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Research Article

BIOTRANSFORMATION OF LIMONENE BY FREELY SUSPENDED AND IMMOBILISED CELLS OF NIGELLA SATIVA

RASHEED-UZ-ZAFAR, AFSHAN KAUSAR

Faculty of pharmacy, Jamia Hamdard, New Delhi, India. Email: afshan.ksr@gmail.com

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ABSTRACT

Seeds of *Nigella sativa* possess rarest kind of phytoconstituents, giving potential clues of presence of unique enzymes. Cultured plant cells are valuable source of enzymes to transform exogenous chemicals. As a preliminary attempt to explore the enzymes involved, biotransformation potential of *Nigella sativa* was checked for the first time on limonene. Cell suspension cultures and the immobilized cells were established, from primary static callus cultures of seeds of *Nigella sativa*. Limonene at 0.2 µl $ml^{-1}$when fed to system resulted in the production of carveol, limonene-1,2-diol , p-mentha-2,8-diene-1-ol-trans and carvone in an average concentrations (% v/v ; calculated at 24 h, 48 h and 72 h) 16.36, 16.3, 4.26 and 2.7 respectively. Similar transformations took place with immobilized cells but in higher yields than that in freely suspended cells. The average increase in % yields were 1.53 for limonene-1,2-diol, 2.63 for p-mentha-2,8-diene-1-ol-trans, 6.16 for carveol and 6.80 for carvone.

Keywords: Biotransformation, Cell culture, Immobilization, *Nigella sativa*, Enzyme, Limonene.

INTRODUCTION

Plant cell cultures are the source of enzymes for carrying out potential biotransformation of exogenously administered chemicals to the cultured cells.[1,2] Recently , the application of cultured cells for the conversion of cheap & plentiful substances into rare & expensive substances, utilizing multienzyme reactions has been the subject of many papers.[3,4] Biotransformation study of exogenous chemicals using plant cells has other potential advantagestoo. Even if the transformation does not lead to the formation of higher value substances, as it can't be predicted, the type of products biotransformed suggests the class of enzymes involved and constitutes a preliminary step towards the elucidation of biosynthetic pathways. Thus biotransformation of chemicals using in-vitro cultured plant cells can be considered as a low cost model to study biosynthetic pathways. Seeds of *Nigella sativa* are found to be possessed with rarest kind of phytochemicals.[5,6,7] The unusual phytochemical profile suggests a strong possibility of the presence of some unusual enzyme machinery in the seeds of *Nigella sativa* , which can be used for some potential biotransformation. It prompted us for using *Nigella sativa* to effect the desired transformations. Biotransformation of any compound using cultured cells of *Nigella sativa* has not previously been reported. Limonene, the most abundant monocyclic monoterpene is readily available, cheap and highly optical pure.[8] Its simple carbon skeleton makes it prone for transformations. The large price differences between limonene and its oxygenated derivatives and the diverse properties of closely related monoterpene, important in flavour and perfume industries justify the demand of conversion of limonene into products of higher value. Besides, classical chemical oxidation procedures for conversion of limonene are not quite successful due to similar electronic properties of allylic methylene and allylic methyl groups in limonene molecule.[9,10] So, for the obvious reasons biotransformation of limonene using cultured cells of *Nigella sativa* is carried out. Here, along with freely suspended cells, immobilized cells are also employed for carrying out biotransformation. Immobilisation which confines a catalytically active enzyme or cell within a reactor system, when employed in biotransformation studies, offer advantages of better resistance to shear damage, repeated usage over a prolonged period, better yields, recovery of the cell mass and products. So, the employment of Immobilisation technique here might help in the examination of its strength and the weakness when used as a technique for deploying cultured nigella cells in reactor.

MATERIALS AND METHODS

Chemicals

(R)-(+)-Limonene, 97 % was purchased from Sigma-Aldrich. Dichloromethane, HPLC grade was purchased from Merck specialities private limited, Mumbai. Dimethyl sulphoxide was from S D fine-chem limited. All other chemicals used in tissue culture were of analytical grade.

Cell culture

Seeds of *Nigella sativa* were surface sterilized with 1 % (v/v) sodium hypochlorite (contact time 7 mins). Primary seed callus from whole seed was grown and maintained in Murashige & Skoog's (MS) medium supplemented with different combinations of plant growth hormones. Various combinations used were 1.0 mg 2,4-dichlorophenoxyacetic acid (2,4-D) L^{-1} + 1.0 mg Kinetin (Kin) L^{-1}, 1.0 mg Naphthaleneacetic acid (NAA) L^{-1} + 1.0 mg 2,4-D L^{-1}, 1.0 mg indole acetic acid (IAA) L^{-1} + 1.0 mg Kin L^{-1} , 1.0 mg benzyl adenine L^{-1} (BA) +1.0 mg 2,4-D L^{-1}, 1.0 mg Kin L^{-1} + 1.0 mg NAA L^{-1}, 1.0 mg 2,4-D L^{-1} + 1.0 mg Kin L^{-1} + 1.0 mg BA L^{-1} and 1.0 mg Kin L^{-1} + 1.0 mg BA L^{-1} + 1.0 mg NAA L^{-1}. Callus cultures were subcultured at every 30 day interval to fresh medium. 100 mg ascorbic acid L^{-1} was introduced into media in second passage of callus growth. Suspension cultures (5 g fresh weight of callus in 50 ml medium) were initiated from fourth generation of callus in same media (without agar) containing 1.0 mg IAA L^{-1} + 1.0 mg Kin L^{-1}. Suspension cultures were incubated for 3 weeks in 250 ml conical flasks at 25 ± 2 ^{o}C on an orbital shaker (100 rpm) with a light intensity of 1600 lux for 16 hr a day.

Preparation of the immobilized cells

After leaving the suspension cultures for one week, cells were collected by passing the suspensions through nylon cloth (20 mesh). Packed cells (5.0 cm^3) were suspended in 4 % Na alginate (50 ml), the suspension was added dropwise to 0.1 M $CaCl_2$ solution. Beads were left for 15 minutes in the same solution for stabilization. The formed beads (mean diameter 3.0 mm) were collected using nylon cloth and then cultured in the same medium under same conditions that of suspension cultures. All procedures were carried out under sterile conditions.

Biotransformation

200 µl of limonene was dissolved in 10 ml DMSO for the uniform dispersion of limonene in aqueous phase. 400 µl of the prepared solution was injected through a preautoclaved mobile phase filter (0.45 mM pore size) to the suspensions, under aseptic conditions. Blank experiment was carried out by adding no substrate to media containing suspended cells in order to check the presence of compounds in the cell suspensions, under same conditions. Another blank trial consisting of the cultured medium with administered limonene without the addition of cell suspension was conducted under the same conditions in order to check the non-enzymatic modifications of substrate. Flasks containing suspension cultures

and immobilized cells with added limonene and blanks were incubated for three days under the same conditions as that of suspension cultures.

Analysis

After vigorous shaking, 25 ml samples were taken out aseptically from suspensions at 24, 48 and 72 h. Suspensions were centrifuged at 2400 rpm for 5 min. Plant cells and media were extracted with twice the volume of dichloromethane. Extracts were then combined and passed through Whatman no. 1 filter paper. Extracts were reduced to a volume of 5 ml at 25 °C under vaccum using rotary evaporator. Compounds in dichloromethane extract were identified using GC MS with Rtx-5MS column (60 m length, 0.25 mm i.d. and 0.25 µm df). All samples were analysed in duplicates. Each extract (1 µl) was injected into a splitless mode (270 °C injection temperature). Helium (purity 99.999 %) at a linear velocity of 28.7 cm/s was used as the carrier gas. The oven temperature was programmed from 80 to 180 and finally to 280 °C with an initial hold time of 2 min. to 6 min. and a final hold time 20 min. The identifications were complete by comparison of retention times and MS using wiley spectra library and by interpretation of mass fragmentation patterns. MS fragmentation patterns were, limonene-1,2-diol ([170] 27, 41, 43, 67, 71, 93, 108, 123, 137, 152), carvone ([150] 38, 39, 54, 67, 82, 93, 108), carveol ([152] 27, 41, 55, 69, 83, 84, 109, 119, 134) and p-mentha-2,8-dien-1-ol-trans (+) ([152] 39, 41, 43, 67, 79, 91, 109, 119, 134, 152).

RESULTS AND DISCUSSIONS

The biotransformation results are summarised in Table 1. *Nigella sativa* seed cultures did not produce any terpenes. Limonene when fed to the cultures resulted in the production of carveol, limonene-1,2-diol, p-mentha-2,8-dien-1-ol-trans (+) and carvone in order of decreasing concentrations.

Table 1: Shows the profile of biotransformed products of limonene in freely suspended cells system and in immobilized cells system of *Nigella sativa* at different time intervals.

Products	F 24 hrs [%Y]	I 24 hrs [%Y]	F 48 hrs [%Y]	I 48 hrs [%Y]	F 72 hrs [%Y]	I 72 hrs [%Y]
Carveol	13.7	20	20	26	15.4	21.6
Limonene-1,2-diol	12.5	13.9	22.4	24.0	14.0	15.6
p-Mentha-2,8-diene-1-ol	5.6	8.5	3.6	6.0	3.9	6.0
Carvone	2.7	9.5	0	0	0	0

F: freely suspended cells system I: immobilized cells system

[%Y]: yield (%) of products relative to concentration of limonene administered (0.2 µl ml^{-1}).

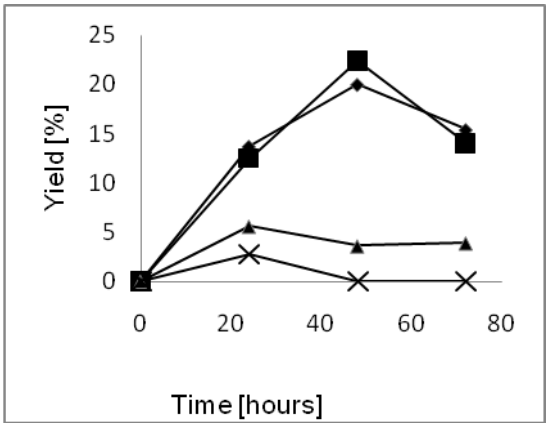

Fig. 1: Concentration of biotransformed products in suspension cultures of Nigella sativa after feeding limonene. Carveol ◆, limonene-1,2-diol ■, p-mentha-2,8-dien-1-ol-trans ▲, carvone X.

* Yield (%) of products relative to concentration of limonene administered (0.2 µl ml^{-1})

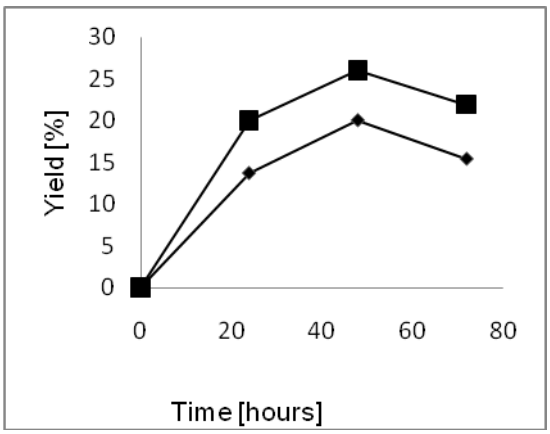

Fig 2 Concentrations of carveol in freely suspended and immobilized cells of N. sativa. Freely suspended cells (—◆—) , immobilized cells (—■—).

* Yield (%) of products relative to concentration of limonene administered (0.2 µl ml^{-1}).

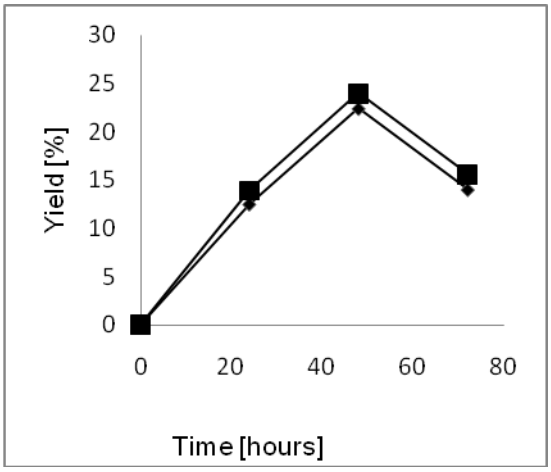

Fig. 3: Concentrations of limonene-1,2-diol in freely suspended and immobilized cells of *N. sativa.* Freely suspended cells (◆—), immobilized cells (■—).

* Yield (%) of products relative to concentration of limonene administered (0.2 µl ml^{-1}).

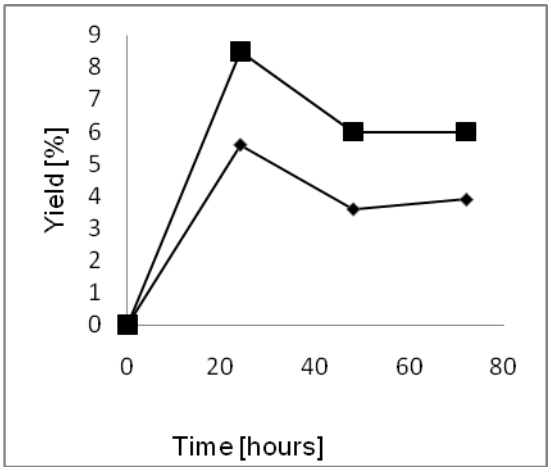

Fig. 4: Concentrations of p-mentha-2,8-diene-1-ol-trans (+) in freely suspended and immobilized cells of *N. sativa.* Freely suspended cells (◆—), immobilized cells (■—).

* Yield (%) of products relative to concentration of limonene administered (0.2 µl ml^{-1}).

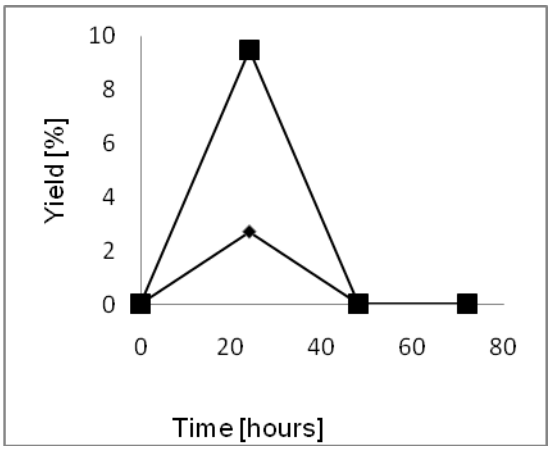

Fig. 5: Concentrations of carvone in freely suspended and immobilized cells of *N. sativa.* Freely suspended cells (◆—), immobilized cells (■—).

* Yield (%) of products relative to concentration of limonene administered (0.2 µl ml^{-1}).

The concentrations of biotransformed products over the initial 72 h after feeding 0.2 µl limonene ml^{-1} are shown in figure 1. Products decreased in concentration or attained a constant level after reaching to their peak concentrations, depicting the general characteristic of enzymatic reactions. Same biotransformation reactions but at a higher yield rate were found to occur with immobilized cells. Figs 2-5 represent the comparative yields of all the products at different time intervals in freely suspended cells and in immobilized cells.

Among all the products, carvone showed the maximum increase of 6.8 % in yield with immobilized cells as compared to that with freely suspended cells. The average increase in % yields were 1.53 for limonene-1,2-diol, 2.63 for p-mentha-2,8-diene-1-ol-trans, 6.16 for carveol and 6.80 for carvone. All the bioconversions were initiated from a common regioselective hydroxylation of limonene. Formation of carveol and carvone is in accordance with the biosynthetic pathways in plants starting from limonene. In light of literature data, there is strong possibility of involvement of cytochrome P-450 enzyme in the first step of bioconversion sequence. The biosynthetic P-450s that have been characterised to date were shown to have an extremely high regiospecificity, but low substrate enantioselectivity.[11] Formation of carvone, found in low concentration only at 24 h might either be due to auto-oxidation of carveol or due to enzymatic conversion with lesser affinity for it. Because of the total disappearance of carvone at 48 and 72 h, possibility of reduction of carvone back to carveol can also not be neglected. Formation of diol is supposed to occur via the formation of corresponding epoxide. The formation of p-mentha-2,8-dien-1-ol-trans (+) as a biotransformed product of limonene strengthens the probability of formation of an intermediate epoxide. p-mentha-2,8-dien-1-ol, an important intermediate in the synthesis of menthol and cannabinoids is chemically prepared from limonene by rearrangement of its epoxide.[12] Role of an intermediate epoxide is postulated which by its rearrangement via an array of enzymatic conversions leads to the formation of enantioselective p-mentha-2,8-dien-1-ol-trans (+). The same epoxide via some epoxide hydrolase produces limone-1,2-diol. Among enzymes, epoxide hydrolases are probably one of the most versatile biocatalysts which carry out hydrolysis of epoxides producing corresponding vicinal diols.[13] Fig 6 depicts the most probable scheme of reactions taking place in bioconversion process.

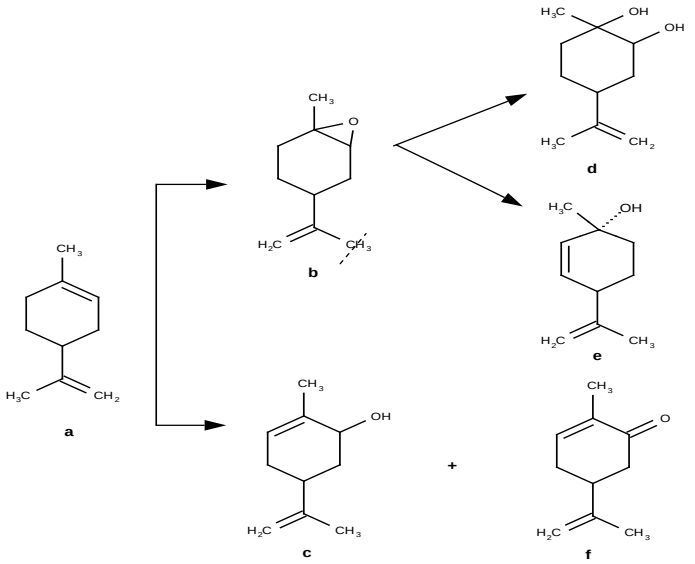

Fig. 6: Shows the scheme of bioconversion of limonene using cultured cells of *Nigella sativa*. (a) limonene, (b) limonene epoxide, (c) limonene-1,2-diol, (d) p-mentha-2,8-dien-1-ol (+) trans, (e) carveol, (f) carvone.

All the biotransformed products point towards the common regioselective site of attack at limonene in the initial step of bioconversion. The hydroxylation occurs regioselectively at endocyclic C=C double bond and/or its allylic position i.e. 6th carbon of limonene. Further reactions are supposed to be initiated by oxireductases. Bioconversion reactions encompass hydrolyzation, cyclic cleavage, oxidation including hydroxylation and ketonization.

CONCLUSION

Biotransformation potential of cultured cells of *Nigella sativa* has not been described so far. It was checked for the first time for limonene biotransformation. Carveol, limonene-1,2-diol, p-mentha-2,8-dien-1-ol-trans (+) and carvone, in order of decreasing concentrations, were found as the biotransformed products. Thus it can be postulated that the cultured cells of *Nigella sativa* possess distinctive biocatalytic activities towards limonene and have the abilities to introduce oxygenated functional groups regioselectively into alkenes for its assymetrization. Cultured cells of *Nigella sativa* possess enzymes for hydrolyzation, cyclic cleavage, oxidation including hydroxylation and ketonization. Bioconversion was found maximum at 48 hours. The immobilization resulted in improvement of the yields in all bioconversion reactions. To obtain a deeper insight into mechanistic interpretation of bioconversions studies and of enzymes, isolation and purification of ezymes involved are needed.

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