Production of β -Phenethyl Alcohol from Styrene by *Pseudomonas* 305-STR-1-4

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The productivity of β -phenethyl alcohol (β -PA) from styrene by *Pseudomonas* 305-STR-1-4 was examined under various conditions. Under the optimum condition, 3.27 mg/ml of β -PA was produced from 20 mg/ml of styrene. The metabolic inhibitor, pyrazole, aided in effectively producing β -PA more reliably and promptly.

β -PA was extracted from the culture broth and purified to a semi-viscous, light-yellow liquid with a rose-like fragrance. Recovery was 90% and purity was 99.4%. IR spectrum of this liquid was identical with that of authentic sample.

The mechanism of β -PA production by strain 305-STR-1-4 was proposed based on the results of this experiment.

Pseudomonas 305-STR-1-4, which was isolated from soil as a styrene assimilating bacterium, was shown to convert styrene into the product presumed as β -phenethyl alcohol (β -PA), as reported in previous paper.¹⁾ Sielicki *et al.*²⁾ obtained a mixed population of bacteria capable of utilizing styrene, and they detected β -PA in the incubation mixture. There is no other paper indicating microbial formation of β -PA from styrene.

The mechanism of microbial hydroxylation of styrene at the β -position of the side chain is not known. Two hypothetical mechanisms, however, can be proposed.

One is the addition of H_2O (*Mechanism I* in Fig. 1). The enzyme catalyzing this reaction is called hydratase.³⁾ More than 10 hydratases, such as fumarase, aconitase and enolase, have been found. Only one paper describes the hydration of alkene, in which *n*-decane-1-ol was anaerobically produced from *n*-1-decene with resting cells of *Candida* species.⁴⁾

The other mechanism is two-step oxidation via epoxide (*Mechanism II* in Fig. 1). Azoulay *et al.*⁵⁾ reported that crude enzyme of *Pseudomonas* catalyzed the production of *n*-heptane-1-ol from *n*-1-heptene, and they presumed epoxide as a metabolic intermediate. ω -Hydroxylation system of *Pseudomonas ole-*

ovans,^{6~8)} which forms a hydroxyl group at the terminal carbon of paraffins, is known to produce epoxides in the presence of olefins. This system requires NADH and molecular oxygen. Although this microorganism can produce epoxides, the epoxides can not be converted to alcohols. Styrene⁹⁾ was not epoxidized in this system.

In mammalian tissue, hepatic microsomes¹⁰⁾ produce epoxides from olefins, such as *n*-1-octene, *t*-*n*-4-octene, 3-2-ethylpentene, secobarbital, indene, naphthalene and styrene, and epoxides are transformed to the respective glycols by epoxide hydratase. Ohtsuji and Ikeda¹¹⁾ reported that styrene injected in a rat was metabolized via styrene oxide and styrene glycol.

The present authors have examined bacterial production of β -PA from styrene. This paper deals with the effect of various culture conditions on β -PA production and the presumable mechanism of its production.

MATERIALS AND METHODS

Materials. Styrene, medium components and other chemicals were obtained from commercial sources.

Microorganisms. Strain 305-STR-1-4 used in this

FIG. 1. Proposed Mechanisms for β -Phenethyl Alcohol Production by Strain 305-STR-1-4.

investigation is one strain of styrene assimilating bacteria which were isolated from soils and identified as *Pseudomonas* species, as described in the previous paper.

Cultural conditions. In all cases except special description, cultivations were carried out as follows. Two basal media (*Medium 1* and *Medium 2*), whose composition is shown in Table I, were used. Styrene was added to 50 ml of basal medium in a 500 ml flask at a concentration of 20 mg/ml. The medium was then inoculated with seed culture (2%, v/v) and reciprocally

TABLE I. COMPOSITION OF BASAL MEDIUM

The medium was made 1 liter with tap water. Initial pH was adjusted to 7.0 by 0.5 N NaOH or 0.5 N HCl after sterilization of medium at 120°C for 20 min.

Components	Medium 1	Medium 2
Nitrogen sources		
Polypeptone	1 g	—
Urea	—	5 g
NH ₄ NO ₃	1 g	—
Phosphates		
Na ₂ HPO ₄ · 12H ₂ O	10 g	1.5 g
KH ₂ PO ₄	5.5 g	1.5 g
Minerals		
MgSO ₄ · 7H ₂ O	0.2 g	0.2 g
CaCl ₂ · 2H ₂ O	10 mg	10 mg
FeSO ₄ · 7H ₂ O	1 mg	1 mg
Other nutrients		
Yeast extract	0.1 g	—
Corn steep liquor	0.1 g	0.2 g

shaken (120 rev/min, 7 cm amplitude) at 30°C for 3 days. The seed culture was prepared by incubating the strain in nutrient broth at 30°C for 24 hr.

Determination of cell growth. The culture broth was centrifuged at 10,000 rpm for 10 min after removal of excess styrene under reduced pressure. The precipitate was resuspended in an appropriate volume of distilled water. Cell concentration was determined by measuring the turbidity of the suspension at 610 nm with a Hitachi model 200-10 Spectrophotometer.

Gas liquid chromatography. Gas liquid chromatography (GLC) was performed by a Hitachi Gas Chromatograph 063 (10% PEG-20M, 60~80 mesh, 3 mm × 3 m, helium: 50 ml/min, 180°C, flame ionization detector). The retention time of styrene, styrene oxide, α -phenethyl alcohol (α -PA) and β -PA was 0.8, 1.85, 2.9 and 4.1 respectively. The quantity of styrene, styrene oxide and β -PA was calculated with a Shimadzu Chromatopac E-1-A, on the basis of peak area comparison with α -PA as the internal standard.

Infrared spectrum. The infrared (IR) absorption spectrum was taken with a Hitachi Infrared Spectrophotometer 215.

RESULTS

Culture conditions of β -phenethyl alcohol production

Concentration of styrene

Figure 2 shows the results of cultivation at

FIG. 2. Effect of Styrene Concentration on Production of β -Phenethyl Alcohol.

Cultivation was carried out in *Medium 1* at 30°C. ●—●, β -phenethyl alcohol (mg/ml); ○—○, growth (OD at 610 nm).

TABLE II. EFFECT OF NITROGEN SOURCES AND PHOSPHATE ON β -PHENETHYL ALCOHOL PRODUCTION

Nitrogen ^a sources	Phosphate ^b concentration	Final pH	Growth (OD at 610 nm)	β -PA (mg/ml)
NH ₄ NO ₃ (1 g/liter)	High	7.6	4.1	2.17
NH ₄ NO ₃ (1 g/liter) + polypeptone (1 g/liter)	Low	5.6	1.6	1.62
NH ₄ NO ₃ (2 g/liter)	Low	5.3	0.9	1.10
KNO ₃ (3 g/liter)	Low	7.8	1.1	1.95
Polypeptone (2 g/liter)	Low	6.8	1.0	1.80
Urea (5 g/liter)	High	6.8	2.3	2.74
	Low	6.6	2.4	3.00

^a Instead of NH₄NO₃ (1 g/liter) and polypeptone (1 g/liter) in *Medium 1*, the nitrogen source listed in the table was used.

^b High: Na₂HPO₄·12H₂O, 10 g/liter; KH₂PO₄, 5.5 g/liter
Low: Na₂HPO₄·12H₂O, 1.5 g/liter; KH₂PO₄, 1.5 g/liter.

FIG. 3. Effect of Initial pH on Production of β -Phenethyl Alcohol.

Cultivation was carried out in *Medium 1* at 30°C. ●—●, β -phenethyl alcohol (mg/ml); ○—○, growth (OD at 610 nm); □—□, final pH.

various concentrations of styrene. At the concentration of styrene under 2 mg/ml, β -PA was not accumulated at all. The amount of β -PA increased as the concentration of styrene increased up to 20 mg/ml. Styrene at 50 mg/ml produced a repressive effect to β -PA production and bacterial growth. The yield of β -PA based on styrene showed a tendency to decrease as the concentration of styrene increased. The optimal concentration of styrene for β -PA

production was around 20 mg/ml.

Initial pH

The pH dependence of β -PA production was examined in the range of 6.0 to 8.5 adjusted by 0.5 N NaOH or 0.5 N HCl. The optimal pH was around 7.0 as shown in Fig. 3.

Composition of basal medium

Nitrogen sources and phosphate. The effect of nitrogen sources listed in Table II was examined. When urea was used as a nitrogen source, the production of β -PA was highest. In this case pH of the medium did not change significantly although the concentration of phosphate was low.

Corn steep liquor. As shown in Table III, corn steep liquor (CSL) could be used as a substitute for yeast extract, and its optimal concentration for β -PA production was from 0.2 to 0.5 g/liter.

TABLE III. EFFECT OF CORN STEEP LIQUOR ON PRODUCTION OF β -PHENETHYL ALCOHOL

Cultivation was carried out at 30°C for 3 days in *Medium 2* with varied amount of corn steep liquor.

Corn steep liquor (g/liter)	Yeast extract (g/liter)	Final pH	Growth (OD at 610 nm)	β -PA (mg/ml)
0	0	6.6	1.2	2.09
0.1	0	6.7	1.4	2.32
0.2	0	6.8	2.1	3.00
0.5	0	6.8	2.2	3.17
1.0	0	7.0	2.2	2.67
0.1	0.1	6.7	2.1	2.83

Aeration and agitation

Effect of aeration and agitation on β -PA production was investigated by changing the volume of *Medium* 1 in the 500 ml flask. As the amount of β -PA accumulated in 50 ml and 100 ml of the medium was 2.93 mg/ml and

2.23 mg/ml, respectively, higher aeration was favorable for β -PA production.

Effect of metabolic inhibitors

The effect of metabolic inhibitors listed in Table IV on the production of β -PA was examined. These are known to inhibit enzymes, such as alcohol dehydrogenase,¹²⁻¹⁸⁾ enzymes of β -oxidation system, β -PA dehydrogenase¹⁶⁾ and protocatechuic acid oxygenase.¹⁸⁾ The experiment was performed in a test tube (15×160 mm) stoppered with a sponge plug. As shown in Table IV, extreme increase of β -PA accumulation was observed, when pyrazole¹⁶⁾ was added at a concentration of 0.1 mg/ml; 1.64 mg/ml of β -PA was produced under the effect of pyrazole, where as only 0.6 mg/ml was produced without pyrazole. On the other hand, 1 mg/ml of pyrazole diminished the production of β -PA and caused an accumulation of styrene oxide, whose identity was presumed by comparing the retention time with authentic sample in GLC.

Subsequently, the effect of pyrazole concentration and the time of addition was examined, as shown in Table V. The pyrazole addition at the time of inoculation was more effective than that at 24 hr of incubation. The optimal concentration was around 0.1 mg/ml.

Time course of β -PA fermentation

The production of β -PA in *Medium* 2 as a function of incubation time is shown in Fig. 4. Styrene disappeared within 48 hr, and the production of β -PA reached maximum at 48 hr of incubation in the same manner as cell growth. The addition of 0.1 mg/ml pyrazole promoted the accumulation of β -PA and retarded cell growth. The use of pyrazole enables β -PA to be produced more reliably and promptly.

The amount of β -PA produced during 48 hr incubation was 3.27 mg/ml, and that was 3.59 mg/ml when pyrazole was added.

Separation and purification of β -PA

As shown in Fig. 5, β -PA was separated and purified from 500 ml of 3-day-old broth which

TABLE IV. EFFECT OF METABOLIC INHIBITORS ON PRODUCTION OF β -PHENETHYL ALCOHOL

Incubation was carried out in a test tube (15×160 mm) with 2 ml of *Medium* 1, 40 mg of styrene and metabolic inhibitor. The tube was stoppered with a sponge plug. After inoculation, the test tube was reciprocally shaken at 30°C for 24 hr.

Metabolic inhibitors	(mg/ml)	Products (mg/ml)	
		Styrene oxide	β -PA
α,α' -Dipyridyl	1.0	0.00	0.00
	0.1	0.00	0.33
<i>o</i> -Phenanthroline	1.0	0.00	0.00
	0.1	0.00	0.00
EDTA	1.0	0.00	0.00
	0.1	0.00	0.42
NaN ₃	1.0	0.00	0.00
	0.1	0.00	0.00
KCN	1.0	0.00	0.00
	0.1	0.00	0.00
CH ₂ ICOOH	1.0	0.00	0.00
	0.1	0.00	0.02
CaCl ₂	1.0	0.00	0.00
	0.1	0.00	0.60
NH ₂ OH·HCl	1.0	0.00	0.00
	0.1	0.00	0.00
PCMB	1.0	0.00	0.00
	0.1	0.00	0.00
Barbital	1.0	0.00	0.52
	0.1	0.00	0.64
<i>o</i> -Phenylphenol	1.0	0.00	0.00
	0.1	0.00	0.57
Folic acid	1.0	0.00	0.63
	0.1	0.00	0.54
Pyrazole	1.0	0.09	0.29
	0.1	0.00	1.64
Salicylic acid	1.0	0.00	0.05
	0.1	0.00	0.46
2,4-Dinitrophenol	1.0	0.00	0.00
	0.1	0.00	0.34
Acrylic acid	1.0	0.00	0.00
	0.1	0.00	0.15
Na-Benzotate	1.0	0.00	0.31
	0.1	0.00	0.34
Pyrocatechol	1.0	0.00	0.00
	0.1	0.00	0.29
Control	—	0.00	0.60

TABLE V. EFFECT OF PYRAZOLE ON THE PRODUCTION OF β -PHENETHYL ALCOHOL

Incubation was carried out in *Medium 1* at 30°C for 3 days (72 hr). Varied amount of pyrazole was added at the time of inoculation or 24 hr later.

Pyrazole		Final pH	Growth (OD at 610 nm)	Product (mg/ml)	
Concentration (mg/ml)	Addition time (hr)			Styrene oxide	β -PA
Control		7.1	4.22	0.00	2.33
0.01	0	7.1	4.20	0.00	2.96
	24	7.2	4.20	0.00	2.43
0.02	0	7.0	4.00	0.00	3.16
	24	7.1	4.15	0.00	2.61
0.05	0	6.7	2.70	0.00	3.26
	24	7.1	4.20	0.00	3.05
0.10	0	6.8	2.52	0.18	3.35
	24	6.9	4.13	0.00	2.94
1.00	0	7.0	1.23	0.34	1.05
	24	6.8	2.87	0.15	1.13

contained 2.30 mg/ml of β -PA, estimated by GLC. After centrifugation, the supernatant was extracted twice with 200 ml of ethyl acetate and the extract was washed twice with 100 ml of 0.1 N ammonia. After dehydrated with Na_2SO_4 and decolorized with charcoal, the ethyl acetate extract was concentrated by an evaporator to give 1.08 mg of a solvent free, semi-viscous, light-yellow liquid with a rose-like fragrance. IR spectrum of this liquid was identical to that of authentic β -PA, and the

purity was estimated to be 99.4% in GLC. The all over recovery by this procedure was about 90%.

Production mechanism of β -phenethyl alcohol

Reaction with resting cells

Resting cells were prepared by growth in *Medium 1* containing 10 mg/ml styrene for 40 hr and washed twice with 0.01 M phosphate buffer (pH 7.5). The β -PA production from

FIG. 4. Time Course of β -Phenethyl Alcohol Production.

—, *Medium 2*; ----, *Medium 2* + pyrazole (0.1 mg/ml); ●, β -phenethyl alcohol (mg/ml); ○, growth (OD at 610 nm); △, styrene; □, pH.

FIG. 5. Separation and Purification of β -Phenethyl Alcohol.

TABLE VI. EFFECT OF OXYGEN ON REACTION WITH RESTING CELLS

Conditions			Residual substrates and products ($\mu\text{g/ml}$)					
			Styrene		Styrene oxide		β -PA	
Cells ^a	Substrates ^b	Oxygen	2 hr	18 hr	2 hr	18 hr	2 hr	18 hr
No cells	Styrene	+	498	370	0	0	0	0
	Styrene oxide	+	—	—	498	272	0	0
Heated cells	Styrene	+	498	415	0	0	0	0
	Styrene oxide	+	—	—	493	265	0	0
Untreated cells	No substrate	+	—	—	0	0	0	0
	Styrene	— ^c	500	420	0	0	0	3
	Styrene	+	430	231	0	1	2	54
	Styrene oxide	— ^c	—	—	114	95	80	83
		+	—	—	25	24	85	100

^a Resting cells were prepared by growth in *Medium* 1 containing 10 mg/ml styrene and washed twice with 0.01 M phosphate buffer. Reaction was carried out as described in Fig. 6.

^b 500 $\mu\text{g/ml}$.

^c Air in the test tube was purged by nitrogen gas.

FIG. 6. Formation of β -Phenethyl Alcohol by Resting Cells.

One milliliter of 0.01 M phosphate buffer (pH 7.5) containing 500 $\mu\text{g/ml}$ of substrate was poured into a test tube (12 $\times$ 100 mm). The final cell concentration was 2.0 as OD at 610 nm. The tube was sealed tightly and shaken reciprocally at 30°C during the reaction.

styrene or styrene oxide by resting cells was demonstrated, as shown in Fig. 6. β -PA production from styrene started after 3 hr of incubation and increased linearly. β -PA production from styrene oxide started from the beginning of the reaction and increased linearly.

Effect of oxygen

As shown in Table VI, the effect of oxygen on β -PA production from styrene and styrene oxide was examined. With no cells or heated cells, neither β -PA from styrene or styrene oxide nor styrene oxide from styrene was produced. Styrene oxide in these mixtures decreased gradually, probably because of spontaneous transformation to styrene glycol (not detected yet). The removal of oxygen from the reaction system by nitrogen substitution diminished the production of β -PA from styrene. However the production of β -PA from styrene oxide was not affected significantly by nitrogen substitution.

TABLE VII. ASSIMILABILITY OF VARIOUS SUBSTRATES

Incubation was carried out in *Medium* 1 containing 1 mg/ml of substrates listed below at 30°C for 40 hr.

Substrates	Final pH	Growth (OD at 610 nm)	β -PA ($\mu\text{g/ml}$)
Ethyl benzene	7.0	0.40	0
Styrene	6.9	0.72	150
Styrene oxide	6.9	0.65	500
Styrene glycol	7.0	0.27	0
α -Phenethyl alcohol	7.0	0.15	0
β -Phenethyl alcohol	6.7	0.57	0
Na-Benzozate	7.3	0.63	0
No substrate	7.0	0.20	0

Assimilability of some substrates

Strain 305-STR-1-4 grew in medium with ethyl benzene, styrene, styrene oxide, β -PA and benzoic acid, as the carbon and energy sources (Table VII). Styrene glycol and α -PA was not assimilated. From 1 mg/ml of styrene and styrene oxide, 150 and 500 μ g/ml, respectively, of β -PA was produced.

DISCUSSION

In the beginning of the investigation, *Mechanism I* (Fig. 1) was the expected mode of β -PA production from styrene by strain 305-STR-1-4, as reported by Iida and Iizuka⁴⁾ for *n*-decane-1-ol formation. As the investigation proceeded, however, this mechanism was excluded, and *Mechanism II* (Fig. 1), two-step reaction via styrene oxide, was proposed as follows. The product suggested as styrene oxide was detected in the culture broth and the reaction mixture for the production of β -PA from styrene. Styrene oxide was assimilated and β -PA was produced from styrene oxide by growing cells and resting cells. These results suggest that styrene oxide is an intermediate between styrene and β -PA. Molecular oxygen was required, as the aerobic condition was desirable for β -PA production, and β -PA production was hindered in the anaerobic condition, when styrene was used as a substrate for cultivation and reaction with resting cells. The β -PA production from styrene oxide was not affected significantly by removal of molecular oxygen.

Therefore, it is concluded that β -PA is produced from styrene by *Mechanism II*, or in detail *Mechanism II-1* and *Mechanism II-2* (Fig. 1). In *Mechanism II-1*, 1 mol of oxygen and 1 mol of hydrogen donor may be consumed stoichiometrically to metabolize 1 mol of styrene. One mol of styrene oxide produced in *Mechanism II-1* is reduced with 1 mol of hydrogen donor and reductase in *Mechanism II-2*. Mechanisms similar to *Mechanism II* were proposed in *n*-heptane-1-ol formation form *n*-1-heptene by Azoulay *et al.*⁵⁾ *Pseudomonas oleovorans*^{6~8)} produces epoxides

from olefins with molecular oxygen and NADH in ω -hydroxylation system, but can not transform epoxides to alcohols. In ω -hydroxylation system, styrene was not epoxidized at the double bond or the aromatic ring. Sielicki *et al.*²⁾ reported that a mixed population of soil bacteria was able to utilize styrene and produce β -PA, but its mechanism was not discussed. In mammalian tissues,¹⁰⁾ although styrene is epoxidized with molecular oxygen and NADPH by the hepatic microsome system, the produced styrene oxide was hydrated to styrene glycol.

Among 18 metabolic inhibitors added to the medium, only pyrazole was active in accumulating β -PA or styrene oxide. Reynier¹⁰⁾ reported that pyrazole and its derivatives inhibited alcohol dehydrogenase and β -phenethyl alcohol dehydrogenase. In strain 305-STR-1-4, it seems that lower concentration (below 0.1 mg/ml) of pyrazole stimulates the production of β -PA because of the inhibition of β -PA dehydrogenation and slight inhibition of styrene oxide reduction. Higher concentration of pyrazole may inhibit styrene oxide reductase (*Mechanism II-2*, Fig. 1), since styrene oxide is accumulated and β -PA is not produced significantly.

For the purpose of increasing β -PA production, two approaches are under way; one is investigation on detailed mechanism of production, and the other is examination of more effective systems of β -PA production.

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REFERENCES

- 1) K. Shirai and K. Hisatsuka, *Agric. Biol. Chem.*, **43**, 1595 (1979).
- 2) M. Sielicki, D. D. Focht and J. P. Martin, *Appl. Environ. Microbiol.*, **35**, 124 (1978).
- 3) B. G. Malmstrom, "The Enzymes," Vol. 5, 2nd ed., Academic Press Inc., New York, 1961, p. 455.
- 4) M. Iida and H. Iizuka, *Z. Allgem. Mikrobiol.*, **10**, 245 (1970).
- 5) E. Azoulay, J. Chouteau and G. Davidovics, *Biochim. Biophys. Acta*, **77**, 554 (1963).

- 6) S. W. May and B. J. Abbott, *Biochem. Biophys. Res. Commun.*, **48**, 1230 (1973).
 - 7) S. W. May and B. J. Abbott, *J. Biol. Chem.*, **248**, 1725 (1973).
 - 8) B. J. Abbott and C. T. Hou, *Appl. Microbiol.*, **26**, 86 (1973).
 - 9) S. W. May, B. J. Abbott and R. D. Schwartz, *Am. Chem. Soc. Div. Pet. Chem. Prepr.*, **19**, 713 (1974).
 - 10) E. W. Maynert, R. L. Foreman and T. Watabe, *J. Biol. Chem.*, **245**, 5234 (1970).
 - 11) H. Ohtsuji and M. Ikeda, *Toxicol. Appl. Pharmacol.*, **18**, 321 (1971).
 - 12) H. Sund and H. Theorell, "The Enzyme," Vol. 7, 2nd ed., Academic Press Inc., New York, 1963, p. 26.
 - 13) A. H. Conney, *Pharmacol. Rev.*, **19**, 317 (1967).
 - 14) H. J. Rehm and J. Baltes, *Z. Lebersm. Unters. Forsch.*, **137**, 295 (1968).
 - 15) W. H. Vogel, R. Snyder and R. P. Schulman, *Proc. Soc. Exp. Biol. Med.*, **115**, 545 (1964).
 - 16) M. Reynier, *Agressologie*, **11**, 401 (1970).
 - 17) S. Grisolia, J. Mendeson and D. A. Diederich, *FEBS Lett.*, **11**, 140 (1970).
 - 18) R. B. Cain and N. J. Cartwright, *Biochim. Biophys. Acta*, **37**, 197 (1960).
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