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Production of Fumaric Acid Using Rice Bran and Subsequent Conversion to Succinic Acid Through a Two-Step Process

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Abstract

The fungal production of fumaric acid using rice bran and subsequent bacterial conversion of succinic acid using fungal culture broth were investigated. Since the rice bran contains abundant proteins, amino acids, vitamins, and minerals, it is suitable material that fungi use as a nitrogen source. The effective concentration of rice bran to produce fumaric acid was 5 g/L. A large amount of rice bran caused excessive fungal growth rather than enhance fumaric acid production. In addition, we could produce fumaric acid without the addition of zinc and iron. Fungal culture broth containing approx 25 g/L of fumaric acid was directly employed for succinic acid conversion. The amount of glycerol and yeast extract required for succinic acid conversion was reduced to 70 and 30%, respectively, compared with the amounts cited in previous studies.

Index Entry: Fumaric acid; succinic acid; two-step process; fungal culture broth; rice bran.

Introduction

Succinic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle and also as one of the fermentation products of anaerobic metabolism. It has been considered an important chemical because it can be used for the precursor of 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone as well as for application in polymers, foods, pharmaceuticals, and cosmetics *(1,2)*. Currently, succinic acid is produced commercially through chemical synthesis. However, the production of

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succinic acid by biological processes has recently been the focus of attention as an alternative chemical feedstock.

For the biological production of succinic acid, various studies, such as those on bioconversion of fumaric acid to succinic acid *(3–6)* and production from carbohydrate *(7–10)*, have been conducted. Although succinic acid was largely produced by anaerobic bacteria, succinic acid yield was low. In addition, there are some problems with succinic acid fermentation, such as the requirement of high-purity CO_2 gas because the related microorganism is a strict anaerobe, byproduct (acetic acid) formation, and product inhibition *(7,8)*. Although some investigations of the conversion of fumaric acid into succinic acid have been reported *(3–6)*, succinic acid production from fungal culture broth containing fumaric acid has rarely been reported so far.

In the present study, we evaluated a two-step process for succinic acid production. The first process was fumaric acid production by *Rhizopus* sp. using rice bran, and the second process was succinic acid production by *Enterococcus faecalis* RKY1 *(5–7)* using fungal culture broth obtained in the first process. We investigated the effects of rice bran on fumaric acid production and optimized the culture medium for fumaric acid fermentation. Furthermore, we optimized the culture conditions for succinic acid conversion from fumaric acid produced by the first process.

Materials and Methods

Microorganisms and Inocula

Rhizopus sp., a newly isolated fungus from brown rice, was used for producing fumaric acid. It was cultivated and maintained on potato dextrose agar slants. Spores from a 5-d-old slant at 35°C were suspended in sterile water. This spore suspension (approx 8×10^5 spores/mL) was inoculated into fermentation medium at a level of 0.3% (v/v).

E. faecalis RKY1 was used for converting the aforementioned fungal culture broth into succinic acid. Stock cultures were stored in the culture medium with 50% (v/v) glycerol at –20°C. The growth medium consisted of 10 g/L of glycerol, 20 g/L of fumaric acid, 18 g/L of Na_2CO_3, 15 g/L of yeast extract, and 5 g/L of K_2HPO_4. To prepare the inoculum, 1 mL of the glycerol stock culture was transferred into a 20-mL vial containing 15 mL of growth medium and cultivated at 38°C, 200 rpm for 6 h.

Production of Fumaric Acid

To optimize the culture medium for fumaric acid production, we investigated the effects of rice bran concentrations and various carbon sources. When rice bran was used as a nitrogen source, the effects of additional elements (phosphate, magnesium, zinc, and iron) on fumaric acid production were also investigated. The medium previously reported by Zhou et al. *(11)* was used as the basal medium. Fermentations were performed in 250-mL Erlenmeyer flasks containing 100 mL of medium.

Fumaric acid for succinic acid conversion was prepared using a 2.5-L jar fermentor (KF-2.5L; Korea Fermentor, Incheon, Korea). The pH was adjusted to 4.5 with $CaCO_3$ or 5 *N* Na_2CO_3.

Succinic Acid Conversion

The fungal culture broth containing fumaric acid was used as the medium for succinic acid conversion. Prior to the use of fungal culture broth, the fungi were removed by filtration through Whatman No. 1 filter paper, and then the pH of the broth was adjusted to 7.0. Glycerol, nitrogen source, and phosphate were aseptically added to pretreated fungal culture broth. We investigated the effects of added glycerol concentrations as hydrogen donor and corn steep liquor (CSL) concentrations as nitrogen source. Furthermore, we investigated the inhibition of gradually concentrated fungal culture broth on succinic acid conversion. To study the inhibition of fungal culture broth on succinic acid conversion, we concentrated fungal culture broth with a rotary vacuum evaporator (N-1NW; Eyela, Kyoto, Japan) at 70°C. Two, three, and four times concentrated broth were tested.

Analytical Methods

Glucose concentration was measured bya glucose oxidase–peroxidase method using a glucose E-kit (Young-Dong Pharmaceuticals, Seoul, Korea). Cell growth was measured as optical density at 660 nm (OD_{660}) with an ultraviolet (UV) spectrophotometer (UV-160A; Shimadzu, Tokyo, Japan). Dry cell weight was determined using precalculated standard curves by substituting OD_{660} values.

Succinic acid and fumaric acid concentrations were quantified using a high-performance liquid chromatography (Waters, Milford, MA) with an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA) and a UV detector set at 210 and 260 nm, respectively. The mobile phase was 0.008 *N* H_2SO_4 at a flow rate of 0.6 mL/min. The temperature of the column was maintained at 38°C.

Results and Discussion

Production of Fumaric Acid

To investigate the effects of rice bran concentrations on fumaric acid production, various concentrations of rice bran ranging from 0 to 6 g/L were added to the medium. As shown in Fig. 1, the amount of glucose consumed was almost proportional to the rice bran concentration in the range of the experiment. When 6 g/L of rice bran was added, glucose was completely metabolized. Thus, it seemed that 1 g of rice bran was necessary to convert 9 g of glucose to fumaric acid. When a rice bran concentration above 6 g/L was used, fungal growth was predominant but an increase in fumaric acid production was not observed (data not shown). Since the culture pH dropped as fumaric acid was produced, it was necessary to add

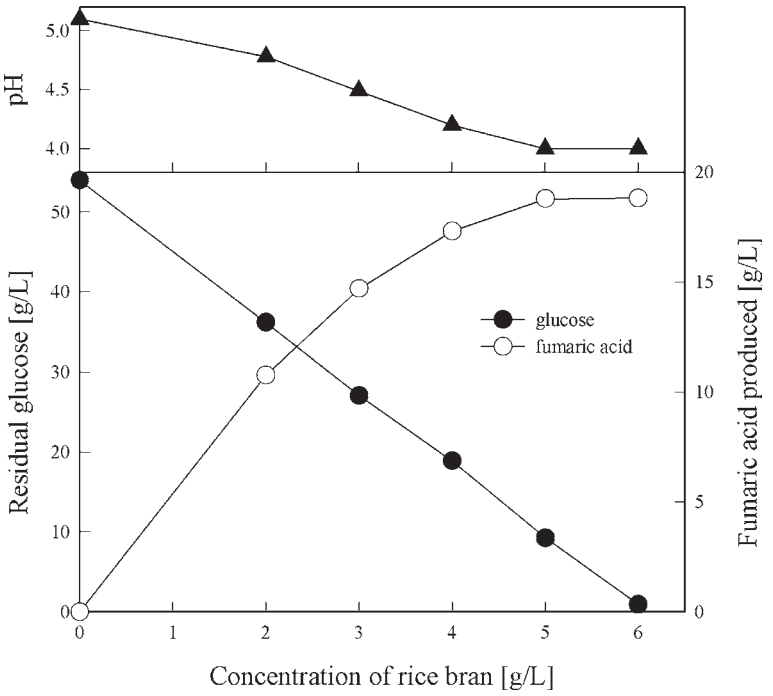

Fig. 1. Effect of rice bran concentration on fumaric acid production. The culture conditions were as follows: 250-mL flask containing 100 mL of medium at 35°C, 200 rpm for 4 d; 50 g/L of initial glucose; 15 g/L of $CaCO_3$; 0.6 g/L of KH_2PO_4; 0.5 g/L of $MgSO_4 \cdot 7H_2O$; 0.0179 g/L of $ZnSO_4 \cdot 7H_2O$; 0.498 mg/L of $FeSO_4 \cdot 7H_2O$.

more $CaCO_3$ to control the culture pH as a neutralizing agent. However, the amount of $CaCo_3$ did not influence fumaric acid production. Therefore, the optimal rice bran concentration for fumaric acid production was found to be 5 g/L.

To determine the optimal carbon source and its concentration on fumaric acid production, various carbon sources and their concentrations for fumaric acid production were examined. Figure 2 shows that *Rhizopus* sp. could metabolize glucose, fructose, maltose, and starch into fumaric acid. In view of industrial utilization, starch and rice bran might be used for economical carbon and nitrogen sources in the production of fumaric acid. Figure 3 shows that fumaric acid production was severely inhibited by the addition of high glucose concentration. Consequently, the optimum carbon source was found to be glucose, and maximum fumaric acid was obtained at 50 g/L of glucose.

Because rice bran contains various minerals, the effect of trace elements on fumaric acid production was investigated. Since the basal medium contained 0.6 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.0179 g/L of $ZnSO_4 \cdot 7H_2O$, and 0.498 mg/L of $FeSO_4 \cdot 7H_2O$, 16 trials were carried out to determine the effect of four minerals; the results are shown in Table 1.

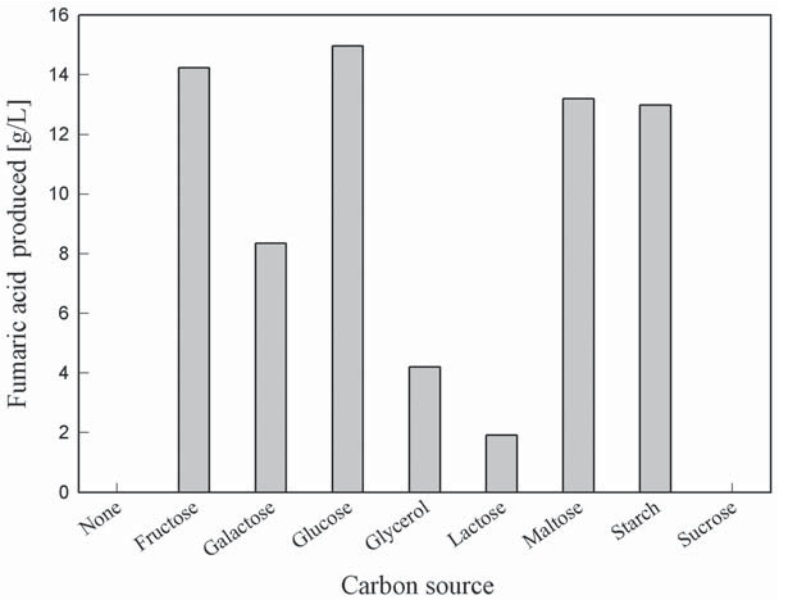

Fig. 2. Effect of different carbon sources on fumaric acid production. The culture conditions were as follows: 250-mL flask containing 100 mL of medium at 35°C, 200 rpm for 3 d; 50 g/L of initial carbon source; 5 g/L of rice bran; 15 g/L of $CaCO_3$; 0.6 g/L of KH_2PO_4; 0.5 g/L of $MgSO_4 \cdot 7H_2O$; 0.0179 g/L of $ZnSO_4 \cdot 7H_2O$; 0.498 mg/L of $FeSO_4 \cdot 7H_2O$.

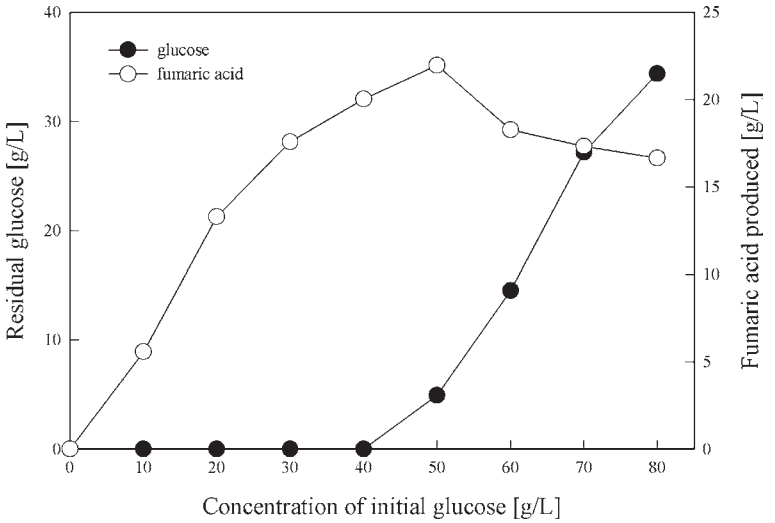

Fig. 3. Effect of initial glucose concentration on fumaric acid production. The culture conditions were as follows: 250-mL flask containing 100 mL of medium at 35°C, 200 rpm for 5 d; 5 g/L of rice bran; 15 g/L of $CaCO_3$; 0.6 g/L of KH_2PO_4; 0.5 g/L of $MgSO_4 \cdot 7H_2O$; 0.0179 g/L of $ZnSO_4 \cdot 7H_2O$; 0.498 mg/L of $FeSO_4 \cdot 7H_2O$.

Table 1
Effect of Phosphate, Magnesium, Zinc, and Iron on Fumaric Acid Production Using Rice Bran[a]

Trial	KH_2PO_4 (0.6 g/L)	$MgSO_4 \cdot 7H_2O$ (0.5 g/L)	$ZnSO_4 \cdot 7H_2O$ (0.0179 g/L)	$FeSO_4 \cdot 7H_2O$ (0.498 mg/L)	Residual glucose (g/L)	Fumaric acid produced (g/L)
1	○	○	○	○	1.19	22.49
2	○	○	○	×	2.09	22.72
3	○	○	×	○	3.56	21.69
4	○	×	○	○	2.35	21.76
5	×	○	○	○	6.45	7.78
6	○	○	×	×	2.67	22.71
7	○	×	○	×	1.51	22.30
8	○	×	×	○	5.85	21.33
9	×	○	○	×	10.16	8.28
10	×	○	×	○	5.80	6.65
11	×	×	○	○	6.64	9.49
12	○	×	×	×	6.02	19.84
13	×	○	×	×	5.42	7.77
14	×	×	○	×	12.66	8.21
15	×	×	×	○	16.44	6.34
16	×	×	×	×	12.47	8.58

[a] Culture conditions: 250-mL flask containing 100 mL of medium at 35°C, 200 rpm for 5 d; 50 g/L of initial glucose; 5 g/L of rice bran; 15 g/L of $CaCO_3$. ○, added ; ×, not added.

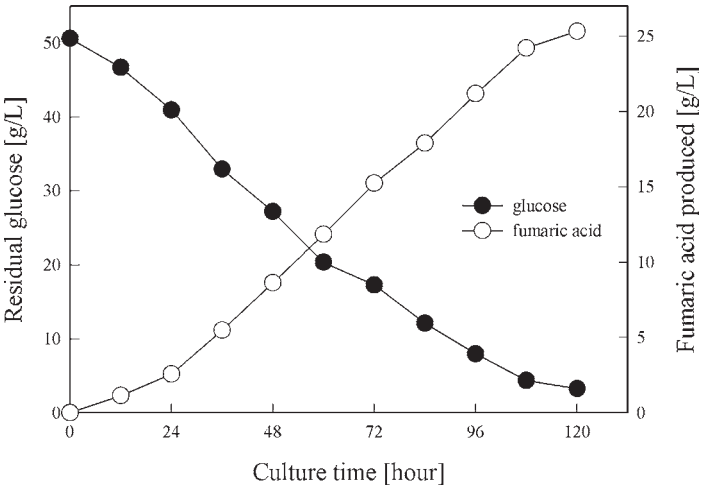

Fig. 4. Typical time course of fumaric acid production in fermentor by *Rhizopus* sp. The culture conditions were as follows: 2.5-L jar fermentor containinga 1-L working volume at 35°C, 200 rpm, 1 vvm, pH 4.5 adjusted with 5 *N* Na_2CO_3; 50 g/L of glucose; 5 g/L of rice bran; 1 g/L of KH_2PO_4; 0.5 g/L of $MgSO_4 \cdot 7H_2O$.

With the use of rice bran as a nitrogen source, we studied the effect of phosphate, magnesium, zinc, and iron on fumaric acid production. Although magnesium, zinc, and iron did not cause any effect (Mg^{2+}; trials 1 and 4, Zn^{2+}; trials 1 and 3, Fe^{2+}; trials 1 and 2), phosphate was crucial to fumaric acid production (trials 12 and 16).

Batch fermentations were carried out using rice bran and glucose as nitrogen and carbon sources, respectively. Figure 4 shows the profile of fumaric acid production in a 2.5-L jar fermentor. The fumaric acid concentration reached 25.3 g/L. The yield (fumaric acid produced/glucose consumed) and the productivity were 52% and 0.21 g/(L·h), respectively. For the following experiment, the fungal culture broth was used as a medium for the bioconversion of fumaric acid into succinic acid.

Bioconversion of Fumaric Acid into Succinic Acid

For the bioconversion experiments, we directly employed fungal culture broth containing fumaric acid as a basal medium. We optimized the culture medium for the production of succinic acid. Figure 5 represents the effects of glycerol on succinic acid production, fumaric acid consumption, and cell growth. When glycerol was not added to the culture medium, only 17 g/L the succinic acid was produced from the fungal culture broth. The quantity of glycerol added in the culture medium could be reduced to 70% compared with that of previous studies *(4–6)*. This result may imply that other hydrogen donors exist in fungal culture broth derived from fumaric acid fermentation.

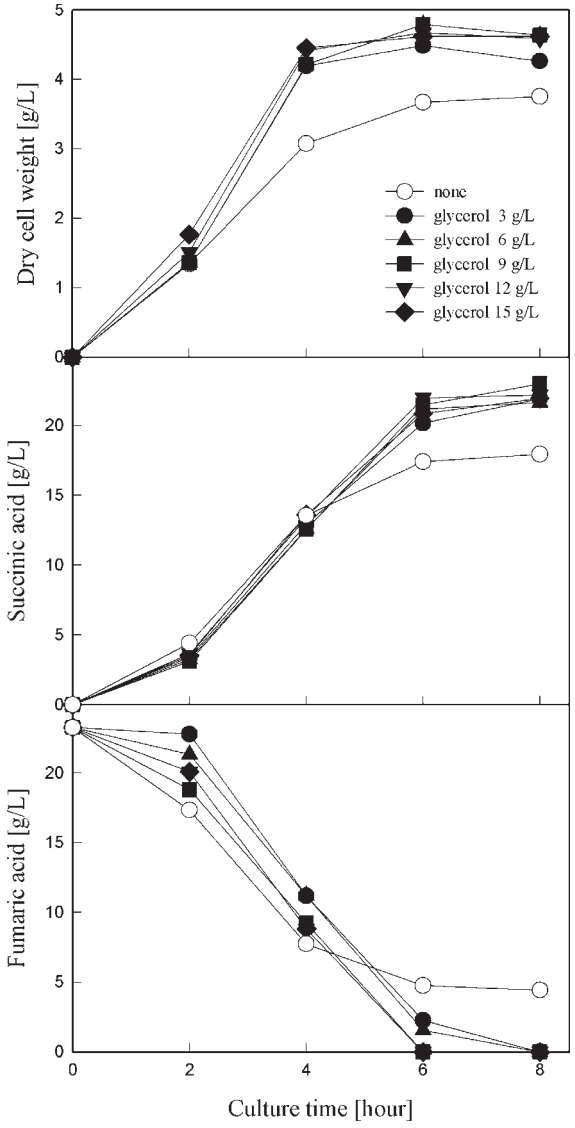

Fig. 5. Effect of glycerol concentration on succinic acid production, fumaric acid consumption, and cell growth. The culture conditions were as follows: 50-mL vial (40 mL of medium); 15 g/L of yeast extract; 5 g/L of K_2HPO_4; initial pH of 7.0.

To investigate the effect of yeast extract concentrations on succinic acid production, fumaric acid consumption, and cell growth, *E. faecalis* RKY1 was grown on culture medium containing from 0 to 20 g/L of yeast extract. As shown in Fig. 6, cell growth increased with increasing yeast extract. Succinic acid production and fumaric acid consumption were also enhanced by an increase in yeast extract up to 10 g/L, but they were not

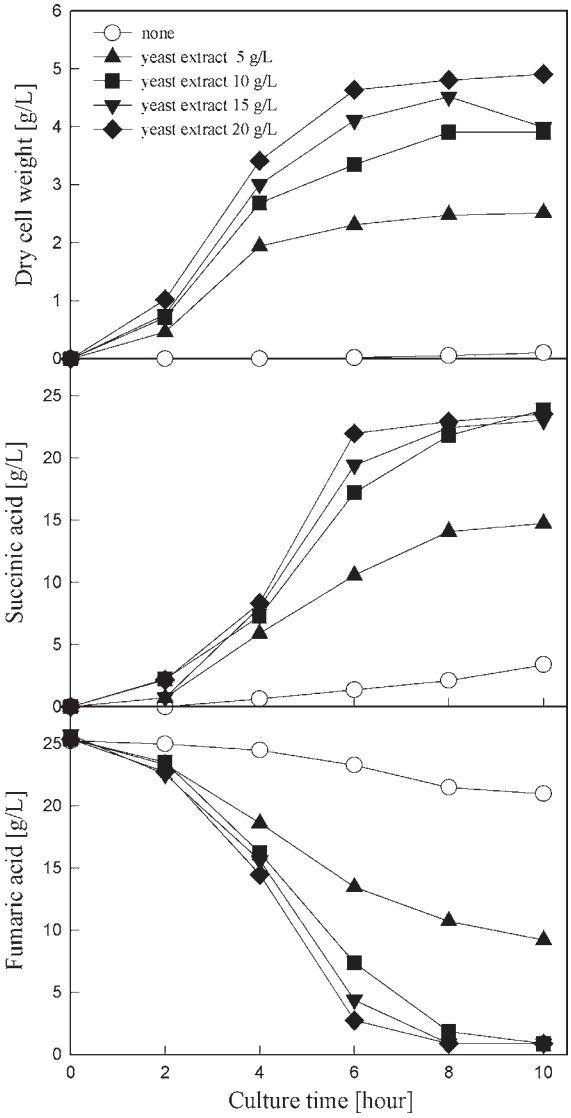

Fig. 6. Effect of yeast extract concentration on succinic acid production, fumaric acid consumption, and cell growth. The culture conditions were as follows: 50-mL vial (40 mL of medium); 3 g/L of glycerol; 5 g/L of K_2HPO_4; initial pH of 7.0.

significantly affected by yeast extract beyond 10 g/L. The amount of yeast extract could be reduced to approx 30% of that of previous reports *(4–6)* by using fungal culture broth. In this experiment, we could efficiently produce succinic acid from fungal culture broth containing approx 25 g/L of fumaric acid through the novel two-step bioconversion process. The maximum bioconversion yield and productivity were 97% and 2.4 g/(L·h), respectively.

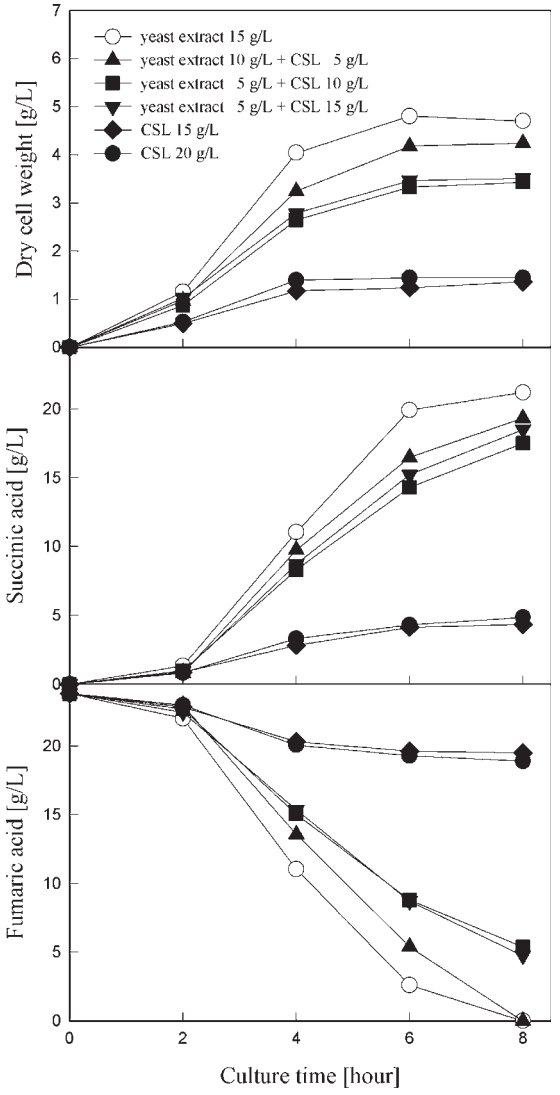

Fig. 7. Effect of addition of CSL on succinic acid production, fumaric acid consumption, and cell growth. The culture conditions were as follows: 50-mL vial (40 mL of medium); 3 g/L of glycerol; 5 g/L of K_2HPO_4; initial pH of 7.0.

Because yeast extract is very expensive, we studied the effect of CSL supplement in order to reduce the amount of yeast extract. CSL has been the focus of attention as an alternative nitrogen source *(8,12)*. As shown in Fig. 7, the bacterial conversion was very poor when only CSL was used as a nitrogen source. Even if yeast extract had a significant effect on cell growth, when 5 g/L of yeast extract and 15 g/L of CSL were used as nitrogen sources, a bioconversion yield and productivity of 95% and 2.2 g/(L·h), respectively, could be obtained.

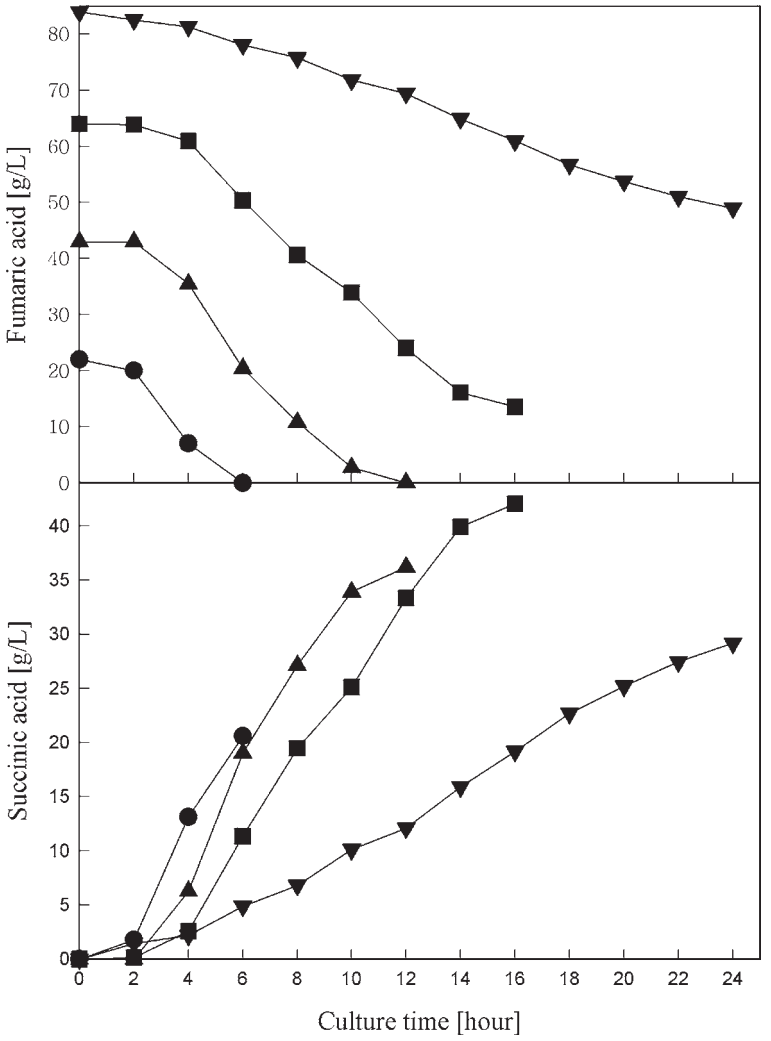

Fig. 8. Effect of broth concentration on succinic acid production and fumaric acid consumption. The culture conditions were as follows: 50-mL vial (40 mL of medium); 15 g/L of yeast extra; 5 g/L of K_2HPO_4; initial pH of 7.0. (–●–), Original broth; (–▲–), two-fold concentrated broth; (–■–), three-fold concentrated broth; (–▼–), four-fold concentrated broth.

Finally, we investigated the inhibition of concentrated fungal culture broth on succinic acid production and fumaric acid consumption. As shown in Fig. 8, we found that concentrated fungal culture broth slightly inhibited the bacterial conversion. Succinic acid could be efficiently produced from fungal culture broth until it was concentrated to three-fold (64 g/L of fumaric acid). However, the conversion time needed was severely prolonged when it was concentrated to more than four-fold (84 g/L of fumaric acid). Since *E. faecalis* RKY1 could efficiently convert fumaric acid

(to 100 g/L) into succinic acid *(5–6)*, the succinic acid conversion by concentrated fungal broth was not inhibited because the fumaric acid in the fungal culture broth was concentrated, but because other components in the broth were concentrated. Further investigations should be focused on the identification of inhibiting compounds in the fungal culture broth.

Conclusions

A novel two-step process was developed to produce fumaric acid using *Rhizopus* sp. and subsequently to convert the fungal culture broth containing fumaric acid into succinic acid using *E. faecalis* RKY1. Rice bran is a byproduct of the rice-milling process, however, the interest in rice bran has been focused on various fields of microbial propagation and enzyme and ethanol production because it contains various nutrients *(13–15)*. Therefore, rice bran was used as an additive to produce fumaric acid. First, we produced fumaric acid using rice bran and glucose. Fumaric acid was efficiently produced when 5 g/L of rice bran and 50 g/L of initial glucose were used as nitrogen and carbon sources, respectively, without the addition of zinc and iron. For the bioconversion of fumaric acid into succinic acid, the fungal culture broth was directly employed. Fumaric acid in fungal culture broth was efficiently converted into succinic acid with the addition of only 3 g/L of glycerol, 5 g/L of yeast extract, and 15 g/L of CSL. The bioconversion yield and productivity from fumaric acid to succinic acid were 95% and 2.2 g/(L·h), respectively. Our results suggest that this novel two-step process allows the respective production capacity to be controlled according to the commercial demand of fumaric acid and succinic acid.

Acknowledgment

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