Pyruvic Acid Production from 1,2-Propanediol by Thiamin-requiring *Acinetobacter* sp. 80-M

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For the purpose of producing pyruvic acid from 1,2-propanediol (PD), three PD-utilizing and thiamin-requiring microorganisms were isolated from soil. All the isolated strains were found to be pyruvic acid producers. Among them, strain 80-M was the best producer and was identified as an *Acinetobacter* sp. With this bacterium, the conditions were optimized for pyruvic acid production from PD by two methods: growing cell and resting cell methods. The amount of thiamin added to the medium remarkably affected the pyruvic acid production using either method. Under optimal conditions, 14.6 and 10.0 mg/ml of pyruvate as a sodium salt were produced from 20 mg/ml of PD by the growing and resting cell methods, respectively. In the resting cell system, only PD-grown cells showed a significant pyruvate productivity from PD.

There have been some reports on the microbial production of pyruvic acid from glucose,^{1~3)} gluconate,⁴⁾ lactate⁵⁾ and 1,2-propanediol (PD).⁶⁾ However, the amounts of pyruvic acid accumulated were unsatisfactory.

Only a few studies have used thiamin-requiring microorganisms, which produce pyruvic acid when cultivated in the presence of thiamin at a low concentration.^{2,4)} The cause of this pyruvate accumulation is most likely that thiamin diphosphate-dependent pyruvate dehydrogenase, responsible for pyruvate degradation, hardly functions owing to the coenzyme deficiency. Therefore, we considered that there might be thiamin-requiring microorganisms having high pyruvic acid productivity, and tried to isolate such organisms from natural sources. In the present work, we choose PD as a raw material for pyruvic acid production since the compound, an alcohol having the same carbon number as pyruvic acid, is one of the cheap petrochemicals.

The present paper deals with the isolation and identification of a PD-utilizing and thiamin-requiring bacterium which produced large amounts of pyruvic acid from PD, and

the optimization of the conditions for pyruvic acid production by two different methods, *i.e.*, a growing cell method and resting cell method, using the bacterium.

MATERIALS AND METHODS

Organisms. PD-utilizing and thiamin-requiring microorganisms were isolated from soil samples in Kyoto.

Chemicals. Kongo-amino-san® was the kind gift of Ajinomoto Co., Inc., Japan. Other chemicals were commercial products of reagent grade.

Media. The following media were used during this work. Medium P-I: 20 g PD, 3 g NH₄NO₃, 2 g K₂HPO₄, 1 g KCl, 0.2 g MgSO₄·7H₂O, 10 mg FeSO₄·7H₂O, 10 mg MnSO₄·4~6H₂O, 20 g CaCO₃ (precipitated) and 10 µg thiamin·HCl in 1 liter of distilled water, pH 7.0. Medium P-II: 3 g Polypepton (Daigo) replaced NH₄NO₃ in medium P-I. Medium G: 20 g glucose replaced PD in medium P-II and CaCO₃ was omitted. For a slant-culture medium 2% agar was added, and for a plate-culture medium 2% agar was added and CaCO₃ omitted. CaCO₃ was separately sterilized by dry heating. For the preservation of strains a slant-culture medium of medium G was used.

Isolation of PD-utilizing, thiamin-requiring organisms. Soil samples were cultivated with shaking at 28°C for 2 days in 5 ml of medium P-I. The cultured broth was

streaked on a plate of medium P-I and cultivated at 28°C for 3~4 days. The colonies were transferred to two different plates of medium P-I with and without thiamin. The strains, which grew only on the plate of the medium with thiamin, were transferred to slants of medium G and cultivated at 28°C for 2~3 days.

Cultivation for pyruvate production. Organisms were precultured in 5 ml of medium P-I or P-II at 28°C for 1 day with shaking (350 rpm). The precultured broth (1% (v/v) of main culture medium) was inoculated into the main culture medium. Cultivation was carried out at 28°C for 4 days with reciprocal shaking (350 rpm for test tubes and 120 rpm for flasks).

Preparation of resting cells of strain 80-M. The organism was precultured at 28°C for 1 day in 5 ml of medium P-II containing 0.5% instead of 0.3% Polypepton. The whole precultured broth was transferred to a 500-ml shaking flask containing 100 ml of the same medium and cultivated at 28°C for 2 days with shaking. The cultured broth was centrifuged at $800 \times g$ for 5 min at 0°C to remove CaCO_3 and then the supernatant was centrifuged at $10,000 \times g$ for 10 min at 0°C. The cells harvested were washed once with 0.85% NaCl solution, and suspended in the NaCl solution. The cell suspension, which was frozen at -20°C until use, was employed for resting cell reactions. One unit of OD_{610} of the suspension corresponded to 0.267 mg (as dry weight)/ml of cells.

Pyruvate production of resting cells. The standard reaction mixture contained 200 mg PD, 200 mg CaCO_3 (precipitated), 1 mmol potassium phosphate buffer (pH 7.0) and 26 mg (as dry weight) cells in a total volume of 10 ml in a 24-mm ϕ test tube. The reaction was performed at 28°C for 24 hr with reciprocal shaking (350 rpm).

Determination of pyruvic acid. Pyruvic acid produced in the cultured broth or reaction mixture was determined by the method of Friedemann and Haugen,⁷⁾ with sodium pyruvate as the standard.

Determination of growth. After 2 N HCl was added to the cultured broth to dissolve CaCO_3 (2 ml of the acid against 0.1 g CaCO_3), the optical density was measured at 610 nm.

RESULTS

Isolation of PD-utilizing and thiamin-requiring microorganisms

From about 300 PD-utilizing isolates from soil, three strains were found to require thiamin for growth. The strains were tested for pyruvic acid production in medium P-I. As a result, all three strains were found to produce

TABLE I. TAXONOMICAL STUDIES OF STRAIN 80-M

Morphological characteristics
Shape and size of cells: rods, $0.73 \sim 0.81 \times 1.5 \sim 3.7 \mu\text{m}$, predominantly in pairs
Motility: non-motile
Spores: not formed
Gram stain: negative
Aminopeptidase reaction*: positive
Cultural characteristics
Colonies on nutrient agar: white to pale orange, non-chromogenic, circular, smooth convex, and glistening
Liquefaction of gelatin: negative
Physiological characteristics
Aerobic growth: strictly aerobic
Reduction of nitrate: positive
VP test: negative
MR test: negative
Indole production: negative
Hydrogen sulfide production: negative
Hydrolysis of starch: negative
Urease reaction: positive
Oxidase reaction: negative
Catalase reaction: positive
Litmus milk: positive
Temperature for growth: 5~40°C; pH for growth: 5.5~9.0
Penicillin resistance: resistant to 10 units/ml of penicillin
Requirements for growth: thiamin
OF test: negative
Acid formation from carbohydrates: Acid was formed from galactose, mannose and dextrin.

* See ref. 8.

100 to 600 $\mu\text{g}/\text{ml}$ of a sodium salt. From these, strain 80-M, the best producer, was used for the following experiments after improving the productivity by mono-colony screening.

Identification of strain 80-M

The results of taxonomical studies of strain 80-M are summarized in Table I. From these results, we concluded that strain 80-M belongs to the genus *Acinetobacter* according to Bergey's Manual, 8th Ed.⁹⁾

Culture conditions for pyruvate production from PD by strain 80-M

To improve the pyruvate production from PD by strain 80-M, the following culture

FIG. 1. Effect of Thiamin on the Growth of Strain 80-M and Its Pyruvate Production from PD.

The organism was cultivated at 28°C for 4 days in a 24-mm ϕ test tube containing 10 ml of medium P-II, where the thiamin-HCl concentration was varied as indicated. ●, growth; ○, pyruvate produced; △, pH.

TABLE II. EFFECT OF NITROGEN SOURCE ON THE PYRUVATE PRODUCTION

The organism was cultivated at 28°C for 3 days in a 500-ml flask containing 100 ml of medium P-I.

Nitrogen source (0.3%)	Growth (OD ₆₁₀)	Pyruvate (mg/ml as Na salt)
None	0	0
NH ₄ NO ₃	2.83	3.18
NH ₄ Cl	0.83	0.13
(NH ₄) ₂ SO ₄	1.05	0.13
NaNO ₃	1.35	3.11
Urea	0.33	0
Polypepton	3.25	4.66
Casamino acids	2.13	0
Meat extract	2.72	0
Yeast extract	7.60	0

conditions were examined.

(a) *Effect of thiamin concentration.* As shown in Fig. 1, the growth of strain 80-M increased with increased amounts of thiamin-HCl up to 5 µg/liter, above which there was almost no change in growth. On the other hand, the level of pyruvate was maximal on addition of 10 µg/liter of thiamin-HCl; the addition of more than 20 µg/liter of

FIG. 2. Effect of PD and Polypepton Concentrations on the Growth and Pyruvate Production.

The PD and Polypepton concentrations of medium P-II were varied as indicated. Other culture conditions were the same as in Fig. 1. Polypepton concentration (%): ○, 0.2; ●, 0.3; △, 0.5; ▲, 0.75.

thiamin-HCl markedly decreased the pyruvate production.

(b) *Effect of CaCO₃.* The addition of more than 2% CaCO₃ was necessary for the best pyruvate production.

(c) *Effect of nitrogen source.* Among the nitrogen sources tested as shown in Table II, Polypepton was the most effective for pyruvate production. Nitrates such as NH₄NO₃ and NaNO₃ were also effective. In the following experiments, Polypepton was used as a nitrogen source.

(d) *Effect of PD and Polypepton concentrations.* The effect of PD and Polypepton concentrations on the growth and pyruvate production was examined. As shown in Fig. 2, the maximum level of pyruvate was achieved in a medium containing 2% PD and 0.75% Polypepton. The pyruvate production was suppressed when either of the substances was added to a concentration higher than these respective values.

(e) *Time course of pyruvate production.* Under the optimal conditions obtained in the above experiments, the time course of the pyruvate production was examined. As illustrated in Fig. 3, the growth increased slowly up

FIG. 3. Time Course of the Pyruvate Production from PD.

The organism was cultivated at 28°C in a 24-mm ϕ test tube containing 10 ml of medium P-II, where the Polypepton concentration was changed to 0.5%. ●, growth; ○, pyruvate produced; △, pH.

TABLE III. EFFECT OF METAL SALTS ON THE PYRUVATE PRODUCTION

The organism was cultivated at 28°C for 4 days in a 16.5-mm ϕ test tube containing 5 ml of medium P-II with 0.5% instead of 0.3% Polypepton. Metal salts were added to the medium at concentrations of 10 μ g/ml.

Metal salt	Growth (OD ₆₁₀)	Pyruvate (mg/ml as Na salt)
None	7.00	6.85
CaCl ₂ ·6H ₂ O	7.20	6.63
ZnSO ₄ ·7H ₂ O	6.52	3.12
CuSO ₄ ·5H ₂ O	7.03	7.62
CoCl ₂ ·6H ₂ O	7.08	8.53
AlCl ₃ ·6H ₂ O	7.21	3.50
H ₃ BO ₃	7.23	6.70
Na ₂ MoO ₄ ·2H ₂ O	7.26	7.39

to 5 to 6 days of incubation. The pyruvate production reached its maximum at the late logarithmic phase of growth with a subsequent gradual decrease in pyruvate.

(f) *Effect of metal salts.* Among the metal salts tested, the addition of CoCl₂ increased the pyruvate production by about 25% (Table III).

(g) *Effect of organic nutrients.* As indicated in Table IV, Kongo-amino-san, an acid-hydrolyzed soybean protein, had a stimulatory

TABLE IV. EFFECT OF ORGANIC NUTRIENTS ON THE PYRUVATE PRODUCTION

The organism was cultivated at 28°C for 4 days in a 16.5-mm ϕ test tube containing 5 ml of medium P-II with 0.5% instead of 0.3% Polypepton. Organic nutrients were added to the medium at concentrations of 1 mg/ml.

Organic nutrient	Growth (OD ₆₁₀)	Pyruvate (mg/ml as Na salt)
None	7.82	5.79
Corn steep liquor	8.20	0.18
Yeast extract	26.4	0.27
Meat extract	16.4	0.53
Malt extract	8.83	2.67
Casamino acids	7.64	2.04
Kongo-amino-san	8.40	7.79
Cotton seed flour	8.61	0.98
Kongo-amino-san + CoCl ₂ ·6H ₂ O (10 μ g/ml)	8.32	14.6

effect on pyruvate production, while other nutrients markedly decreased it. When 1 mg/ml of Kongo-amino-san and 10 μ g/ml of CoCl₂·6H₂O were both added to a medium, 14.6 mg/ml of pyruvate as sodium salt was produced.

Reaction conditions for pyruvate production by resting cells of strain 80-M

In a preliminary experiment, resting cells of strain 80-M were also found to produce pyruvate from PD. Therefore, the reaction conditions for the pyruvate production by resting cells were examined.

(a) *Effect of thiamin concentration in the culture medium.* The effect of the thiamin concentration in the culture medium on the pyruvate productivity of resting cells were examined. As shown in Fig. 4, resting cells grown on 10 μ g/liter of thiamin·HCl had the highest productivity.

(b) *Effect of incubation time and cell and PD concentrations.* As indicated in Fig. 5(A), the pyruvate production increased with an incubation time of up to 24 hr. The optimal cell concentration was 2.6 and 8 mg (as dry weight)/ml in 24 and 48 hr reactions, respec-

FIG. 4. Effect of Thiamin Concentration in the Culture Medium on the Pyruvate Production by Resting Cells of Strain 80-M.

The cultivation and reaction were carried out as described in MATERIALS AND METHODS except that the concentration of thiamin-HCl in the culture medium was varied as indicated.

FIG. 5. Effect of Incubation Time and Cell and PD Concentrations on the Pyruvate Production by Resting Cells.

The reaction conditions were the same as in Fig. 4 except that the incubation time (A), cell concentration (B) and PD concentration (C) were varied as indicated, respectively. In (B), incubation was carried out for 24 (●) and 48 hr (○).

tively (Fig. 5(B)). The optimal PD concentration was 20 mg/ml (Fig. 5(C)).

(c) *Effect of buffers and pH.* The effect of buffers with different pH values was examined. As shown in Fig. 6, the optimal pH for pyruvate production was around 8.5. In particular, a glycine-NaOH buffer of pH 8.5 had the best effect on the pyruvate production (10 mg/ml of pyruvate as sodium salt). It was confirmed that no pyruvate production occurred from glycine.

(d) *Effect of aeration.* The effect of aeration on the pyruvate production was tested by

FIG. 6. Effect of Buffers and pH on the Pyruvate Production by Resting Cells.

The reaction was carried out at 28°C for 24 hr in 5 ml of the reaction mixture having the same composition as in Fig. 4 except that 3.6 mg (as dry weight)/ml of cells and 0.1 M of the indicated buffer were used.

Buffer: ×, Tris-maleate buffer; △, potassium phosphate buffer; ▲, KH₂PO₄-Na₂B₄O₇ buffer; ●, Tris-HCl buffer; □, NH₄OH-NH₄Cl buffer; ○, glycine-NaOH buffer.

FIG. 7. Effect of Aeration on the Pyruvate Production by Resting Cells.

The reaction was carried out at 28°C for 24 hr in a 16.5-mm φ (●) or 24-mm φ test tube (○) containing the indicated volume of reaction mixture with the same composition as in Fig. 4 except that 0.1 M glycine-NaOH buffer of pH 8.5 and 3.9 mg (as dry weight)/ml of cells were used.

changing the volume of the reaction mixture. As shown in Fig. 7, vigorous aeration brought about the production of large amounts of pyruvate.

(e) *Pyruvate production from PD of resting cells grown on various carbon sources.* The pyruvate production of resting cells grown on various carbon sources was examined to see

TABLE V. PYRUVATE PRODUCTION FROM PD
BY RESTING CELLS GROWN ON
VARIOUS CARBON SOURCES

The organism was cultivated in medium P-II containing Polypepton (0.5%) and the indicated carbon source (2%). Four milliliters of the reaction mixture, having the same composition as in Fig. 7, was incubated in a 24-mm ϕ test tube at 28°C for 24 hr.

Cells grown on	Pyruvate (mg/ml as Na salt)
None	0.99
1,2-Propanediol	8.99
1,3-Propanediol	0.36
Glucose	0
Ethanol	0
Glycerol	0.99
Sucrose	0
Fructose	0

whether the enzyme system of the pyruvate production from PD in the organism was inducibly or constitutively produced. Table V indicates that only the cells grown on PD showed a marked pyruvate productivity, while those grown on other carbon sources showed no or low productivity.

DISCUSSION

Interestingly, all the thiamin-requiring strains isolated from soil have been found to be pyruvate producers, as we expected, although the number of strains was small. Using strain 80-M, the present work also confirmed that thiamin plays an important role as a regulating factor for pyruvate production in both the growing cell and resting cell methods: the organism showed maximum pyruvate productivity when cultivated at 10 μ g/ml of thiamin·HCl, while at higher concentrations of the vitamin, the productivity decreased significantly.

A method for pyruvate production using resting cells was demonstrated here for the first time. When the two methods, growing cell and resting cell, were compared, the maximum amount of pyruvate produced was larger using the growing cell method. However, the py-

ruvate produced was more stable in case of the resting cell method. This may be because such substances as thiamin, amino acids and vitamin B6 compounds which are closely related to the pyruvate metabolism, were absent in the reaction mixtures in the resting cell system. The maximum amounts of pyruvate obtained using the growing cell and resting cell methods were 14.6 and 10.0 mg/ml as a sodium salt, respectively: the molar conversion ratios of PD to pyruvate were 50 and 34%, respectively. These values are higher than those obtained by a growing cell method using *Corynebacterium* sp.⁶⁾

Some papers^{10~14)} have appeared concerning the aerobic metabolism of PD in bacteria. These papers suggest the oxidation sequence of PD to pyruvate under strongly aerobic conditions as follows: PD $\rightarrow$ acetol (or lactaldehyde) $\rightarrow$ lactate $\rightarrow$ pyruvate. The enzyme, PD dehydrogenase, is known to be induced by PD.^{10,12,13)} The present work has demonstrated that a remarkable amount of pyruvate was produced from PD only by PD-grown cells, suggesting the existence of an inducible PD-dehydrogenating enzyme in strain 80-M as well.

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