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Biotransformation of limonene by *Pseudomonas putida*

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Abstract From a study of three fungal and 15 bacterial strains, it was observed that *Pseudomonas putida* MTCC 1072 oxidized limonene with the highest efficiency of. Fermentation of limonene by *P. putida* MTCC 1072 was conducted for 120 h at 30 °C at a fixed pH of 5.0. Major bioconversion products were isolated and characterized by Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy, and by elemental analysis. The bioconversion products were identified as perillyl alcohol and *p*-menth-1-ene-6,8-diol, and under optimum conditions the yields were 36% and 44%, respectively (a rate kinetic model indicated corresponding limiting yields of 44% and 56%). No further degradation of the products was observed using this bacteria.

Introduction

Microorganisms and their enzymes have proven to be versatile biocatalysts (Jones et al. 1993) and are extensively used for biotransformations of various terpenoids (Kieslich et al. 1986; Trudgill 1990). Unlike traditional chemical processes, which require extreme temperatures and pressures, microbial conversions take place under mild conditions and, in some instances, the products are formed stereoselectively. A large variety of enzymes occur in several microorganisms (such as bacteria, yeast and fungi) which are effective in biotransformations of various terpenoids and can be used *in vivo*. In addition, whole cells are generally much less expensive compared

to purified enzymes, and, in some cases, enzymes are more stable within the cell, thus extending the life of the biocatalyst (Knox and Cleffe 1984). Another advantage of using whole cells is that the addition of purified co-factor is not required, since it already contained within the cell. The microbial transformation processes have therefore been explored for terpenoids with a view to achieving desired conversions, optical resolution of product, as well as an understanding of the metabolic pathway for biodegradation of terpenoids.

The majority of microbial transformations of terpenoids have been performed on monoterpenoids, which are the main constituents of many essential oils. Among various monoterpenes, limonene (4-isopropenyl-1-methyl cyclohexene) is a widely available monoterpene hydrocarbon and a major component in oils from citrus peel (Braddock and Cadwallader 1992). The characteristic organoleptic properties of limonene and its usage in food and other applications have led to extensive work on its synthesis and microbial conversions (Dhavlikar and Bhattacharyya 1966; Kraidman et al. 1969; Cadwallader et al. 1989; Tan and Day 1998). However, most studies dealing with microbial conversion of limonene have reported low yields of products due to volatility of the substrate and the toxicity of limonene to most of the microorganisms (Bowen 1975; Uribe and Pena 1990). The present study was therefore aimed at the screening of microorganisms, and in the course of the survey a strain of *Pseudomonas putida* MTCC 1072 was shown to successfully metabolize limonene to perillyl alcohol and *p*-menth-1-ene-6,8-diol. Among the two bioconversion products, perillyl alcohol is of particular importance, since it has been reported by several researchers that perillyl alcohol derived from lavender (*Lavandula angustifolia*) has chemopreventive properties against liver, mammary and lung carcinogenesis (Reddy et al. 1997; Bardon et al. 1998). Hence the biosynthesis of perillyl alcohol from limonene, as an important anticancer drug, is quite important. The impact of several parameters on this biotransformation has been investigated and optimized. A rate kinetic model has also been used to analyze the data.

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Materials and methods

Materials

Limonene, obtained locally, was double distilled and further purified by silica gel (60–120 mesh, obtained from Loba Chemie, Mumbai, India) chromatography. The purity of limonene was found to be 98.4% as measured by gas-liquid chromatography (GLC). Silica gel G for thin layer chromatography (TLC) was obtained from Tara Chemicals (Calcutta, India). Deuterated chloroform (CDCl_3) for ^1H - and ^{13}C -NMR spectroscopy was obtained from Aldrich Chemicals (Milwaukee, Wis., USA) and all solvents were of analytical grade.

Microorganisms

The microbial strains *Aspergillus niger* MTCC 961, *Candida tropicalis* MTCC 230, *Pseudomonas aeruginosa* MTCC 1034, *Pseudomonas putida* MTCC 102, *Pseudomonas putida* MTCC 1072, *Pseudomonas putida* MTCC 1194, and *Rhodococcus rhodochrous* MTCC 289 used in this study were obtained from the Institute of Microbial Technology (Chandigarh, India). The fungal strain *Rhizopus nigricans* No. 282 and the ten unidentified soil bacteria were isolated and provided by Process Development and Analytical Control Laboratory (Calcutta, India) and were used as supplied. The microorganisms were maintained on agar slants, stored at 5 °C and subcultured periodically.

Screening methods

A loop of inocula from an agar slant was transferred to 250-ml Erlenmeyer flasks containing 100 ml of the three different media and cultured on a shaker at 30–35 °C for 24–48 h. To the resulting culture broth, 0.2% (v/v) limonene was added as substrate using sterile technique. The flasks were shaken at 30 °C for 5 days. To recover the biotransformation products, the reaction mixture was centrifuged at 12,800 *g* at 4 °C for 10 min. The biomass was discarded and the supernatant was extracted with ethyl acetate (3×25 ml). The combined organic extract was washed with distilled water (3×10 ml), dried over anhydrous sodium sulphate and filtered using Whatman No.1 filter paper. The solvent was then removed under reduced pressure to obtain crude reaction products, which were subsequently examined by TLC and GLC. Microorganisms were screened for their ability to degrade limonene.

Culture media

Medium A contained 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose in distilled water (pH 7.0 for yeast). Medium B contained 0.1% beef extract, 0.2% yeast extract, 0.5% peptone and 0.5% sodium chloride in distilled water (pH 5.0 for bacteria). Medium C contained 5.0% sucrose, 0.2% NaNO_3 , 0.1% K_2HPO_4 , 0.05% KCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% yeast extracts in distilled water (pH 7.0 for fungi and yeasts). The pH of the media were adjusted by adding either HCl (1 N) or NaOH (30%, w/v) solution prior to sterilization. The culture media were autoclaved at 15 psi pressure for 15 min, and the substrate media were sterilized.

Fermentation procedure

Fermentations were carried out in twenty 250-ml Erlenmeyer flasks, containing 100 ml of medium B and the screened microorganism *P. putida* MTCC 1072. The flasks were divided into five sets with four containing 0.1% (v/v), four containing 0.2% (v/v), four containing 0.3% (v/v), four containing 0.4% (v/v) and four containing 0.5% (v/v) of limonene to determine the optimum concentration. The flasks were incubated for 5 days at 30 °C. Simulta-

neously, a control experiment was carried out without microorganisms by adding substrate directly into the sterile broth.

Extraction of products

Extraction of bioconversion products of limonene after treatment with *P. putida* MTCC 1072 was carried out after removing the bacterial cells by centrifugation (12,800 *g*, 10 min), and the supernatant was extracted with ethyl acetate (3×25 ml). The combined extract was washed with distilled water (3×10 ml), dried over anhydrous sodium sulfate, and filtered using Whatman No.1 filter paper. The solvent was removed under reduced pressure to obtain crude reaction products.

Purification of products

The crude reaction products were subjected to preparative TLC (PTLC) to isolate pure reaction product for identification. For PTLC, glass plates (20×20 cm) precoated with silica gel G (0.5 mm thickness) and activated at 120 °C for 1 h were used. Plates were developed in the solvent system *n*-hexane-diethyl ether (50:50, v/v) and visualized by iodine absorption. After visualization in iodine, bands were cut out and immediately placed in chloroform (15 ml). The residue, containing silica gel G, was discarded by centrifugation, and the chloroform extracts were combined and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give pure reaction products.

Analyses of products

Analyses of products were done by GLC. A 1- μl aliquot was analyzed by GLC with a Hewlett Packard HP5890 A Gas Chromatograph (Hewlett Packard, Penn., USA) equipped with a flame ionization detector (FID). A 10% DEGS (HP) column was used and operated isothermally at 140 °C. Injector and detector temperatures were set at 200 °C and 210 °C, respectively. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min.

Qualitative analysis of the reaction products was carried out by TLC on glass plates, using a 0.2-mm layer of silica gel G. The TLC solvent system was *n*-hexane-diethyl ether (50:50, v/v), and the bands were visualized by iodine absorption (R_f 0.27 and 0.42 for the two bioconversion products). Control flasks were also extracted using the same procedure described above and analyzed by TLC.

Chemical structures of the bioconversion products were identified by Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. FTIR spectra were obtained on a Perkin-Elmer 1600 Fourier transform spectrometer (Conn., USA) on NaCl plates. ^1H and ^{13}C -NMR spectra were obtained in deuterated chloroform (CDCl_3) with a Bruker AM-300 L spectrometer (Rheins, Germany) operating at 300 MHz. For ^1H -NMR, the following abbreviations were used: *br* broad, *s* singlet, *d* doublet, *t* triplet and *m* multiplet. The reference compound was tetramethylsilane (TMS). Elemental analyses were performed with a Perkin Elmer 240 instrument.

Results

Selection of strains

Fungi, yeasts and bacteria were tested for their ability to metabolize limonene. In the fungal strains *Aspergillus niger* MTCC 961 and *Rhizopus nigricans* No. 282, only a small amount of limonene conversion was observed. The only yeast strain tested in this study, *Candida tropicalis* MTCC 230, did not convert limonene at all. Five

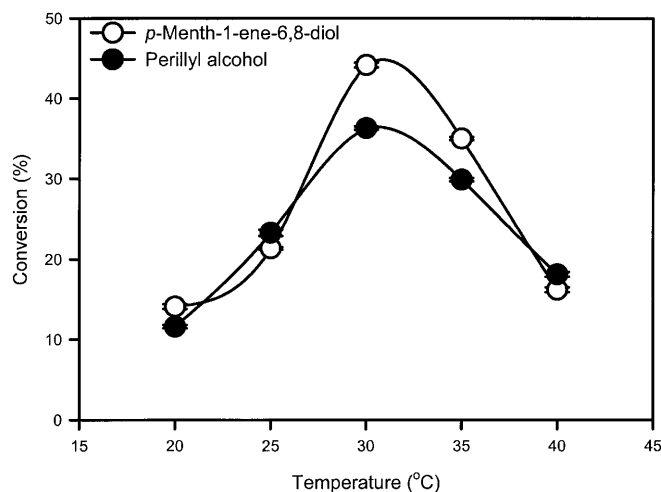


Fig. 1 Growth of *P. putida* MTCC 1072 on limonene and disappearance of growth substrate from the medium. Each assay was conducted at 30 °C

strains of bacteria, *Pseudomonas aeruginosa* MTCC 1034, *Pseudomonas putida* MTCC 102, *Pseudomonas putida* MTCC 1072, *Pseudomonas putida* MTCC 1194 and *Rhodococcus rhodochrous* MTCC 289, and ten unidentified terpene-utilizing bacteria isolated from soils were also tested for the biotransformation of limonene. Some of the bacterial isolates consumed limonene, but in very small amounts (~3%, the products were neither isolated nor identified). Amongst the bacteria, a strain of *Pseudomonas putida* MTCC 1072 was found to transform limonene to aroma compounds in reasonable yields and hence was selected for further study.

Growth of *P. putida* in culture medium

Following inoculation of the growth medium with *P. putida* MTCC 1072, a visible increase in biomass with time was observed. A growth curve was constructed by measuring the dry weight of the biomass formed at intervals (Fig. 1). For measuring the weight of the biomass, samples were filtered through a weighed ashless filter paper (Whatman No 4), which was washed with water and dried to a constant weight at 80 °C. The initial limonene concentration in this experiment was 1.23 μM. The consumption of limonene by the microorganism was evident from the sharp decline in the concentration of limonene during the first 5 days. However, although there was an increase in the quantity of biomass with time (measured up to 7 days), the consumption of limonene by *P. putida* MTCC 1072 remained the same.

Identification of bioconversion products

The biotransformation products were identified by their FTIR, ¹H- and ¹³C-NMR spectra. Two products, namely, *p*-menth-1-ene-6,8-diol and perillyl alcohol, were identified and their spectral data are given below.

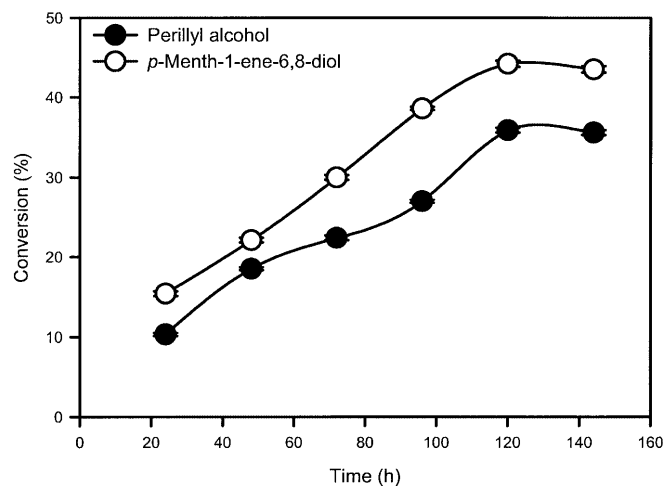


Fig. 2 Time course of microbial conversion of limonene using *P. putida* MTCC 1072 at 30 °C. Samples were analyzed after 24, 48, 72, 96, 120 and 144 h incubation. An average of two sets of data, with error bars corresponding to half the difference between them, are presented

- *p*-menth-1-ene-6,8-diol (*R_f* 0.27). C₁₀H₁₈O₂. FTIR *v*_{max} (cm⁻¹): 3400–3300 (OH), 2925, 1675 (–C=C–), 1160, 815. ¹H-NMR δ TMS (ppm): 1.26 (s, 3H, CH₃), 1.30 (s, 3H, CH₃), 1.76 (s, 3H, CH₃–C=C–), 2.16 (m, 1H, CH), 2.33–2.40 (m, 4H, 2 CH₂), 4.03 (br, 1H, –C–OH, D₂O exchangeable), 4.80 (m, 1H, CH), 5.61 (t, 1H, *J*=1.2 Hz, –C=C–H), 5.68 (br, 1H, –CH–OH, D₂O exchangeable). ¹³C-NMR δ TMS (ppm): 22.53 (CH₃), 25.17 (CH₃), 27.29 (CH₃), 29.14 (CH₂), 32.83 (CH₂), 36.02 (CH), 68.80 (CH–OH), 71.40 (C–OH), 125.21 (CH), 133.13 (C).
- Perillyl alcohol [4-(1-methylethyl)-cyclohex-1-ene-1-methanol] (*R_f* 0.42). C₁₀H₁₆O. FTIR *v*_{max} (cm⁻¹): 3350 (–OH), 2925, 1640 (–C=C–), 1455, 1107, 895. ¹H-NMR δ TMS (ppm): 1.60 (t, 1H, *J*=4.8 Hz, –OH), 1.76 (s, 3H, CH₃–C=C–), 2.26–2.06 (m, 7H), 3.70 (d, 2H, *J*=4.5 Hz, CH₂–OH), 4.74 (d, 2H, *J*=2.0 Hz, –C=CH₂), 5.70 (t, 1H, –C=C–H). ¹³C-NMR δ TMS (ppm): 22.72 (CH₃), 27.24 (CH₂), 29.41 (CH₂), 31.93 (CH₂), 42.81 (CH), 68.92 (CH₂–OH), 110.50 (CH₂), 128.01 (CH), 129.90 (C), 144.74 (C).

Effect of time on bioconversion

The time course of bioconversion of limonene by *P. putida* MTCC 1072 was carried out by analyzing the reaction products at 24, 48, 72, 96, 120 and 144 h and is shown in Fig. 2. Gradual utilization of limonene by the bacteria was observed over the growth period, and the yields of both bioconversion products increased significantly between 24 and 48 h. From Fig. 2 it is also evident that the highest incorporation of perillyl alcohol and *p*-menth-1-ene-6,8-diol (0.00444 and 0.00546 mol/l, respectively) were achieved by about 120 h, after which concentrations of the products remained unchanged, in-

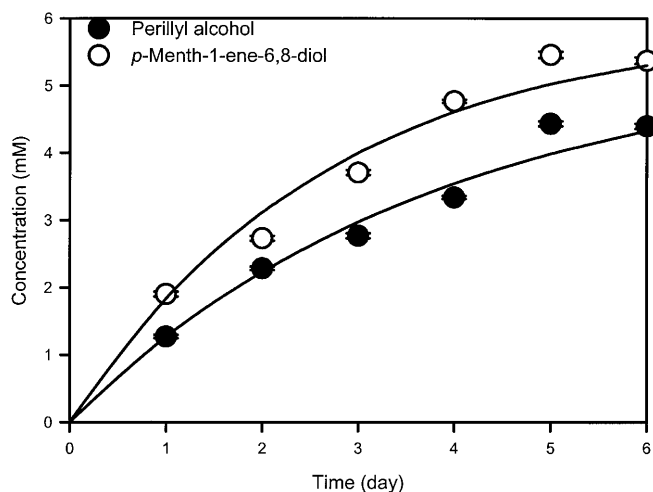


Fig. 3 Kinetics of limonene bioconversion by *P. putida* MTCC 1072 at 30 °C. Samples were analysed after 1, 2, 3, 4, 5 and 6 days. An average of two sets of data, with error bars corresponding to half the difference between them, are presented

dicating no further production or metabolism of products by the microorganism.

A simple first-order-reaction kinetic description (ignoring intermediate complexes) of the yield of the products, perillyl alcohol and *p*-menth-1-ene-6,8 diol, was made for a proper understanding of the time course of the reaction (Fig. 3). The evolution over time of the molar concentration of the products shows that the conversion of limonene to perillyl alcohol and *p*-menth-1-ene-6,8 diol is effectively unidirectional, and a preliminary attempt to model this reaction indicated that the reverse rates are less than 1% that of the forward rates. Using the scheme



one can approximately model the bioconversion of limonene. Though the scheme ignores intermediate complexes and the concentration of the enzyme is not explicitly stated, it can nonetheless be used to get an idea of the overall rate of production of the products and their relative yields. The scheme above may be written as

$$\begin{aligned} \frac{d[P]}{dt} &= r_1([L]_0 - [P] - [D]) \\ \frac{d[D]}{dt} &= r_2([L]_0 - [P] - [D]) \end{aligned} \quad (1)$$

where $[P]$ is the molar concentration of perillyl alcohol, $[D]$ is the molar concentration of *p*-menth-1-ene-6,8-diol, $[L]_0$ (0.01235 mol/l) is the initial molar concentration of limonene. The time evolutions of $[P]$ and $[D]$ from Eq. 1 are χ -squares fitted to the observed data to obtain the reaction frequency values $r_1 = 1.38 \times 10^{-6} \text{ s}^{-1}$ and $r_2 = 1.77 \times 10^{-6} \text{ s}^{-1}$. The equilibrium yield of perillyl alcohol, $r_1 L_0 / (r_1 + r_2)$, is 0.00541 mol/l and that of *p*-menth-1-ene-6,8-diol, $r_2 L_0 / (r_1 + r_2)$, is 0.00694 mol/l, while the time constant of the reaction is 88.08 h.

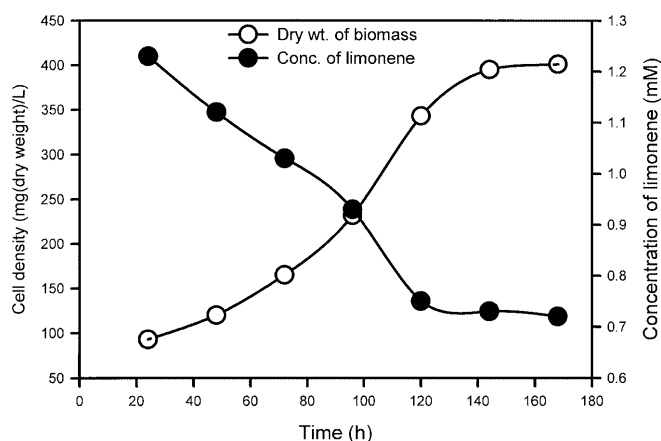


Fig. 4 Effect of temperature on the formation of perillyl alcohol and *p*-menth-1-ene-6,8-diol by *Pseudomonas putida* MTCC 1072. Each assay was conducted for 48 h. An average of two sets of data, with error bars corresponding to half the difference between them, are presented

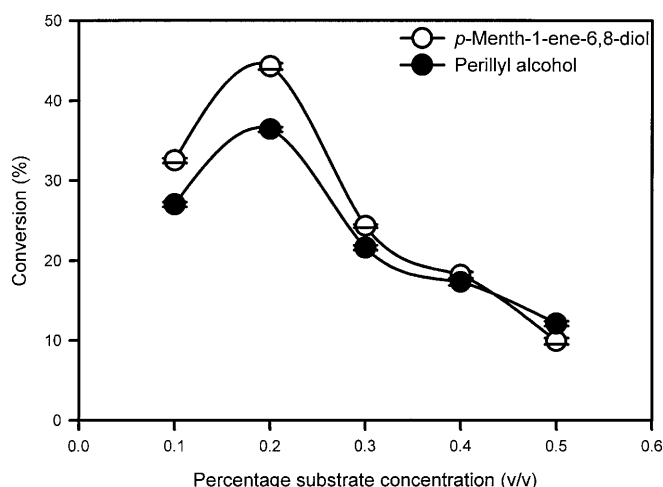


Fig. 5 Effect of limonene concentration on the activity of *P. putida* MTCC 1072. Each assay was conducted at 30 °C for 120 h. An average of two sets of data, with error bars corresponding to half the difference between them, are presented

Effect of temperature on bioconversion

The influence of temperature on the bioconversion of limonene by *P. putida* MTCC 1072 was investigated at different temperatures, at 5 °C increments, over the range of 20–40 °C. Figure 4 shows that formation of the two bioconversion products reached a maximum at 30 °C and started to decrease as the temperature was increased further.

Influence of substrate concentration

Since limonene is toxic to many microorganisms even at low concentrations, it was important to study the influence of limonene concentration on the growth of

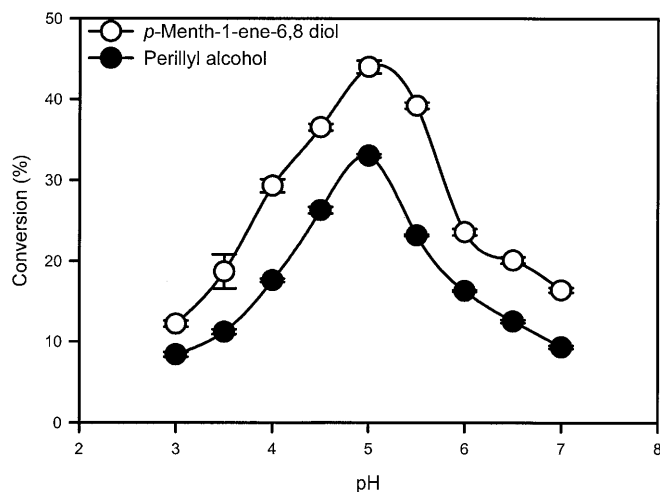


Fig. 6 Effect of pH on the bioconversion of limonene by *P. putida* MTCC 1072. Each assay was conducted at 30 °C for 120 h. An average of two sets of data, with error bars corresponding to half the difference between them, are presented

P. putida MTCC 1072. The influence of limonene concentration was tested by varying its concentrations between 0.1 and 0.5 (v/v) (Fig. 5). The maximum conversion yields (36% and 44% for perillyl alcohol and *p*-menth-1-ene-6,8-diol, respectively) were obtained at 0.2% (v/v). Using a limonene concentration of 0.5% (v/v), the yields of both products were reduced significantly, as depicted in the figure.

Effect of pH on bioconversion

To determine the influence of pH on the course of bioconversion of limonene by *P. putida*, we investigated bio-transformation over the pH range 3.0–7.0 (Fig. 6). The activity of *P. putida* MTCC 1072 toward limonene increased steadily as the acidity of the medium was reduced. The microorganism displayed good activity over a pH range of 4.5–5.5, with the highest activity at pH 5.0.

Discussion

Allylic hydroxylation of limonene is an interesting reaction due to the multiple bioactivities of several of the resulting aroma compounds. In the present study, *P. putida* MTCC 1072 effected allylic hydroxylation of the *exo*-cyclic methyl group (C-7) of limonene to give perillyl alcohol, and hydroxylations at both C-6 (*endo*-cyclic) and C-8 positions to give *p*-menth-1-ene-6,8-diol. The structures of both the bioconversion products were confirmed using various spectral methods.

It has been reported in literature that limonene increase the fluidity of fungal membranes, which leads to a high unspecific membrane permeability, the loss of membrane integrity and thus a decrease of dry matter (Heipieper et al. 1994). The high membrane fluidity may

prevent the maintenance of membrane-bound enzyme complexes involved in the oxidative transformation of terpenes (Masaphy et al. 1995).

The fungal strains *Aspergillus niger* MTCC 961 and *Rhizopus nigricans* No. 282 were able to convert only a small amount of limonene. Yeasts have seldom been considered for hydroxylation purposes, since these unicellular fungi were found to be useful for reduction reactions and as a possible source of lipases (Faber 1992). The yeast *Candida tropicalis* MTCC 230 was used in this study and no conversion of limonene was observed.

The optimum bioconversion temperature in the present study was found to be 30 °C. The decrease in the yields of the bioconversion products with increasing temperature may be attributed to the stability of the substrate and product at low temperature, which minimizes evaporation losses of limonene and volatile bioconversion products (Cadwallader et al. 1989). The consumption of limonene by *P. putida* MTCC 1072 increased with the increasing weight of the biomass (cell dry weight). However, the consumption of limonene remained the same after 5 days, although growth of the cells increased for up to 7 days. This may be attributed to the fall in the catalytic activity of the biomass. The increase in the weight of the biomass with time was possibly due to the utilization of nutrients in the medium (Cadwallader et al. 1989).

The optimum concentration of limonene in the present study was found to be 0.2% (v/v). Several studies have reported an inhibitory effect of limonene on various microorganisms (Bowen 1975; Chang and Oriel 1994). In addition, some researchers have noted that limonene inhibits the energy-producing process (oxidative phosphorylation) in cells, and causes membrane damage in microorganisms (Uribe and Pena 1990). Thus, the decrease in the yields of both bioconversion products with increasing limonene concentration above 0.2% (v/v) is in good agreement with the results of other studies (Chang and Oriel 1994).

The bioconversion pathway of limonene by *P. putida* MTCC 1072 described here allows convenient synthesis of perillyl alcohol and *p*-menth-1-ene-6,8-diol, both of which are widely used as flavor and fragrance compounds. With the growing demand for natural flavors, the biological production of such alcohols is of great commercial interest, and hence may prove to be commercially viable.

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