1–4.4 g/L for the other three cases (Fig. 4B).

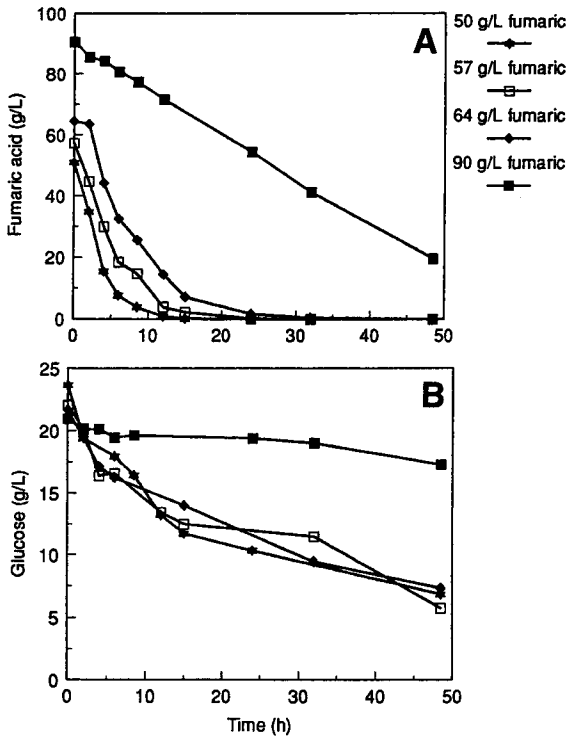

Fig. 3. Profiles of fumaric acid consumption **(A)** and glucose utilization **(B)** in the bioconversion of fumaric acid to succinic acid with various initial fumaric acid concentrations. The initial concentrations of glucose were approx 20 g/L.

Effect of Initial Glucose Concentration

The effect of initial glucose concentration on the bioconversion of fumaric acid to succinic acid was studied with initial glucose levels at 0, 5, 10, 20, and 30 g/L. The initial fumaric acid levels were fixed at around 50 g/L.

The production of succinic acid was similar for those fermentations started with at least 5 g/L glucose (Fig. 5). Their initial volumetric production rates ranged from 4.1 to 4.6 g/L/h in the first 6 h. Maximal concentrations of succinic acid achieved were from 41 to 46 g/L at 32 h. In contrast, the initial succinic acid production rate was 2.0 g/L/h and final succinic acid concentration only reached 27 g/L for the fermentation started with 0 g/L glucose (Fig. 5).

The profiles for fumaric acid and glucose consumption are shown in Fig. 6 and those for L-malic acid and acetic acid accumulation are shown in Fig. 7. L-Malic acid accumulated to 12.6 g/L for the fermentation started without glucose. For fermentations with at least 5 g/L initial glucose levels, L-malic acid eventually approached zero at the end of the incubation. Acetic

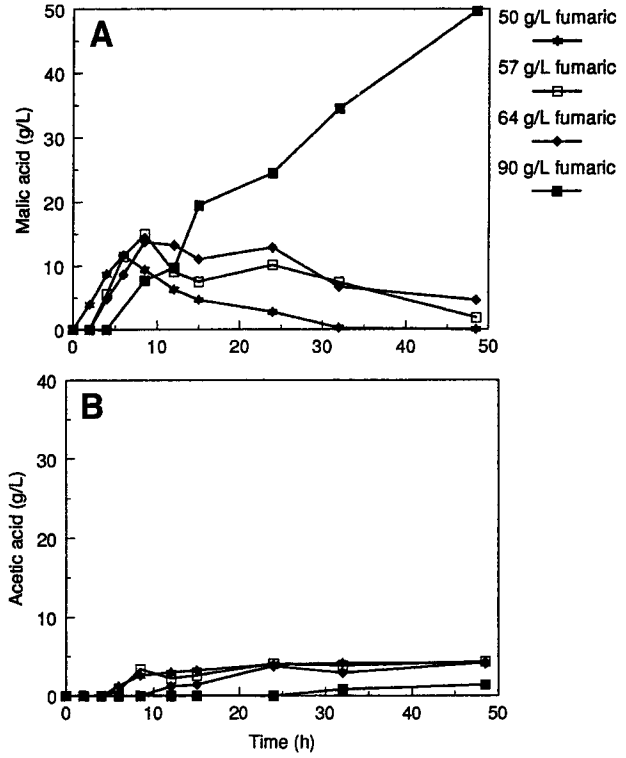

Fig. 4. Profiles of L-malic acid concentration **(A)** and acetic acid accumulation **(B)** in the bioconversion of fumaric acid to succinic acid with various initial fumaric acid concentrations. The initial concentrations of glucose were approx 20 g/L.

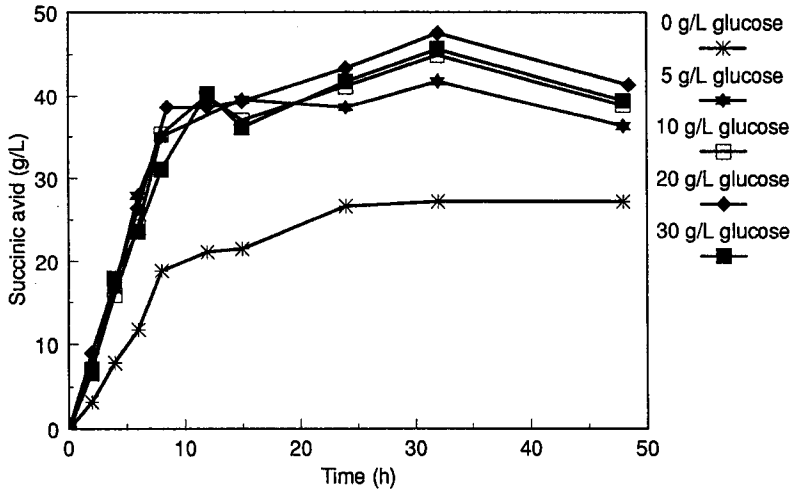

Fig. 5. Succinic acid production with various initial glucose concentrations. The initial concentrations of fumaric acid were approx 50 g/L.

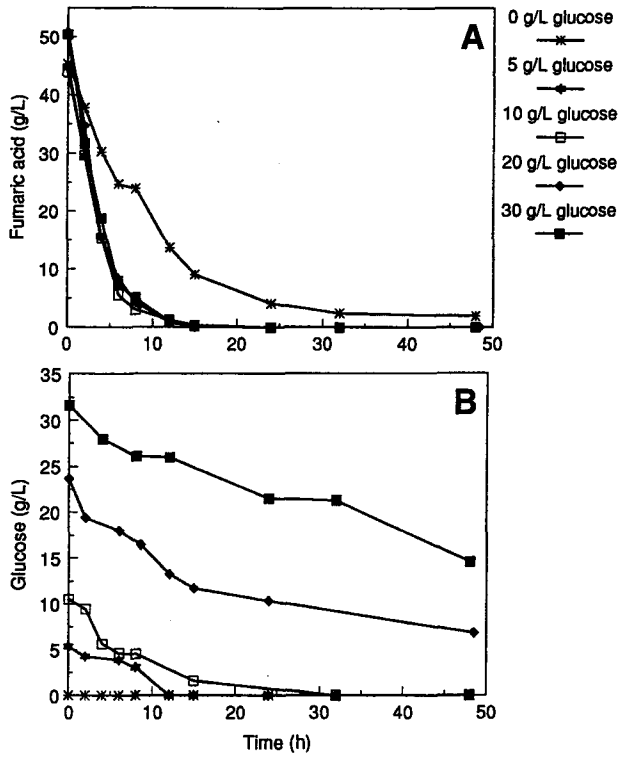

Fig. 6. Profiles of fumaric acid consumption **(A)** and glucose utilization **(B)** in the bioconversion of fumaric acid to succinic acid with various initial glucose concentrations. The initial concentrations of fumaric acid were approx 50 g/L.

acid was not detected for the fermentation started with 0 g/L glucose. For the other fermentations, final acetic acid levels ranged from 2.3 to 4.1 g/L.

The experimental data demonstrated that providing glucose, even at a concentration of 5 g/L, could substantially increase both the production rate and the final concentration of succinic acid in the fumaric acid to succinic acid bioconversion process. Further experiment performed at 2 g/L initial glucose level (with the same initial fumaric acid concentration) only allowed initial succinic acid productivity to reach 3.7 g/L/h, lower than that with 5 g/L initial glucose concentration. A control experiment employing a fermentation medium containing 10 g/L glucose and 0 g/L fumaric acid was also performed. No succinic acid was detected in the fermentation broth. Therefore, glucose alone was not converted to succinic acid. Glucose was possibly used as the hydrogen donor for the conversion of fumaric acid to succinic acid, it provided additional amount of the required cofactor $FADH_2$ to extend the reaction for succinic acid production. Glucose was also converted to acetic acid and possibly to some other cellular metabolites. Biomass was not changed at the end of the fermentation.

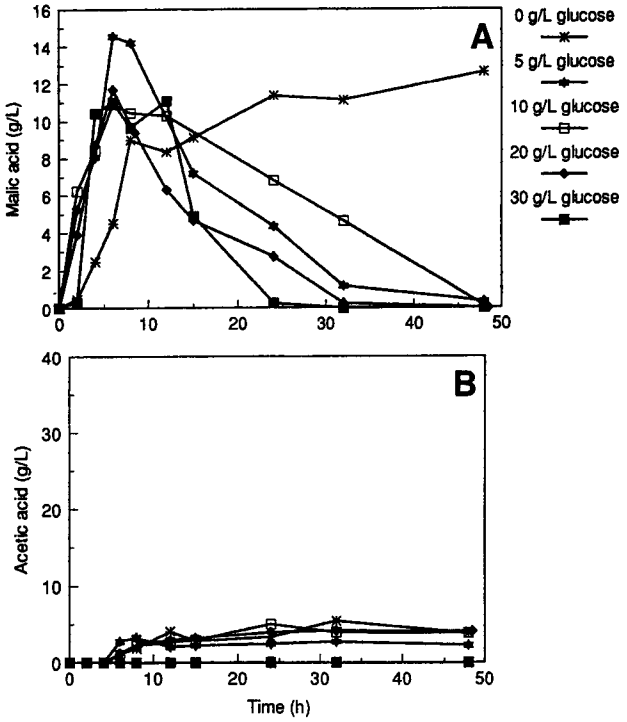

Fig. 7. Profiles of L-malic acid concentration **(A)** and acetic acid accumulation **(B)** in the bioconversion of fumaric acid to succinic acid with various initial glucose concentrations. The initial concentrations of fumaric acid were approx 50 g/L.

CONCLUSIONS

Fumaric acid was efficiently converted to succinic acid employing the recombinant *E. coli* strain DH5α/pGC1002. Initial succinic acid production rate could reach 4.3 g/L/h and final succinic acid concentration achieved 41 to over 60 g/L in 2 d under batch operations. For initial fumaric acid concentration over 64 g/L, substrate inhibition effect could be a problem. At 90 g/L initial fumaric acid level, L-malic acid, instead of succinic acid, became the major fermentation product. Fed-batch operations can be employed to optimize succinic acid bioconversion process. Glucose was observed to promote succinic acid bioconversion from fumaric acid. Over twofold increase in initial production rate was obtained by adding glucose (5–30 g/L) to the fermentation medium. Only slight difference in succinic acid synthesis was seen for initial glucose levels from 5 to 30 g/L. Acetic acid appeared to be a low-concentration byproduct in the bioconversion.

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