Biotransformation of Δ3-carene by *Penicillium nigricans*

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A fungus was isolated from forest soil by selective enrichment method with Δ3-carene as a sole source of carbon and identified as *Penicillium nigricans*. The isolate was capable of transforming Δ3-carene into neutral [dihydrocarvone, carvone, carveol, (+)-trans-p-mentha-5,8-dien-2-ol and (+)-trans-p-mentha-5,8-dien-2-one] and acidic (perillic acid and 2-hydroxy-p-menth-8-ene-7-oic-acid) metabolic compounds. These compounds were identified based on infrared (IR), proton nuclear magnetic resonance (¹H NMR) and mass spectrum (MS) studies. Three pathways have been proposed for the transformation of Δ3-carene into the neutral and acid metabolic compounds based on the study of oxygen consumption by Δ3-carene grown fungal cells. As the different metabolic intermediates of Δ3-carene are much used in the perfume industry, the Δ3-carene, which is abundantly available, can be used as a starting material in the perfume industry by microbial techniques, using this fungal strain.

**Keywords:** Biotransformation, Δ-3-carene, *Penicillium nigricans*, metabolite

**Introduction**

A process where microorganisms convert chemical compounds into useful chemical products is called microbial transformation. Terpenes are natural unsaturated hydrocarbons derived from isoprene units. Monoterpenes are branched chain C-10 hydrocarbons formed from two isoprene units. They are widely distributed in nature and are the main constituents of essential oils. They are used as raw materials for perfume and drug industries¹. Δ3-carene is a bicyclic monoterpen and is present in the commercial Indian terpentine oil². Δ3-carene cannot be stored as it readily undergoes aerial oxidation forming resins³. Attempts are being made to convert this unstable compound into stable useful intermediates that can be used in perfumery, pharmaceutical and plasticizer industries⁴,⁵. Fungi and most bacteria are normally unable to degrade and use the monoterpenes as the sole source of carbon because they have strong antimicrobial activity. However some of them can metabolize monoterpenes when grown in energy rich media⁶. Gehrig and Knight⁷ were the first to observe that fungal spores could transform organic compounds. They reported the conversion of octanoic acid in 2-heptanone by *Penicillium roqueforti*. Joglekar et al.⁸ have isolated the RDL-1 strain capable of utilizing Δ3-carene as a sole source of carbon and transforming it into perillic acid. Biotransformation of Δ3-carene in *Mycobacterium smegmatis* DSM 43061 was studied by Stumpf et al.⁹ and obtained different metabolites like (+)-chaminic acid, (+)-3-carene-5-one and 2-(3-methyl-cyclohexa-3,5-dienyl)propan-2-ol. Investigations on the bacterial transformation of Δ3-carene have been reported to obtain the neutral (carveol, p-menth-8-ene-1, 2-diol, p-menth-8-ene-1-ol-2-one and carvone) and acidic (perillic acid and 2-hydroxy p-menth-8-ene-7oic acid) products⁵. The review of literature indicates that even though a good deal of work on the microbial transformation of terpenes such limonene, pinene, camphene, linalool and citronellal have been reported by several workers, Δ3-carene has not received much attention as far as its biotransformation is concerned. This hydrocarbon was therefore selected for the present study. No work has been reported on the isolation of a fungal culture capable of utilizing Δ3-carene as a sole source of carbon. The objectives of the present study were to isolate a fungal strain capable of using Δ3-carene as a sole source of carbon, identify the different metabolites resulted from the fungal

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bioconversion of Δ3-carene and elucidate the metabolic pathways of Δ3-carene transformation.

Materials and Methods

Chemicals
All the chemicals used for the preparation of media for the maintenance and propagation of microbial culture were of reagent grade and those for the metabolic and enzymatic purpose were of analytical reagent grade. The various compounds used in these studies were obtained from the different commercial firms viz. Sigma, HiMedia, S. D. fine, SRL and Aldrich Co. The sample of Δ3-carene employed in the experimental work was obtained from Camphor and Allied Products Ltd., Bombay.

Analysis of hydrocarbon Δ3-carene
The Δ3-carene sample was purified by column chromatography on neutral silica gel and the purity was checked by thin layer chromatography (TLC). The pure Δ3-carene was then subjected to spectral studies viz. IR, $^1$H NMR and MS for the determination of the structure. IR spectra were scanned using Nicolet Impact 410-FT-IR Spectrometer. $^1$H NMR Spectra were recorded in carbon tetrachloride using Bruker Varian-300 MHzFT-NMR spectrometer. Mass spectra were recorded on MAS SPEC using Electron Impact.

Isolation and identification of the Δ3-carene-transforming fungus
A fungal strain was isolated from the forest soil sample by enrichment culture technique, using Δ3-carene as a sole source of carbon and energy. The Byrde medium supplemented with 0.2% Δ3-carene and 2.5% agar was used. The Byrde medium contained (g/L) KH$_2$PO$_4$ (0.6969), KCl (0.1495), ZnSO$_4$ (0.008), MgSO$_4$ (0.200), FeSO$_4$ (0.02), NH$_4$NO$_3$ (1.00) and MnSO$_4$ (0.006), pH 7.0. The culture was purified through subculturing on the Byrde solid medium. The colonies were finally transferred to the potato dextrose agar (PDA) slants and maintained at 4 °C. The isolate was identified on the basis of morphological and microscopic features. The isolate was further identified by authentic authority (Agarkar Research Institute, Pune).

Fermentation, extraction and separation of products of fungal transformation
After standardizing the conditions, the fermentation of Δ3-carene by *P. nigricans* was carried out in the Byrde medium in 500-ml Erlenmeyer flasks, each containing 100 ml sterile medium (pH 7.0) and a total of 0.6% substrate (by the addition of 0.2% at 0 h, 48 h and 96 h time intervals). Each flask was inoculated with 1% inoculum and incubated for 120 h on a rotary shaker (150 rpm) at room temperature. At the end of the incubation period, the contents of the flasks were pooled together and the broth was acidified with 4N hydrochloric acid (pH 2.0) and extracted thrice with ethyl acetate (broth: solvent, 3:1, v/v). The ethyl acetate layers were pooled together and shaken with keiselguhr to remove suspended cells and was treated with anhydrous sodium sulphate to remove last traces of water. The ethyl acetate layer was subjected to evaporation and the residue obtained was the total fraction. The total fraction was separated into neutral and acidic fractions by taking it in ethyl acetate layer and treating it thrice with aqueous 5% sodium bicarbonate. The organic layer was dried over anhydrous sodium sulphate and filtered. Ethyl acetate was completely removed by evaporation. The residue thus obtained contained neutral fermented products. The neutral fraction after removal of ethyl acetate was separated into various individual compounds by using column chromatography on a neutral silica gel column. The purity of the neutral compounds was monitored by TLC. The sodium bicarbonate extract containing acidic fractions was acidified with 4N hydrochloric acid (pH 2.0) and extracted thrice with ethyl acetate. The extract was dried over anhydrous sodium sulphate and filtered. Ethyl acetate was removed by evaporation and solid acidic fraction was obtained. The total liquid mixture of acids, left after removal of the solid acid fraction, was taken up for the separation by converting to methyl esters with the help of anhydrous methanol and dry hydrochloric acid gas. The methyl esters were then taken in petroleum ether and the individual pure methyl esters were separated by column chromatography on a neutral silica gel column. The purity was ascertained by TLC. The pure neutral, acid and methyl ester fractions were further subjected to IR, $^1$H NMR and Mass spectral studies.

Oxygen uptake studies
The oxygen uptake by whole cells of the isolate grown on Δ3-carene and different intermediates was performed in an oxygraph fitted with a Clark type of oxygen electrode (Hanstech, Germany). The cells were harvested in the early logarithmic phase, and subjected to centrifugation at 10,000 × g for 30 min
and washed twice with 50 mM phosphate buffer of pH 7.0. The final volume made was 1 ml, containing substrate (1μM) and appropriate amount of cells (20 mg/ml dry weight and buffer). Oxygen consumed was measured per min/mg of dry cells. All the values were corrected for endogenous respiration.

**Δ3-carene hydroxylase enzyme assay**

Cell free extracts were prepared from the washed cells suspended in three volumes of 0.05M phosphate buffer, pH 7.0 by sonication for 5 min using an ultrasonic processor model XL 2010 and centrifuged (REMI C-30 BL cooling centrifuge, India) at 15,000 × g for 40 min at 4 °C. The clear supernatant was used for enzyme assay. Δ3-carene hydroxylase was assayed spectrophotometrically by monitoring the decrease in absorbance at 340 nm due to the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH). The reaction mixture contained 0.1 ml of 30 μM NADPH, 2.7 ml of 50 μM potassium phosphate buffer (pH 7.0), 0.1 ml of substrate and 0.1 ml of crude enzyme.

**Results and Discussion**

**Analysis of Δ3-carene**

The Δ3-carene sample was purified by column chromatography and the purity was checked by TLC. The analytical and physical constants of the purified Δ3-carene are summarized in Table 1. It was colorless, free flowing oily liquid with a pleasant terpenic odour.

**Isolation of Δ3-carenetransforming fungus**

A fungus transforming Δ3-carene was isolated from a forest soil by selective enrichment method with Δ3-carene as the sole source of carbon. The isolate was identified as *P. nigricans* based on morphological and microscopic characteristics and further confirmed by the National Fungal Culture collection of India, Agarkar Research Institute Pune. The Byrde medium was chosen for growth because the oxidation of hydrocarbon is usually accompanied by acid production and the presence of carbonate or phosphate in the medium is desirable to buffer it at a favorable hydrogen ion concentration. The RDL-1 strain, *Mycobacterium smegmatis* DSM 43061 and *Enterobacter* sp. were also capable of utilizing Δ3-carene as a sole source of carbon.

**Characterization of metabolic compounds resulted from biotransformation of Δ3-carene by P. nigricans**

The biological transformation of Δ3-carene by *P. nigricans* based on IR, 1H NMR and Mass Spectral studies has resulted in the formation of various metabolic compounds, of which seven could be isolated in pure form and characterized. Two of them are acids and the remaining five are neutral metabolites containing either hydroxyl or carbonyl group or both (Fig. 1; Table 2). One of the acid metabolite was converted into a methyl ester of hydroxy acid. The IR, 1H NMR and Mass spectrum for 2-hydroxy-p-menth-8-en-7-oic-acid methyl ester values are respectively shown on Table. 2. The transformation is thus mostly oxidative and aerobic in nature. This is in keeping with the fact that the *P. nigricans* is an aerobe and requires oxygen for its growth and development. Oxygen appears to have been

<table>
<thead>
<tr>
<th>Table 1—Analytical constants of the purified Δ3-carene</th>
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<tr>
<td><strong>Appearance</strong></td>
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<tr>
<td>Refractive index (N(_{30}^D))</td>
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<tr>
<td>Optical density (α)(_{30}^D)</td>
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<tr>
<td>Density D(_{30}^0)</td>
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<td>Boiling point</td>
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<td>Molecular formula</td>
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<td>IR absorption bands cm(^{-1})</td>
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<td>(^{1})H NMR ppm</td>
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<td>Mass m/z</td>
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Fig. 1—Metabolic products resulted from the biotransformation of Δ3-carene by *Penicillium nigricans*
Elucidation of the pathways of transformation of Δ3-carene

In order to elucidate the oxidative pathway of transformation of Δ3-carene, the neutral and acid metabolites along with the substrate Δ3-carene were subjected to the oxygen uptake studies. The results of the oxygen uptake studies by Δ3-carene grown cells show that the values obtained from the oxidation of the substrate Δ3-carene and its metabolites by these cells are much higher, as compared with the insignificant values obtained using glucose grown cells. These results indicate that the enzyme systems that are operating for the transformation of Δ3-carene seem to be induced ones and not originally present in the fungus *Penicillium nigricans*. The resting cells of the culture grown on Δ3-carene show higher values of oxygen consumption in the case of the acidic transformation products, viz. perillic acid and hydroxy acid, in comparison with the neutral transformation products which show very low oxygen consumption. The oxidation of perillic acid was rapid and much faster than that of hydroxy acid. The neutral fraction was comparatively slowly oxidized by the Δ3-carene grown cells (Table 3). Based on the structures and oxygen uptake study data, the probable biotransformation pathways of Δ3-carene by *P. nigricans* have been presented in three different schemes, accommodating all the neutral and acid metabolites in total (Fig. 2). The

Table 2—Analytical constants of neutral (N1 to N5), acidic (A1 and methylester (ME1)) transformation products

<table>
<thead>
<tr>
<th></th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
<th>A1</th>
<th>ME1</th>
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<tbody>
<tr>
<td><strong>Boiling point</strong></td>
<td>85-90 °C</td>
<td>95 °C</td>
<td>100 °C</td>
<td>86 °C</td>
<td>80-85 °C</td>
<td>123-124 °C</td>
<td>140-145 °C</td>
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<td><strong>/2 mmHg</strong></td>
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<tr>
<td><strong>IR absorption</strong></td>
<td>3079, 2977,</td>
<td>3326, 2925,</td>
<td>3339, 2919,</td>
<td>3400, 1649,</td>
<td>2925, 1725,</td>
<td>3431, 2931,</td>
<td>3437, 2925,</td>
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<tr>
<td><strong>bands cm⁻¹</strong></td>
<td>1709, 1450,</td>
<td>1677, 1460,</td>
<td>1448, 1437,</td>
<td>1070, 1050,</td>
<td>1650, 1320,</td>
<td>1672, 1431,</td>
<td>1726, 1461</td>
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<tr>
<td></td>
<td>1436, 1250,</td>
<td>1036, 887</td>
<td>1000, 897, 790</td>
<td>1230, 1150,</td>
<td>1375</td>
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<td></td>
<td>1115, 905</td>
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<td><strong>¹HNMR δ ppm</strong></td>
<td>4.7, 1.74, 1.02,</td>
<td>7.15, 4.78, 1.77,</td>
<td>6.6, 5.5, 4.8,</td>
<td>5.5, 4.74, 3.88,</td>
<td>5.72, 4.78, 1.75,</td>
<td>6.75, 4.8, 4.75,</td>
<td>7.0, 4.2, 3.2,</td>
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<tr>
<td></td>
<td>1.3-2.7,</td>
<td>1.4-2.6,</td>
<td>1.75, 1.35, 1.5-,</td>
<td>1.75, 1.03,</td>
<td>1.15</td>
<td>2.2-2.8, 1.75,</td>
<td>1.27, 0.9, 1.2-</td>
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<tr>
<td><strong>Mass m/z</strong></td>
<td>152</td>
<td>150</td>
<td>152</td>
<td>151</td>
<td>150</td>
<td>166</td>
<td>182</td>
</tr>
<tr>
<td><strong>Molecular formula</strong></td>
<td>C₁₀H₁₅O₂</td>
<td>C₁₀H₁₅O₂</td>
<td>C₁₀H₁₅O₂</td>
<td>C₁₀H₁₅O₂</td>
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<td>C₁₀H₁₅O₂</td>
<td>C₁₀H₁₅O₂</td>
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<tr>
<td><strong>Molecular weight</strong></td>
<td>152</td>
<td>150</td>
<td>152</td>
<td>151</td>
<td>150</td>
<td>166</td>
<td>182</td>
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</table>

Extensively used to oxidize the organic molecule. A part of the molecule has probably been used as a source of energy, producing carbon dioxide and water. Whatever has been isolated as transformation products might have been formed at intermediate stage or as final accumulations. Some of them were resistant to a further transformation by the same strain. The neutral and the acid products obtained in Δ3-carene fermentation by the *P. nigricans* are similar to other neutral and acid products obtained in the fermentation of limonene by earlier workers 13,14. However, Δ3-carene fermentation by the isolate has not resulted in the intermediate formation of the first ring fission product viz., limonene, the opening of cyclopropane ring. At the same time none of the neutral and the acid products isolated and characterized shows the presence of the cyclopropane ring (broad band in the infrared spectrum at 2900-3100 cm⁻¹ and also peak at 0.82 δ ppm in ¹H NMR spectrum). Surprisingly the cyclopropane ring fission seems to have occurred simultaneously with the oxidation of methyl groups at position 2 or 7, only, which can explain the formation of the different products isolated during the present study.

Fig. 2—Proposed metabolic pathways of the transformation of Δ3-carene by *Penicillium nigricans*. Transformation of Δ3-carene into perillic acid and 7-hydroxy p-menth-8-en-7-oic acid methyl ester (P-I, Pathway-1); Δ3-carene to carveol (2), (+)-trans-p-menth-5,8-dien-3-ol (3), (+)-trans-p-menth-5,8-dien-2-one (4) and then to carveone (5) (P-II) and from Δ3-carene to dihydrocarvone (P-III).
The values were corrected for the endogenous compounds (Fig. 2, P-III) can be explained as mechanism of the formation of the remaining neutral compounds that are different from the ones of the IR, proposed structures would satisfy all the requirements further isomerisation to give carvone (5). The trans-p-mentha-5,8-dien-2-one (4), which undergoes oxidation to the keto group to give (+)-trans-p-mentha-5,8-dien-3-ol (3). The hydroxyl group of compound (3) of the double bond) to give (+)-trans-p-mentha-5,8-dien-2-one (4). The hydroxyl group of Δ3-carene undergoes isomerisation (change in position of the cyclopropane ring) generating a tertiary carbocation leading to the decyclisation of the cyclopropane molecule favors the formation of perillic acid, with the elimination of a proton. Similarly, Δ3-carene was converted to perillic acid and 2-hydroxy Δ3-carene methyl ester (ME) which shows maximum oxygen consumption next to the substrate Δ3-carene, followed by the second metabolite 2-hydroxy p-menth-8-ene-7-oic acid methyl ester which shows the oxygen consumption value much less than perillic acid (Table 3). The formation of perillic acid from Δ3-carene occurs probably in two stages (Fig. 2, P-I). In the first step the methyl group gets oxidized to the carboxyl group. This generation of the acid within the molecule favors the decyclisation of the cyclopropane ring generating a tertiary carbocation leading to the formation of perillic acid, with the elimination of a proton. Similarly, Δ3-carene was converted to perillic acid and 2-hydroxy p-menth-8-ene-7-oic acid by Enterobacter sp. \(^5\). The mechanism of formation of the neutral compounds (Fig. 2, P-II) can be explained as follows. Δ3-carene first undergoes hydroxylation and the cyclopropane ring is cleaved to give the compound carveol (2). In the next step the compound carveol undergoes isomerisation (change in position of the double bond) to give (+)-trans-p-mentha-5,8-dien-3-ol (3). The hydroxyl group of compound (3) undergoes oxidation to the keto group to give (+)-trans-p-mentha-5,8-dien-2-one (4), which undergoes further isomerisation to give carvone (5). The proposed structures would satisfy all the requirements of the IR, \(^1\)H NMR, and Mass Spectrum of the metabolites. The biotransformation of Δ3-carene by Enterobacter sp. also starts by carveol and ends with carveone but passes through two intermediates (p-menth-8-ene-1, 2-diol and p-menth-8-ene-1-ol-2-one) that are different from the ones of \(P. \) nigricans \(^5\). The mechanism of the formation of the remaining neutral compounds (Fig. 2, P-III) can be explained as follows: the substrate Δ3-carene undergoes epoxidation across the double bond to give an epoxide that undergoes ring cleavage to give a keto compound dihydrocarvone. This keto compound is the hydrogenated derivative of compound carvone of P-II. Biotransformation of Δ3-carene was investigated in some other organisms. In rabbits, 3 metabolites viz. m-mentha-4,6-dien-8-ol (major product), 3-caren-9-ol (minor product) and m-cymen-8-ol (in trace amount) were obtained \(^15\). In Mycobacterium smeegmatis DSM 43061, different metabolites like (+)-chaminic acid, (+)-3-caren-5-one and 2-(3-methyl-cyclohexa-3,5-dienylpropan-2-ol \(^6\) were found. In the larvae of Spodoptera litura, the hydrocarbon underwent regio- and stereoselective oxidation to give (+)-(1S, 3S, 4R, 6R, 7S)-3, 4-epoxycaran-9-ol (1-2) \(^16\). As the resulted metabolites are different, the Δ3-carene biotransformation may differ from one organism to another.

**Action of Δ3-carene hydroxylase**

Experiments were also carried out to check the presence of the hydroxylase enzyme. Δ3-carene hydroxylase catalyses the hydroxylation of Δ3-carene to yield carveol (Fig. 2; P-II, step 1). The enzyme Δ3-carene hydroxylase was revealed to act on the substrate Δ3-carene proving that the very first step in the transformation was hydroxylation of Δ3-carene showing that it is a key enzyme in the transformation. The regioselective hydroxylation was also the first step in Δ3-carene biotransformation by the larvae of Spodoptera litura \(^16\).

**Conclusion**

The results of the present investigation bring out the most versatile characters of biological reactions and show how the hydrocarbon Δ3-carene could be degraded in more than one way by \(P. \) nigricans, and the biological method produces compounds entirely different from chemical oxidation. They have helped to know a great deal about the different metabolic intermediates, which are of much use in the perfume industry. Thus Δ3-carene, which is abundantly available, can be biotransformed into acidic and neutral metabolites that can be used in the perfume industry.

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