Rapid Paper

Production of Isobutene by
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In the course of studies on the microbial production of lower olefin hydrocarbons, we found that isobutene was produced by *Rhodotorula* yeasts, especially *R. minuta* var. *texensis* IFO 1102. The addition of L-leucine to a yeast culture stimulated the isobutene production. L-Phenylalanine and L-tyrosine had a synergistic effect with leucine on the production. The biochemical origin of isobutene produced by the yeast was estimated to be 3-methylcrotonyl CoA, an intermediate in the catabolic pathway of L-leucine.

It is well known that ethylene is produced by many microorganisms under aerobic conditions,¹⁾ but there have been few reports on the microbial production of other gaseous hydrocarbons. One of them was on the production of propane, propylene and *n*-butane by the cultivated mushroom, *Agaricus bisporus*²⁾ and the others were on the production of traces of hydrocarbons having 1 to 4 carbon atoms under anaerobic conditions by sewage or enrichment cultures of certain methanogenic bacteria.^{3,4)} From the viewpoint of the global shortage of crude oil deposits, we are attempting to develop a process for the microbial production of gaseous hydrocarbons under aerobic conditions from various reproducible resources and wastes.

As reported previously,^{5,6)} we found that hydrocarbons having 2, 3 and 4 carbon atoms were produced by many kinds of microorganisms under aerobic conditions. In this study, we focused our attention on the production of isobutene by strains of the genus *Rhodotorula*.

MATERIALS AND METHODS

1. Cultivation methods. *Rhodotorula* yeasts were cul-

tivated in 500-ml Erlenmeyer flasks containing 50 ml of medium. Two kinds of medium were used: medium A was 20 g glucose, 3 g meat extract, 5 g peptone, 2 g NaCl, 2 g yeast extract and 1,000 ml deionized water, pH 6.0; medium B was 20 g glucose, 5 g (NH₄)₂SO₄, 1 g KH₂PO₄, 1 g MgSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g NaCl, 0.5 mg *p*-aminobenzoic acid, 0.5 mg thiamine-HCl, 50 mg Na-citrate, 30 mg MnCl₂·4H₂O, 20 mg ZnCl₂, 20 mg FeCl₂·6H₂O, 2 mg CuSO₄·5H₂O, 2 mg CoCl₂·6H₂O, 1 mg Na₂MoO₄·2H₂O, 1 mg K₂B₄O₇·xH₂O and 1,000 ml deionized water, pH 6.5. After inoculation into medium A, the flasks were placed on a rotary shaker (180 rpm with 7-cm strokes) at 25°C for 2 days. When the resting cells were used, the cells were cultivated in medium B for 2 days, collected by centrifugation at 1,000×g for 10 min, and then washed twice with 33 mM Sørensen buffer, pH 6.0. The cells were then resuspended in the same buffer solution.

2. Analytical method for isobutene. Culture broth (1 ml) or resting cell suspension (0.5 ml) was transferred to a sterile test tube (18-mm diameter, 34-ml capacity). The test tube was sealed with a sterile rubber stopper and incubated on a reciprocal shaker at 25°C for 5 to 10 hr. Isobutene contained in 1-ml samples, which were withdrawn from the atmosphere in the sealed test tube, was determined by gas chromatography on an activated alumina column (4 m, 60 to 80 mesh), operated at 150°C with nitrogen (40 ml/min) as carrier gas. The production rate of isobutene (nl/ml culture broth/hr) was calculated by the same methods as previously described.⁵⁾

3. Identification of isobutene. Usually, isobutene was identified by comparison of the retention time of a gas sample with that of an authentic sample. Isobutene was verified with other types of column packing such as Bond-GC/PIC (Wako Pure Chemical Ind. Ltd., Osaka) and Unibeads 2S (Gaskuro Kogyo Inc., Tokyo). Also, it was verified by paper chromatography after absorption into 0.1 M mercuric acetate in methanol from the gas sample.⁷⁾ The solvent system used for paper chromatography was butanol-1/95% ethanol/28% ammonia (8:1:3). Two spots were detected, the major one had the same *R_f* value as that of authentic isobutene and that of the minor one corresponded to that of authentic ethylene. From the above two experiments, we concluded that isobutene was contained in the gas produced by *R. minuta* IFO 1102.

4. Cell concentration. Cell concentration was determined from the optical density at 660 nm.

RESULTS AND DISCUSSION

Table I shows the production rates of isobutene by 18 strains of the genus *Rhodotorula*, including 5 species. The best strain for the production of isobutene was *R. minuta* var.

FIG. 1. Effect of Amino Acids on the Production of Isobutene by *Rhodotorula minuta* IFO 1102.

a) By growing cells: Each amino acid (final concentration of 0.5 g/liter) was added to medium B. After 2 days cultivation, the culture broth (1 ml) was transferred to a sterile test tube. The test tube was sealed with a sterile rubber stopper and incubated on a reciprocal shaker at 25°C for 5 to 10 hr. Isobutene was determined in 1-ml samples of the atmosphere in the sealed test tube by gas chromatography on activated alumina.

b) By resting cells: After 2 days cultivation with medium B, a volume of the broth was centrifuged at $1,000 \times g$ for 10 min and the cells were resuspended in a half volume of 33 mM Sørensen buffer solution, pH 6.0. The cell suspension (0.5 ml) was transferred to a test tube containing the amino acid solution (0.5 ml, final concentration of 0.5 g/liter). The incubation conditions were the same as those in a).

TABLE I. PRODUCTION OF ISOBUTENE BY *Rhodotorula* YEASTS

Each *Rhodotorula* strain was cultivated in medium A for 2 days. Trace denotes a production rate of from 0.01 to 0.05 (nl/ml/hr).

Strain	Production rate (nl/ml/hr)	
<i>Rh. glutinis</i> IFO 0389	Trace	
<i>glutinis</i> IFO 0697	0.3	
<i>glutinis</i> IFO 1501	ND	
<i>glutinis</i> IFO 1503	ND	
<i>graminis</i> IFO 0190	Trace	
<i>marina</i> IFO 0928	ND	
<i>marina</i> IFO 1421	5.5	
<i>minuta</i> IFO 0387	1.7	
<i>minuta</i> IFO 0920	0.5	
<i>minuta</i> IFO 1435	3.0	
<i>minuta</i> var. <i>texensis</i> IFO 0879	1.0	
<i>minuta</i> var. <i>texensis</i> IFO 0932	2.5	
<i>minuta</i> var. <i>texensis</i> IFO 1006	1.6	
<i>minuta</i> var. <i>texensis</i> IFO 1102	16.4	
<i>minuta</i> var. <i>texensis</i> IFO 1434	Trace	
<i>minuta</i> var. <i>texensis</i> IFO 1540	0.3	
<i>minuta</i> var. <i>texensis</i> IFO 1541	1.3	
<i>rubra</i> IFO 0001	ND	

ND means not detected.

texensis IFO 1102 (production rate = 16.4 nl/ml/hr). Isobutene was produced in medium A but not in medium B (data not shown).

Specific rate of isobutene production (nl/ml/OD/hr)

FIG. 2. Effect of the Combination of Amino Acids on the Production of Isobutene by *Rhodotorula minuta* IFO 1102.

R. minuta IFO 1102 was grown in medium B for 2 days. After cultivation, cells were harvested by centrifugation at $1,000 \times g$ for 10 min and resuspended in 33 mM Sørensen buffer solution, pH 6.0. The cell suspension (0.5 ml) was transferred to a test tube containing each amino acid (each final concentration, 0.5 g/liter).

Then, we first tried to add separately 20 species of amino acid to medium B and also to the resting cell system (see Fig. 1). L-Leucine showed a strong effect on the production of isobutene and L-phenylalanine and L-tyrosine showed a slight effect.

Figure 2 shows the effect of the combination of leucine and aromatic amino acids on the

FIG. 3. Additional Effects of L-Phenylalanine on the Production of Isobutene by Intermediates in the L-Leucine Catabolic Pathway.

After 2 days cultivation in medium B, cells were harvested by centrifugation at $1,000 \times g$ for 10 min and resuspended in 33 mM Sørensen buffer solution, pH 6.0. The concentrated cell suspension (0.5 ml) was transferred to a test tube containing the compounds tested (final concentration of 1 mM).

□, without phenylalanine; ▨, with phenylalanine.

production of isobutene. As shown in the figure, the combination of leucine and phenylalanine was the most effective for isobutene production. These results suggest that the isobutene production is closely related to the catabolism of leucine and aromatic amino acids. When leucine and phenylalanine (each 1 g/liter) were added to medium B, the maximum rate of isobutene production was *ca.* 200 nl/ml/hr. Among aromatic compounds we also found that benzoic acid, L-phenylglycine and L-phenylpyruvic acid had a synergistic effect on the isobutene production (data not shown).

Generally, a part of the L-leucine catabolic pathway in microorganisms is as follows:

As shown in Fig. 3, α -ketoisocaproic and isovaleric acids markedly stimulated isobutene production, but 3-methylcrotonic acid did not. Nevertheless, we assume that isobutene is

formed by the decarboxylation of 3-methylcrotonyl CoA in the catabolic pathway of L-leucine, because exogenous 3-methylcrotonic acid might not be incorporated into the cells or the acid might not be activated by CoA. Furthermore, it is noteworthy that phenylalanine shows a synergistic effect with not only leucine, but also α -ketoisocaproic and isovaleric acids.

Aerobic conditions were necessary for the isobutene production from leucine, α -ketoisocaproic acid or isovaleric acid (data not shown). This phenomenon resembles the ethylene production from L-methionine *via* 1-aminocyclopropane-1-carboxylic acid by higher plants.¹⁾

This is the first case of microbial production of isobutene under aerobic conditions and, furthermore, it has become possible to develop a process for the production of isobutene by fermentation from many kinds of biomass resources such as animal feces, agricultural wastes and sewage sludges.

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