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Microbial oxidation of cumene

Ching T. Hou, Thomas A. Seymour and Marvin O. Bagby

Oil Chemical Research, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604, USA

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SUMMARY

A total of 1229 cultures, including 230 actinomycetes, 508 other bacteria, 12 fungi and 479 yeasts were screened for their ability to oxidize the isopropyl side chain of 2-phenyl propane (cumene). Four strains of actinomycetes and six strains of bacteria but no yeasts were found positive in converting 2-phenyl propane to its oxygenated products. Eight strains oxidized cumene through the alkyl side chain producing 2-phenyl-1-propanol. Two *Bacillus* strains oxidized cumene to an oxygenated product. *Pseudomonas oleovorans* NRRL B-3429 exhibited the highest alkyl side chain oxidation activity. The optimum reaction conditions for strain B-3429 are: 25 °C, pH 6.5 and 48 h of reaction. Octane-grown cells of strain B-3429 produced higher product yields (about 7.2-fold) than the glucose-grown cells. Prolonged incubation resulted in an increase in 2-phenyl-1-propionic acid production at the expense of 2-phenyl-1-propanol. The yield of 2-phenyl-1-propanol plus 2-phenyl-1-propionic acid was 5.1%. Reaction in the presence of methanol favored the accumulation of 2-phenyl-1-propionic acid and also increased the total yield. (The yield of 2-phenyl-1-propanol plus 2-phenyl-1-propionic acid was 14.9%.) Structures of the reaction products were confirmed by GC/MS and GC/IR analyses. Products contained 92% R(−) isomer.

INTRODUCTION

Alkylbenzenes are oxidized by microorganisms either at the terminal methyl group or at the aromatic nucleus. As the alkyl chain length increases and the substituents become the major part of the molecule, these compounds are more realistically regarded as substituted alkanes rather than substituted benzenes. Thus, like the n-alkanes, they undergo oxidation to give phenyl alkanoic acids that are then metabolized by beta-oxidation [3,13]. In some cases, the breakdown of the alkyl group alone is sufficient to support growth, and the organism may not always be able to degrade the benzene substituent.

Information on the microbial degradation of alkylbenzenes with short side chains having 2–5 carbon atoms is not extensive. However, a number of products derived from these hydrocarbons by so-called co-oxidation have been described. Ethylbenzene was oxidized to phenyl acetic acid by *Nocardia salmonicolor* [3]. n-Propyl benzene was converted to cinnamic acid or benzoic acid by *Nocardia* sp. grown on hexadecane in the presence of n-propyl benzene [3]. Gibson et al. [4] reported the oxidation of ethylbenzene by *Pseudomonas putida* through positions 2 and 3 of the benzene nucleus to form (+)-*cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol. Jigami et al. [9] showed that the oxidation of isopropyl benzene by *Pseudomonas convexa* S107B1 and *P. desmolytica* S449B1 was initiated on the benzene nucleus at the 2 and 3 positions and not the iso-alkyl side chain. However, using n-propylbenzene they observed [8] something different. These two strains utilized at least two different pathways, one through oxygenation of the methyl group of the n-propyl side chain and the other through oxygenation of the benzene nucleus at the 2 and 3 positions. The authors claimed the coexistence of two different pathways for the metabolism of n-propylbenzene by these strains.

Microbial oxidation of 2-phenyl propane through its isopropyl side chain has not been reported. There are many important drugs or their intermediates that contain 2-aryl propionic acid as their active moiety. For example, stereospecific 2-aryl propionic acid is a member of an important class of nonsteroidal anti-inflammatory drugs [12]. Stereospecific 2-phenoxy propionic acid is an active moiety in certain herbicides [2]. Antihypertensive agents such as captopril also contain a stereospecific 2-acyl-thio-propionic acid moiety [12]. Preparation of these compounds was reported using stereospecific resolution of their corresponding racemic esters by lipases [2,12]. However, the maximum yield for selective resolution using lipases is only 50%, assuming one enantiomer is 100% hydrolyzed. Another approach for preparing these compounds is through direct stereoselective hydroxylation at the isopropyl side chain. For example, Klifford et al. [10] reported the stereospecific hydroxylation of the isopropyl side chain of phenoxy compounds by *Rhodococcus rhodochrous, Rh. erythropolis* and *Nocardia corallina* to give substituted phenoxy propionic acids, an important class of herbicides.

We are interested in the stereospecific oxidation of the isopropyl side chain of aromatic compounds giving products such as 2-phenyl-1-propanol, 2-phenyl-1-propanal and

Correspondence to: Dr Ching T. Hou, National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, IL 61604, USA.

2-phenyl-1-propionic acid by microorganisms or their enzymes. 2-Phenyl propane (cumene) was selected as a model compound to screen for microorganisms that can hydroxylate the isopropyl side chain. A total of 1229 cultures including 230 actinomycetes, 508 other bacteria, 12 filamentous fungi and 479 yeasts were screened for their ability to oxidize cumene. Ten cultures were found to oxidize cumene to its oxygenated products. The reaction products were identified by GC/MS and GC/IR analyses. Octane-grown cells were more active than glucose-grown cells. This paper describes our screening results, product identification and optimum reaction conditions for strain B-3429.

MATERIALS AND METHODS

Microorganisms

All microbial cultures were obtained from the ARS Culture Collection. Bacteria were grown in 50 ml of TGY medium which contained (per liter): tryptone 5 g, yeast extract 5 g, dextrose 1 g, K_2HPO_4 1 g, pH 7.0 at 30 °C, 250 r.p.m. shaking. Yeasts and actinomycetes were grown in 50 ml of PDA medium which contained (per liter): 26 g PDA (Difco Lab., Detroit, MI, USA), pH 5.5 at 25 °C, 150 r.p.m. After 24 h of growth, 10 μl of cumene were added as an inducer. Cultures were also grown in a mineral salts medium containing 5% n-octane as the sole carbon source. The composition of the mineral salts medium and growing conditions were reported previously [1,7].

Chemicals

2-Phenyl propane, 2-phenyl-1-propanol, 2-phenyl-1-propanal, 2-phenyl-1-propionic acid, *o*-, *m*- and *p*-hydroxy-cumene, R(−)-2-phenyl-1-propionic acid, and S(+)-2-phenyl-1-propionic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were reagent grade and were used without further purification.

Microbial oxidation of cumene

Two-day-old cultures (OD at 650 nm ≥3) were harvested by centrifugation and washed once with 25 mM sodium phosphate buffer, pH 6.5. Cells were resuspended in the same buffer solution to a density of OD 4 at 650 nm, and then a 2-ml portion of the suspension was put into a small vial. Sixty μl (425 μmol) of cumene were added, and the vial was sealed with a stopper and incubated in a water bath shaker at 25 °C, 250 r.p.m. for a period of time. At the end of the incubation, the reaction mixture was acidified to pH 2 and extracted with an equal volume of diethyl ether. The ether extract was analyzed with a gas chromatograph.

Analysis of products

The reaction products were analyzed by gas chromatography (GC). Samples were injected into a Hewlett Packard (Rolling Meadows, IL, USA) model 5890 series II gas chromatograph equipped with flame ionization detector, a Supelco SPB-1™ capillary column (Supelco, Bellefonte, PA, USA) 15 m, i.d. 0.32 mm, 0.25 μm thickness and a Hewlett Packard 3392A integrator. GC was run with a temperature gradient initially at 80 °C for 7 min and then a gradient at 5 °C min^{-1} to 120 °C, with a gas flow rate of 50 ml min^{-1} (split ratio 50:1). A typical gas chromatogram is shown in Fig. 1. A linear relationship between the amounts of authentic 2-phenyl-1-propanol or 2-phenyl-1-propionic acid and their GC peak areas was established for quantitative analyses of the reaction products. Experiments were run in triplicate, and the average of the replicates are reported. The variation of each run was within 5%. The same gas chromatograph equipped with a Supelco 20% beta-cyclodextrin capillary column 60 m, i.d. 0.25 mm, 0.25 μm thickness was used for chiral analysis. The chiral GC was run isothermally at 150 °C with split vent flow rate of 65 ml min^{-1}. Mass spectra were obtained with a Perkin Elmer (Norwalk, CT, USA) Sigma 3B Capillary GC coupled to a Hewlett Packard 5970 Series Mass Selective Detector. GC/IR spectra were acquired on a Hewlett Packard 5970A series GC and a Matteson Instruments (Secaucus, NJ, USA) Infrared Spectrophotometer using a Chemist's Workbench for data analysis. Products were characterized by comparison of their GC/MS and GC/IR spectra with those of authentic samples.

Purification of reaction products

For isolation and purification of reaction products, the reaction mixture was centrifuged at 6000 × *g* for 15 min to separate the cells. The supernatants were acidified to pH 2 with dilute hydrochloric acid and then passed through a C18 solid-phase extraction cartridge (Alltech, Newark, NJ, USA). Methanol was used to elute a 1-ml fraction. 2-Phenyl-1-propionic acid was purified from the methanol extract on a Spectra Physics SP8800 HPLC unit fitted with a Supelcosil LC-18, 15 cm × 4.6 mm column. Fractions were eluted with 55% MeOH in 0.25 mM sodium phosphate, pH 7.0 buffer system and detected by UV absorbance at 254 nm with a Spectoflo 757 UV/vis detector (ABI Analytical Instruments,

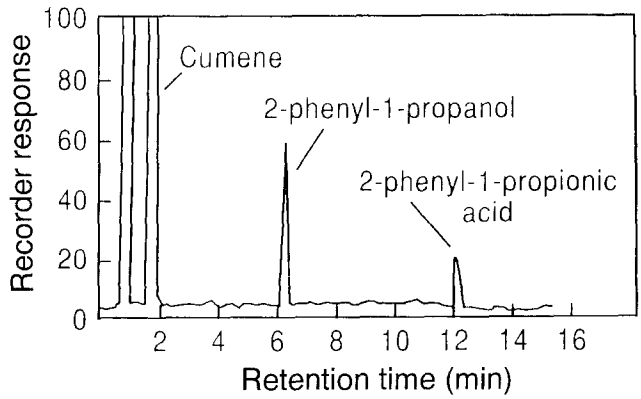

Fig. 1. Gas chromatogram of cumene oxidation products by a resting cell suspension of glucose-grown strain B-3429. GC conditions: FID, Supelco SPB™-1 capillary column 15 m, i.d. 0.32 mm, 0.25 μm thickness. Initial oven temperature 80 °C for 7 min, then gradient at 5 °C min^{-1} to 120 °C. 2-Phenyl-1-propionic acid peak was from authentic sample to indicate the retention time.

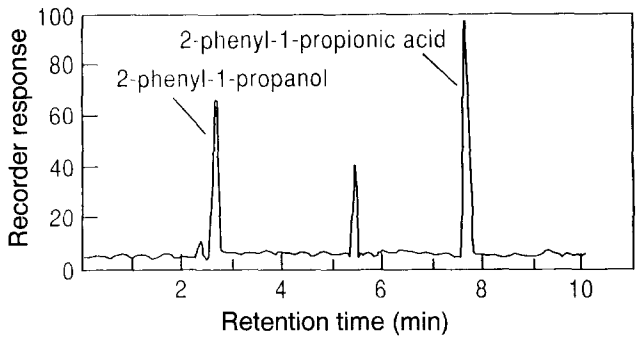

Fig. 2. Purification of cumene bio-oxidation products by HPLC. Column: Supelco C18 reverse phase column 15 cm. Solvents: 45% 25 mM sodium phosphate buffer pH 7.0 and 55% methanol. Flow rate: 1 ml min^{-1}. One half-ml fractions were collected. Detection: Absorption at 254 nm. The peak at retention time 5.5 min was not identified.

Ramsey, NJ, USA). Fig. 2 shows the purification of 2-phenyl-1-propanol and 2-phenyl-1-propionic acid by HPLC.

RESULTS AND DISCUSSION

Selected strains (1229) including actinomycetes, other bacteria, yeasts, and filamentous fungi were screened for their ability to oxidize the isopropyl side chain of cumene stereospecifically. The genera and species covered are the same as those discussed in our previous paper on lipase activity screening [5]. Among these 1229 cultures screened, only two actinomycetes and six other bacterial strains oxidized the isopropyl side chain of cumene (Table 1). Two strains of *Pseudomonas oleovorans* (NRRL B-3429 and B-14683) showed the highest cumene alkyl side chain oxidizing activity, producing 2-phenyl-1-propanol. Two *Bacillus* strains oxidized cumene to an unidentified oxygenated product (see below).

TABLE 1

Microorganisms positive for cumene oxidation

Microorganisms	Oxidation products (μmol)
Pseudomonas putida NRRL B-1245	0.17
Pseudomonas sp. NRRL B-11330	0.19
Pseudomonas oleovorans NRRL B-3429	3.04
Pseudomonas oleovorans NRRL B-14683	2.64
Rhodococcus rhodochrous NRRL B-2153	0.17
Rhodococcus erythropolis NRRL B-16531	0.52
Nocardia globerula NRRL B-2769	0.70
Amycolatopsis rugosa NRRL B-2295	0.17
Bacillus laterosporus NRRL B-4250	34.4*
Bacillus polymyxa NRRL B-130	11.32*

Product is 2-phenyl-1-propanol except * which is an unidentified oxygenated product. The amount was estimated from parent ion (m/z 136) and a 2-phenyl-1-propanol standard curve.
Product is in μmoles per 2 ml reaction mixture (cell density $OD_{650\ nm}$ = 4).

Identification of reaction products

The products of alkyl side chain oxidation were shown to be 2-phenyl-1-propanol and 2-phenyl-1-propionic acid. These were characterized by comparison with authentic samples. GC of products mixed with authentic samples showed one single peak. Electron impact GC/MS of the reaction products is shown in Fig. 3(A). The heaviest mass is 136. The fragment 105 represents M−31 (or minus CH_2OH). Other fragments agreed well with those obtained from an authentic 2-phenyl-1-propanol mass spectrum. The GC/IR spectrum of the alcohol shows bands at 3670 and 3634 cm^{-1} (OH) and 1038 cm^{-1} (C—O). The GC/IR spectrum of the acid shows bands at 3574 cm^{-1} (OH) and 1775 cm^{-1} (C=O). These bands match those of authentic 2-phenyl propanol and 2-phenyl-1-propionic acid, respectively. The product from *Bacillus* was analyzed with GC and GC/MS. This product has a GC retention time of 8 min that is different from both 2-phenyl-1-propanol and 2-phenyl-1-propionic acid. EI mass spectrum of this compound gave a parent ion at Mz 136 (Fig. 3(B)) but gave a fragmentation pattern differing from that obtained with 2-phenyl-1-propanol. This fragmentation pattern was also different from those of aromatic ring oxidation products i.e. *o*-, *m*- or *p*-hydroxycumene. Further identification of this product is currently under investigation.

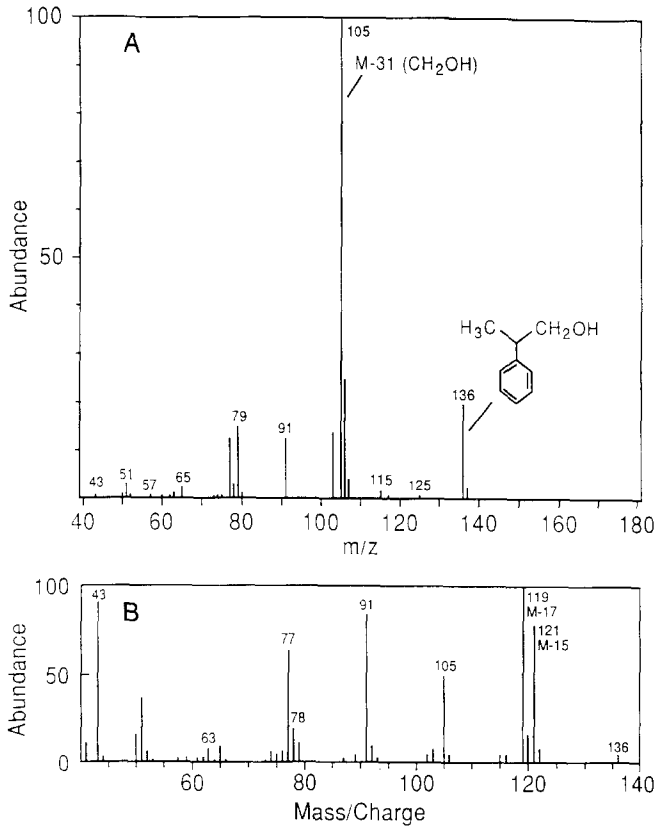

Fig. 3. (A) EI GC/MS of cumene oxidation product by strain B-3429. (B) EI GC/MS of cumene oxidation product by *Bacillus* sp. NRRL B-4250.

Time course of cumene oxidation

The time course for cumene alkyl side chain oxidation by glucose-grown strain B-3429 is shown in Fig. 4. Product (2-phenyl-1-propanol) accumulated slowly and peaked at 48 h of incubation. 2-Phenyl-1-propionic acid was not detected.

Effect of pH on the oxidation of cumene

The effect of pH on the production of 2-phenyl-1-propanol by resting cell suspension of glucose-grown strain B-3429 was studied in 0.05 M buffer solutions: sodium phosphate buffer pH 5.5–7.5; Tris-HCl for pH 7.5–9.0. The maximum activity was obtained at pH 6.5 [relative activity: pH 5.5 (0%); pH 6.0 (20%); pH 6.5 (100%); pH 7.0 (75%); pH 8.0 (12%); pH 8.5 (0%); and pH 9.0 (0%)].

Effect of temperature on the oxidation of cumene

The temperature dependence of the conversion of cumene by strain B-3429 was studied from 15 to 45 °C. The optimum temperature for production of 2-phenyl-1-propanol was 25 °C; oxidation activity did not occur at temperatures above 40 °C [relative activity: 15 °C (0%); 20 °C (50%); 25 °C (100%); 30 °C (66%); 35 °C (17%); 40 °C (0%)].

Effect of cell concentration

The effect of cell concentration on cumene alkyl side chain oxidation by strain B-3429 was studied from 0.5 to 8 $OD_{650\ nm}$ units (1 OD unit = 1.38 mg dry cells ml^{-1}). The production of 2-phenyl-1-propanol was proportional to cell concentration up to 4 $OD_{650\ nm}$ units. Relative activities are, $OD_{650\ nm}$ units: 0.5 (10%); 1.0 (30%); 2.0 (50%); 3.0 (80%); 3.5 (92%); 4.0 (100%); 5.0 (98%); 8.0 (76%).

Effect of substrate concentration

Oxidation of cumene alkyl side chain was also influenced by substrate concentrations. The optimum concentration of substrate cumene for the maximum production of 2-phenyl-1-propanol was 60 μl (425 μmoles) per 2 ml reaction mixture. Higher cumene concentrations inhibit product formation. Relative activities are, μmoles cumene in 2 ml reaction mixture: 106 (54%); 212 (75%); 425 (100%); 900 (30%); 2000 (16%); and 4900 (11%).

n-Octane-grown cells

Because the glucose-grown cells have relatively low cumene–oxidation yield (about 0.7%), we investigated hydrocarbon-grown cells. Strain B-3429 grew well on n-octane as the sole carbon source. In 40–50 h, the cell concentration reached 3 $OD_{650\ nm}$ units. A time course study on cumene oxidation using octane-grown cells is shown in Fig. 5. The maximum yield of 2-phenyl-1-propanol was observed at around 20 h. 2-Phenyl-1-propionic acid also was produced and continued to accumulate as 2-phenyl propanol yield decreased during 72 h of incubation. Total yield of products (5.1%) was much higher than that obtained from glucose-grown cells (about 7.2-fold). This is probably because the octane monooxygenase system of strain B-3429 is also active for the hydroxylation of the cumene alkyl side chain. Prolonged incubation increased 2-phenyl-1-propionic acid accumulation at the expense of 2-phenyl-1-propanol.

Effect of various alcohols on the oxidation of cumene

It is known that a main reason for slowdown of the monooxygenase reaction is the depletion of cofactor NADH. The cofactor NADH can be regenerated by an alcohol dehydrogenase reaction [6]. Therefore, the effect of methanol, isopropanol, and 1-octanol on the oxidation of the cumene alkyl side chain was studied with n-octane-grown cells in the hope that the alcohol would be oxidized by alcohol dehydrogenase and thus regenerate NADH from NAD for the monooxygenase reaction. The yield of 2-phenyl-1-propionic acid increased dramatically in the presence of methanol (Table 2). 2-Phenyl-1-propionic acid was the major product. The total yield also increased from 5.1% (control) to 14.9%. 1-Octanol was found to be inhibitory. Also, methanol stimulated the production of 2-phenyl-1-propionic acid at the alcohol dehydrogenase step. When 2-phenyl-1-propanol was used as substrate in the presence of methanol, 2-phenyl-1-propionic acid yield increased (Table 3). Again,

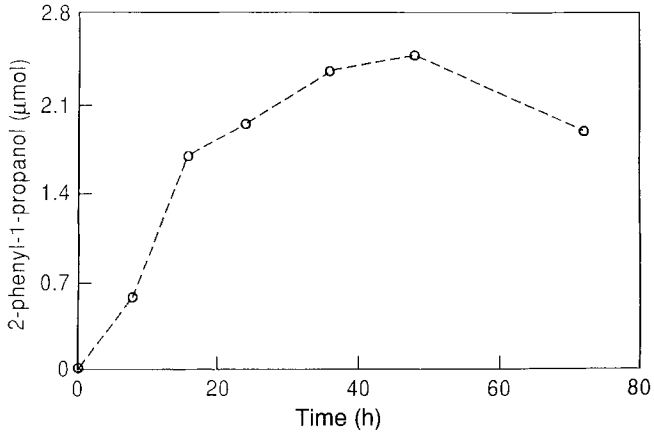

Fig. 4. Time course of the production of 2-phenyl-1-propanol from cumene by a resting cell suspension of glucose-grown strain B-3429. Product is in μmoles per 2 ml reaction mixture (cell density $OD_{650\ nm}$ = 4).

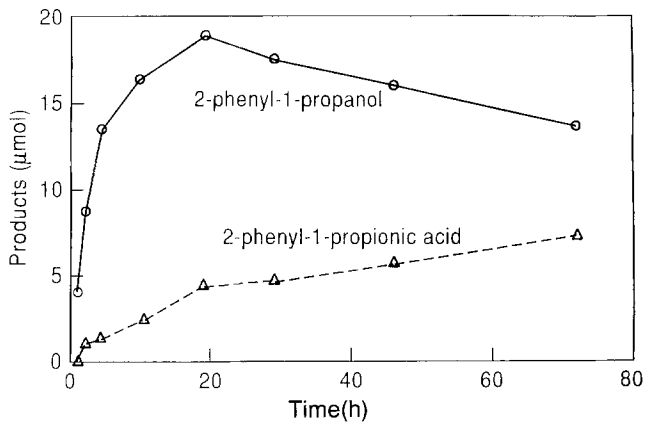

Fig. 5. Time course of cumene oxidation by resting cells of octane-grown strain B-3429.

TABLE 2

Effect of alchols on the oxidation of cumene by a resting cell suspension of octane-grown *Pseudomonas* strain B-3429

Alcohols	Products formed (μmol)	
	2-phenyl-1-propanol	2-phenyl-1-propionic acid
Cumene	15.5	6.5
Cumene + methanol	5.7	57.7
Cumene + 1-octanol	7.1	3.2
Cumene + isopropanol	10.4	13.5

Products are in μmoles per 2 ml reaction mixture (cell density ($OD_{650\ nm}$ = 4).

TABLE 3

Effect of methanol and octanol on the conversion of 2-phenyl-1-propanol to 2-phenyl-1-propionic acid by resting cells of octane-grown *Pseudomonas* strain B-3429

Tests	2-pheny-1-propionic acid formed (μmoles per 2 ml reaction mixture)
2-phenyl-1-propanol 425 μmol	79.9
2-phenyl-1-propanol + 20 μl methanol	100.5
2-phenyl-1-propanol + 20 μl 1-octanol	13.1

1-octanol inhibited the reaction. The mechanism for methanol stimulation is not clear. However, it could be explained as the result of producing additional reducing equivalents for the initial oxygen incorporation step and as an inducer of the alcohol dehydrogenase which catalyzes the subsequent step of the reaction. Recently, Kuge et al. reported the oxidation of 2-phenyl propionic acid to 2-(*p*-hydroxyphenyl) propionic acid by *Streptomyces rimosus* [11]. We did not see *p*-hydroxylation products in the strain B-3429 system.

Stereochemistry

Figure 6 shows the gas chromatogram of cumene oxidation products obtained from strain B-3429 on a beta-cyclodextrin column. The 2-phenyl-1-propionic acid (75 min) is resolved into two peaks. Comparison to authentic enantiomerically pure samples of the acid shows the product to be about 92% in (R)-(−) form. Moreover, when the products are spiked with (S)-(+)-2-phenyl-1-propionic acid, the smaller peak is enhanced. Addition of the (R)-(−) isomer enhances the larger peak. The product, 2-phenyl-1-propanol, showed one peak at 45 min on the GC cyclodextrin chiral column. It might also be the R(−) enantiomer. Without authentic pure 2-phenyl-1-propanol enantiomers, we are not sure of its absolute configuration. Because the process represents two reaction steps, i.e. oxygenation and dehydrogenation,

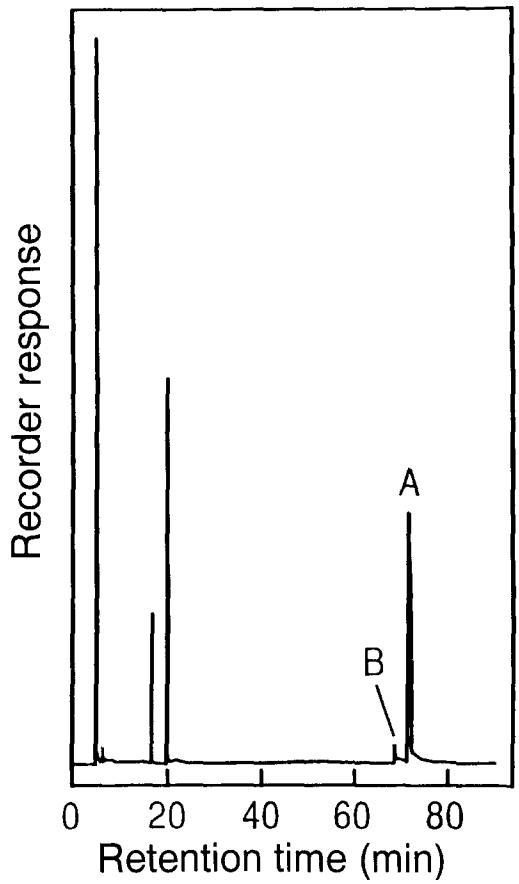

Fig. 6. GC chiral analysis of 2-phenyl-1-propionic acid produced from cumene by octane-grown strain B-3429. GC conditions: Supelco 20% beta-cyclodextrin capillary column 60 m, 0.25 mm i.d., 0.25 μm thickness, carrier gas flow rate 65 ml min^{-1}. Oven temperature at 130 °C isothermally. The peaks at retention time around 20 min are not identified.
A: R(−)-2-phenyl-1-propionic acid.
B: S(+)-2-phenyl-1-propionic acid.

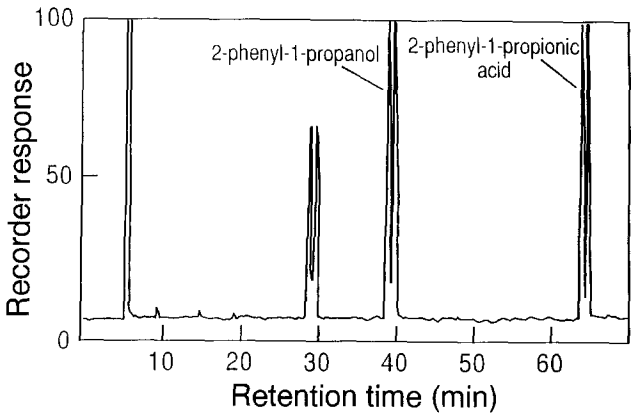

Fig. 7. GC chiral analysis of 2-phenyl-1-propionic acid produced from 2-phenyl-1-propanol by resting cells of octane-grown strain B-3429. For GC conditions see Fig. 6, except oven temperature at 150 °C isothermally.

we examined the second step of the reaction by using 2-phenyl-1-propanol (racemic) as substrate. With the racemic 2-phenyl-1-propanol as substrate, the product acid was racemic (Fig. 7). The peaks at retention time around 29 min, although not confirmed, are possibly 2-phenyl-1-propanal. This result indicated that the second step of the cumene oxidation catalyzed by alcohol dehydrogenase is a non-stereospecific reaction. This clearly showed that the 92% purity of 2-phenyl-1-propionic acid obtained by strain B-3429 is attributed to the first step of the reaction catalyzed by monooxygenase.

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