CHAPTER IV

DISCUSSION
As seen in the previous chapter, the biological transformation of \(\Delta_3\)-carene with the fungal strain *Penicillium nigricans*, 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 remaining five are neutral metabolites containing either hydroxyl or carbonyl group or both. The transformation is thus mostly oxidative and aerobic in nature. This is in keeping with the fact that the *Penicillium nigricans* is an aerobe and requires oxygen for its growth and development. Oxygen appears to have been extensively used to oxidize the organic molecule. A part of the molecule has probably been used as 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. Comparatively some of them are resistant to further transformation by the same strain. Attempts are made to determine the structures of these intermediates based on spectral studies and also to elucidate the pathway of transformation of \(\Delta_3\)-carene with the help of oxygen uptake studies.

After complete elution of unconverted \(\Delta_3\)-carene in the petroleum ether, the first metabolite that is eluted in 5% ether in petroleum ether is \(N_1\) fraction having a molecular formula \(C_{10}H_{16}O\), based on the molecular ion peak in the
mass spectrum. It is neutral compound showing an infrared band for ketone group at 1709 cm$^{-1}$ (fig. 3a) which is not found in the infrared spectrum of Δ3-carene (fig. 1a), =C-H Stretching at 3079 cm$^{-1}$, the aliphatic –CH stretching appears at 2977 cm$^{-1}$ and C=C stretching at 1450 cm$^{-1}$.

The $^1$H NMR spectrum (fig. 3b) shows >C=CH$_2$ protons as singlet at 4.7 δ ppm, the methyl protons appear as singlet at 1.74 δ ppm, another methyl protons appears as singlet at 1.02 δ ppm and the other CH$_2$/CH protons appear as multiplet from 1.3 to 2.7 δ ppm. It is further confirmed by its mass spectrum (fig. 3c), which shows molecular ion peak at m/z 152.

Thus, based on the IR, $^1$H NMR and Mass Spectral data & by comparision with the literature, neutral fraction N$_1$ having the molecular formula C$_{10}$H$_{16}$O is tentatively identified as dihydrocarvone with the following structure

(1) DIHYDROCARVONE

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The third fraction N\textsubscript{2} was eluted from the column in 10% ether in petroleum ether. The infrared spectrum (fig 4a) peak shows band at 3326 cm\textsuperscript{-1} for C=CH, aliphatic –CH stretching band at 2925 cm\textsuperscript{-1}, the carbonyl functional ketonic group at 1677cm\textsuperscript{-1} (-C=O) and a band for C=C is appears at 1436 cm\textsuperscript{-1}.

The \textsuperscript{1}H NMR (fig. 4b) indicates the C=CH protons as singlet at 7.15 δ ppm, >C=CH\textsubscript{2} protons appear as doublet at 4.78 δ ppm, 2-CH\textsubscript{3} and 6-CH\textsubscript{3} protons appear as singlets at 1.77δ ppm and 1.18 δ ppm and the –CH\textsubscript{2}/ CH protons appears as multiplet from 1.4-2.6 δ ppm. It is further confirmed by its mass spectrum (fig. 4c), which shows the molecular ion peak at m/z 150.

Based on the IR, \textsuperscript{1}H NMR and mass spectral data and comparison with literature spectral data, the neutral fraction N\textsubscript{2} is identified as a carvone (2), having the molecular formula C\textsubscript{10}H\textsubscript{14}O, with the following structure.

![Carvone Structure](2) CARVONE
The fourth fraction designated as N₃ was eluted with 25% ether in petroleum ether. The infrared spectrum (fig. 5a) indicated the presence of hydroxyl group at 3339 cm⁻¹, -CH aliphatic stretching at 2919 cm⁻¹, both 1647 and 887 cm⁻¹ due to the isopropenyl group and C=C band at 1448 cm⁻¹.

The ¹H NMR spectrum (fig. 5b) shows -OH protons as singlet at 6.6 δ ppm, C=CH proton appears as singlet at 5.5 δ ppm, C=CH₂ protons appear as singlet at 4.8 δ ppm, methyl protons as singlet at 1.75 δ ppm, another terminal methyl protons as singlet at 1.35 δ ppm and other usual CH₂/CH protons appear as multiplet as 1.5-2.3 δ ppm. Further it was confirmed by its mass spectrum (fig. 5c), which shows molecular ion peak at m/z 152.

Based on IR, ¹H NMR and mass spectral data and comparison with literature spectral data the neutral fraction N₃ having molecular formula C₁₀H₁₆O was identified as carveol (3) with the following structure:

![Carveol Structure](image)

(3) CARVEOL
The fifth fraction designated as N₄ was eluted with 25% benzene in ether. The infrared spectrum (fig 6a) showed a broad peak at 3400 cm⁻¹ for the presence of –OH functional group, a band at 2920 for the aliphatic –CH stretching.

The \(^1\)H NMR spectrum (fig. 6b) shows –CH=CH– protons as broad singlet at 5.58 ppm, -C=CH₂ singlet at 4.74 δ ppm, -CH-OH protons as singlet at 3.88 δ ppm, -CH₃-C=CH₂ protons as singlet at 1.75 δ ppm and -CH-CH₃ protons as doublet at 1.03 δ ppm. Further the mass spectrum (fig. 6c) shows molecular ion peak at m/z 151.

Based on IR, \(^1\)H NMR and mass spectral data and by comparison with the literature spectrum\(^{155}\) the neutral fraction N₅, having molecular formula C₁₀H₁₅O was identified as (4) (+)-trans-p-mentha-5,8-dien-2-ol and the following structure.

(4) (+)-trans-p-mentha-5,8-dien-2-ol
The sixth fraction designated as N₅ was eluted with benzene. The infrared spectrum (fig. 7a) indicated the presence of –CH stretching at 2925 cm⁻¹ and –C=O carbonyl group at 1725 cm⁻¹.

The ¹H NMR spectrum (fig. 7b) shows –CH=CH- protons as singlet at 5.72 δ ppm, CH₃-C=CH₂ protons as singlet at 4.78 δ ppm, -CH₃-C=CH₂ protons as singlet at 1.75δ ppm and –CH-CH₃ protons as doublet at 1.15 δ ppm. Further, it is confirmed by its mass spectrum (fig. 7c) which shows the molecular ion peak at m/z 150.

Thus, based on the IR, ¹H NMR and Mass Spectral data and comparison with the literature spectrum ¹⁵⁵ the neutral fraction N₅ was identified as (+)-trans-p-mentha-5,8-dien-2-one, (5) having molecular formula C₁₀H₁₄O, with the following structure.

\[
\text{(5) (+)-trans-p-mentha-5,8-dien-2-one}
\]
The total acid fraction on keeping in refrigerator resulted in the separation of a colorless crystallized solid with m.p = 123-124°C, which was designated as $A_1$.

The Infrared spectrum (fig. 8a) indicated the presence of $-\text{OH}$ functional group at 3431 cm$^{-1}$, $-\text{CH}$ stretching band at 2931 cm$^{-1}$, carbonyl functional group at 1672 cm$^{-1}$ and the presence of $C=\text{C}$ is appears at 1431 cm$^{-1}$,

The $^1\text{H NMR}$ spectrum (fig. 8b) shows $-\text{OH}$ proton of carboxylic acid as singlet at 6.75 δ ppm, $=\text{CH}$ appears as singlet at 4.8 δ ppm, $=\text{CH}_2$ protons appears as singlet at 4.75 δ ppm, other $-\text{CH}_2$ and $-\text{CH}$ as multiplet from 2-2-2.8 and the terminal $-\text{CH}_3$ appears at 1.75 δ ppm as singlet. Further it is confirmed by its mass spectrum (fig. 8c), which shows the molecular ion peak at m/z 166.

Based on the melting point, IR, $^1\text{H NMR}$ and Mass Spectral data and by comparison with authentic spectra, the acid fraction $A_1$ was identified as (6) perilllic acid having molecular formula $C_{10}H_{14}O_2$ with the following structure

(6) PERILLIC ACID
The remaining acid fraction was converted to methyl ester. The total methyl ester fraction on thin layer chromatography showed the presence of one major fraction, associated with trace quantities of 2 other methyl esters. This mixture was subjected to column chromatography on neutral silica gel. The major fraction was eluted in 10% ether in petroleum ether and designated as ME1.

The infrared spectrum (fig. 9a) also shows the presence of -OH functional group at 3427 cm\(^{-1}\), aliphatic -CH stretching at 2925 cm\(^{-1}\), carbonyl (C=O) functional group at 1726 cm\(^{-1}\) and C=C stretching at 1461 cm\(^{-1}\).

The \(^1\)H NMR spectrum (fig.9b) shows the presence of -OH proton as singlet at 7.00 \(\delta\) ppm, -C=CH\(_2\) protons appear as singlet at 4.2 \(\delta\) ppm, ester -CH\(_3\) protons appear as singlet at 3.2 \(\delta\) ppm, terminal methyl protons as singlet at 1.2 and 0.9 \(\delta\) ppm and the other -CH\(_2\) / CH protons appear as multiplet from 1.2-1.8 \(\delta\) ppm. Further, the mass spectrum (fig.9c) shows the presence of molecular ion peak at m/z 182, corresponding to the molecular formula C\(_{11}\)H\(_{18}\)O\(_2\).

Based on the IR, \(^1\)H NMR and mass spectral data the methyl ester fraction ME1 was tentatively identified as (7) methyl ester of hydroxy acid with the molecular formula C\(_{11}\)H\(_{18}\)O\(_2\) with the following structure.
Thus one can see clearly the formation of five neutral and two acid products for Δ3-carene by fermentation with *Penicillium nigricans*. (fig.9)

Looking back into the literature it is evident that the neutral and the acid products obtained in Δ3-carene fermentation by the *Penicillium nigricans* are similar to some of the neutral and the acid products obtained in fermentation of limonene by earlier workers. However Δ3-carene fermentation 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 cyclopropane ring (broad band in 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 studies.
Fig. 9: Products obtained by the transformation of Δ3-carene by *Penicillium nigricans.*
In order to elucidate the oxidative pathway of transformation of Δ3-carene, the substrates as well as the various neutral and acid products isolated were subjected to the oxygen uptake studies by using oxygraph fitted with Clark type of oxygen electrode (Hanstech Germany).

The results of the oxygen uptake studies by Δ3-carene grown cells show that the values obtained for 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.

Based on the structures and oxygen uptake studies data, the probable biotransformation pathways of Δ3-carene by Penicillium nigricans have been presented in three different schemes, accommodating all the neutral and acid metabolites in total. As seen from the table-15, under chapter-III “Results”, it is evident that the resting cells grown on Δ3-carene shows higher values of oxygen consumption in case of the acidic transformation products viz., perillic acid and hydroxy acid, when compared with the neutral transformation products, which show less oxygen consumption.
The oxidation of perillic acid was rapid and was faster than that of
hydroxy acid (table 15). Based on this data, the oxidation of Δ3-carene to the
acid metabolites could be explained on the basis of the following probable
pathway proposed as shown below (fig. 10), (scheme-1).

**SCHEME – 1**

\[ \text{Δ3-Carene} \rightarrow \text{Perillic acid} \rightarrow \text{Hydroxy acid} \]

Fig. 10: Proposed pathway for perillic acid and 7-hydroxy p-menth-8-ene-
7-oic acid methyl ester from Δ3-carene transformation by

*Penicillium nigricans.*
This pathway indicates that perillic acid is the first metabolite to be formed as it 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. The formation of perillic acid from Δ3-carene occurs probably in two stages (fig. 10). In the first step the methyl group gets oxidized to carboxyl group. This generation of acid with in 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, as shown by earlier workers. The mechanism can be shown to occur as follows. (fig. 11)

![Proposed mechanism of formation of perillic acid from Δ3-carene](image)

The mechanism of formation of some of the neutral compounds can be explained as follows. Δ3-carene (1) first undergoes hydroxylation and then the cyclopropane ring is cleaved to give the compound carveol(2). In the next step the compound carveol undergoes isomerisation (i.e there is a change in position of double bond) to give (+)-trans-p-mentha-5,8-dien-3-ol(3). The
hydroxyl group of compound (3) undergoes oxidation to keto group to give the compound (+)-trans-p-mentha-5,8-dien-2-one (4). Again compound (4) undergoes isomerisation to give compound carvone (5). The proposed structures would satisfy all the requirements of the IR, $^1$H NMR, and Mass Spectrum of the metabolites. The probable pathway proposed for scheme II is given as follows (fig. 12).

Fig.12: Probable pathway proposed for $\Delta^3$-carene to carvone transformation by Penicillium nigricans.
The mechanism of formation of remaining neutral compounds can be explained where in the substrate Δ3-carene undergoes epoxidation across double bond to give an epoxide. This intermediate epoxide then undergoes ring cleavage to give a keto compound dihydrocarvone. This keto compound is the hydrogenated derivative of compound carvone of scheme II, shown in fig. 12.

The probable pathway proposed for scheme III is given as follows (fig. 13).

**SCHEME III**

![Scheme III](image)

**Fig. 13:** Proposed mechanism for formation of neutral compounds from Δ3-carene.
Fig. 14: Probable Pathways of degradation of Δ3-carene by

Penicillium nigricans.

P-I Scheme - I
P-II Scheme - II
P-III Scheme - III
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. 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 existence of these pathways remains to be proven by adequate enzyme studies, which is possible only after improving the yields of the different intermediates.

At this stage the relationship of the acid metabolites with the neutral ones remains rather obscure.

With all the limitations, the results of the present investigation are quite interesting.

In the first place, once again they bring out the most versatile characters of biological reactions and secondly show that somewhat refractory terpene hydrocarbon Δ3-carene could be degraded in more than one ways, and the biological method produces compounds entirely different from chemical oxidation.