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Enzymatic production of pyruvate from fumarate — an application of microbial cyclic-imide-transforming pathway

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Abstract

For the purpose of producing pyruvate from fumarate through microbial cyclic-imide-transforming pathway, various cyclic-imide-utilizing microorganisms were isolated from soil. Among them, strain g31 was the best producer and was identified as *Pseudomonas* sp. With the resting cells of the strain, the conditions were optimized for pyruvate production from fumarate. The cells cultivated in the medium containing 2% (w/v) of fumarate showed the highest production with sufficient yield. The optimized wet-cell concentration, pH and temperature of the reaction were 1% (w/v), pH 6 to 7, and 30°C, respectively. Aeration was found to be an effective factor, and vigorous shaking during the reaction mixture resulted in higher production. Under the optimized reaction conditions, 100 mM of fumarate was almost stoichiometrically converted into pyruvate (94 mM) in 24 h.

Keywords: Pyruvate; Fumarate; Cyclic-imide; TCA cycle

1. Introduction

The commercial demand for pyruvate has been increasing because of its use as an effective precursor in the synthesis of various drugs and agrochemicals as well as a component of cell culture media. Pyruvate has been prepared by both fermentation and enzyme-catalyzed reactions. Fermentation of glucose by a yeast *Torulopsis glabrata* has produced pyruvate in concentrations as high as 770 mM [1–3], but the yield based on added carbon source was relatively low (49%), and isolation of pyruvate from such complex fermentation broth is generally difficult and expensive to perform. Enzyme-catalyzed reactions with L-lactate as a substrate by L-lactate oxidase from *Pedicoccus* [4] or spinach glycolate oxidase transformed into a yeast [5,6] were also investigated. However, by-product hydrogen peroxide decomposes pyruvate to acetate, carbon dioxide and water, and lowered the production yield.

Cyclic-imide is an analog of hydantoin that is a well-known substrate for the enzymatic production of optically active amino acids. During the research of microbial hydantoin transformation, a novel pathway for microbial cyclic-imide transformation was found [7]. This pathway is widely distributed in microorganisms [8] and some microorganisms accumulate pyruvate as a product from succinimide via succinamic acid, succinate, fumarate and malate as successive intermediates. Based on this finding, an

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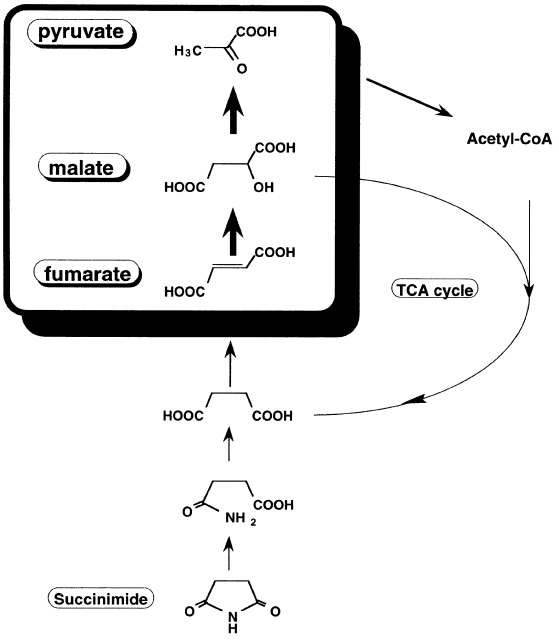

Fig. 1. Pyruvate production from fumarate by microbial cyclic-imide-transforming pathway.

attempt to establish a novel enzymatic process for pyruvate production by microbial cyclic-imide-transforming pathway was made. Fumarate, an intermediate of the microbial cyclic-imide transformation, was selected as a cheap starting material. Fumarate was supposed to be converted into pyruvate by an active metabolic conversion system of cyclic-imide-assimilating microorganism (Fig. 1).

2. Materials and methods

2.1. Isolation of cyclic-imide-utilizing microorganisms

The basal medium consisted of 1 g KH_2PO_4, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g yeast extract and 2 g NH_4Cl in 1 l of tap water, pH 7.0. Soil samples were cultivated with shaking at 28°C for 2 to 4 days in the basal medium supplemented with 0.15% (w/v) succinimide, glutarimide or phthalimide as a sole source of carbon. The cultured broth was streaked on a 2% (w/v) agar plate of the same medium and incubated at 28°C for 2 to 4 days. The colonies were isolated onto the same plate.

2.2. Screening of pyruvate-producing strain

The reaction mixture comprised, in 100 μl, 100 mM sodium fumarate, 100 mM Tris/HCl (pH 7.5) and the wet-cells (1% (w/v)) harvested from the plates for isolation. The reaction mixtures were incubated at 28°C for 24 h, and centrifuged at 4000 × *g* for 10 min. Pyruvate, fumarate, and other organic acids in the resultant supernatants were analyzed on high-performance liquid chromatography (HPLC) at 220 nm, fitted with a TSK-gel QAE-2SW packed column (4.6 × 250 mm; Tosoh, Japan), run at a flow rate of 1.0 ml/min with 67 mM potassium phosphate buffer (pH 6.4) as the eluent.

2.3. Optimization of pyruvate production by the resting cells of strain g31

For the optimization of the culture conditions, the basal medium supplemented with 0.30%, 1.0% or 2.0% (w/v) of glutarimide, succinimide, succinate or fumarate was used. Cultivations were carried out at 28°C for 12 to 100 h. For the optimization of the reaction conditions, the cells cultivated in the basal liquid medium supplemented with 2.0% (w/v) fumarate were used. The composition of the reaction mixture, in 2 ml, was the same as that for screening with the variations of wet-cell concentration (1–10% (w/v)), pH (pH 4.5–6.0, 100 mM acetate/sodium acetate; pH 6.0–8.0, 100 mM potassium phosphate; pH 7.5–9.5, 100 mM Tris/HCl; pH 9.0–11.0, glycine/NaOH), temperature (10–40°C), and shaking speed (0–160 rpm). Reactions were carried out for 12 or 24 h and then centrifuged. The resultant supernatants were analyzed for pyruvate and fumarate by HPLC as described above.

3. Results

3.1. Screening of pyruvate-producer in cyclic-imide-utilizing microorganisms

About 100, 250 and 200 isolates were obtained with succinimide, glutarimide and phthalimide as

Table 1
Effects of carbon sources on the pyruvate production by strain g31

Carbon source	% (w/v)	Pyruvate produced (mM)	Fumarate remained (mM)
Succinimide	2.0	22.5	14.7
	1.0	24.0	20.8
	0.3	2.8	65.4
Succinate	2.0	37.2	7.9
	1.0	25.9	22.5
	0.3	11.3	6.8
Fumarate	2.0	37.4	16.0
	1.0	23.8	10.1
	0.3	24.8	13.5

Cultivations were carried out with the basal medium supplemented with the carbon sources listed in the table. Reactions were carried out under the screening conditions with 100 mM sodium fumarate as a substrate except the reaction time 12 h.

sole carbon sources, respectively. Among them, 22 succinimide-isolates, 26 glutarimide-isolates and 5 phthalimide-isolates produced 5 to 10 mM pyruvate under the screening conditions. Nine glutarimide-isolates produced higher amount of pyruvate more than 10 mM, and one of the strain, g31, showed the highest production (31 mM). The strain g31 was selected for further investigation and identified as *Pseudomonas* sp. based on following taxonomic characteristics: shape, rods; size, 1.2–1.5 × 2.0–3.5 μm; mobility, motile; spores, not formed; Gram stain, negative; colonies on nutrient agar, circular, regular, entire, smooth, buff, low convex, semi-translucent; aerobic growth, obligatory aerobic; catalase, positive; oxidase, positive; OF test, negative.

3.2. Optimization of pyruvate production by the resting cells of strain g31

3.2.1. Culture medium and conditions

To improve the pyruvate production from fumarate by strain g31, carbon source in the culture medium was investigated. Glutarimide, succinimide and intermediates of succinimide metabolism, i.e., succinate and fumarate, were examined at various

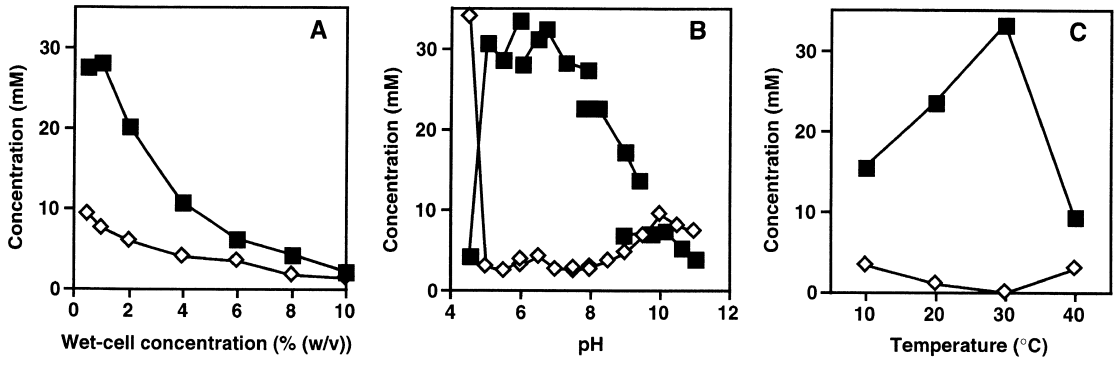

Fig. 2. Optimization of reaction conditions. (A) Wet-cell concentrations. Reactions were carried with various concentrations of wet-cells in 100 mM Tris/HCl (pH 7.5) at 30°C for 12 h. (B) pH. Reactions were carried with 1% (w/v) of wet-cells in various buffers described in Material and methods at 30°C for 12 h. (C) Temperature. Reactions were carried with 1% (w/v) of wet-cells in 100 mM potassium phosphate (pH 7.0) at various temperatures for 12 h. Closed and open square represents pyruvate and fumarate, respectively.

concentrations. Strain g31 showed poor growth with glutarimide, while grew well with succinimide, succinate and fumarate. As shown in Table 1, the cells cultivated in the medium containing 2% (w/v) fumarate showed the highest production. Optimum cultivation time was investigated with the medium containing 2% (w/v) fumarate and found that the cells maintained sufficient activity from 30 to 75 h of cultivation. In the following experiments, the resting cells obtained from 72-h culture broth with 2% (w/v) fumarate were used.

3.2.2. Reaction conditions

The reaction conditions for the pyruvate production from fumarate by the resting cells of strain g31 were examined.

3.2.2.1. Wet-cell concentration. As shown in Fig. 2A, the highest production was obtained with 1% (w/v) wet-cells. The pyruvate production decreased with increasing wet-cell concentration. Under higher wet-cell concentrations, pyruvate did not accumulate so much while fumarate much decreased.

3.2.2.2. Reaction pH. Optimum reaction pH was found to be pH 6.0–7.0 (Fig. 2B). Under acidic condition, fumarate did not transform so much, and under alkaline condition, pyruvate did not accumulate so much while fumarate decreased much.

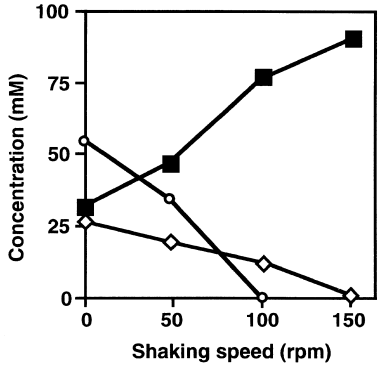

Fig. 3. Effects of aeration on pyruvate production. Reactions were carried with 1% (w/v) of wet-cells in 100 mM potassium phosphate (pH 7.0) at 30°C for 12 h with various shaking speeds. Closed and open square represents pyruvate and fumarate, respectively. Open circle represents malate.

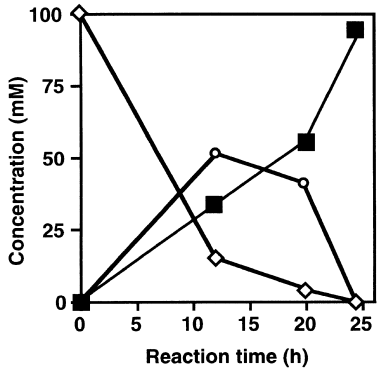

Fig. 4. Preparative production of pyruvate from fumarate. Reaction was carried out in 10 ml under optimized conditions for 24 h. Closed and open square represents pyruvate and fumarate, respectively. Open circle represents malate.

3.2.2.3. Reaction temperature. Optimum reaction temperature was found to be 30°C (Fig. 2C). The pyruvate production increased with increasing temperature, reaching maximum at 30°C, and decreased at 40°C.

3.2.2.4. Effect of aeration. The effect of aeration on the pyruvate production was tested by changing the shaking speed of the reaction mixture. As shown in Fig. 3, vigorous shaking brought about the production of large amounts of pyruvate. With gentle shaking, accumulation of malate, which is a possible intermediate from fumarate to pyruvate, was observed, while with vigorous shaking fumarate was almost completely transformed to pyruvate.

3.3. Preparative production of pyruvate from fumarate under the optimal conditions

The preparative production of pyruvate from fumarate in a total reaction volume of 10 ml was carried out under the optimal conditions: wet-cell concentration, 1% (w/v); pH 7.0, 100 mM potassium phosphate; temperature, 30°C; shaking speed, 100 rpm. As shown in Fig. 4, fumarate was totally transformed in 20 h with the production of both pyruvate and malate, and after 24 h malate was completely transformed to pyruvate, resulting to almost stoichiometric conversion of fumarate to pyruvate: pyruvate production, 94 mM (8.3 mg/ml).

4. Discussion

Pyruvate was effectively produced from fumarate by an active metabolic conversion system of a cyclic-imide-assimilating microorganism. Fumarate was transformed to pyruvate via malate through the similar reactions with those found in TCA cycle.

To obtain a high accumulation of pyruvate, controls of wet-cell concentration, pH and temperature of the reaction were important. Sufficient supply of oxygen by vigorous shaking was required for complete conversion especially for that of malate, an intermediate, to pyruvate.

This novel process for pyruvate production is a promising one in that it uses cheap starting material fumarate and brings high yield that is hardly achieved by fermentation methods.

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