Biotransformation of Myrcene by *Pseudomonas putida* PTCC 1694

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S u m m a r y

Terpineol and linalool are sources of fragrances providing an unique volatile terpenoid alcohol of low toxicity, and thus are widely used in the perfumery industry. They are also being applied in folk medicine and in aromatherapy, as well as important chemical constituents of the essential oil of many plants. Previous studies have implicated the biotransformation of limonene by *Pseudomonas putida*. The objective of this research was to study the pathways involved in biotransformation of myrcene by *Pseudomonas putida*. The culture preparation was done by using such variables as different microbial methods and incubation periods to obtain maximum cells of *P. putida* for myrcene biotransformation. It was found that myrcene was converted to dihydrolinalool, *cis*-β-dihydroterpineol, linalool and *cis*-ocimene-8-oxo in high percentages. The biotransformation products were identified by theoretical study (TS), Fourier-transform infrared spectroscopy (FT-IR), ultraviolet visible (UV), gas chromatography (GC), nuclear magnetic resonance (NMR) and gas chromatography/mass spectroscopy (GC-MS). Comparison of different incubation times showed that 120 h which was more effective, the major products were dihydrolinalool (4.1%), *cis*-β-dihydroterpineol (67.6%) and linalool (25.8%). The main compounds comprised 97.5%. The incubation period of 72 h yielded dihydrolinalool (16.7%), *cis*-ocimene-8-oxo (61.6%),

trans-β-dihydroterpineol (8.4%) and β-cadinene (3.5%), with main compounds comprising 86.7%. Incubation for 30 h yielded dihydrolinalool (59.5%), *cis*-β-dihydroterpineol (25.0%), hexadecanoic acid (12.5%), and the main compounds comprising 97.0%.

Key words: *biotransformation, bioconversion, Pseudomonas putida, fungi, dihydrolinalool*

INTRODUCTION

Recently, several reviews concerning the opportunities of microbial transformation of terpenes have been published [1-7]. Microorganisms are able to oxidate, reduce, perform hydrolytic reactions, dehydrations as well as formation of C–C bonds and several degradation reactions [8]. The plants of *Pseudomonas* genus are able to completely mineralize terpenes using carbon as a source of energy. Biotransformation of limonene by *Pseudomonas putida* was performed by Chatterjee and Bhattachayya [9]. *P. putida* bioconversion products were identified as perillyl alcohol and *p*-menth-1-ene-6,8-diol. The characteristic organoleptic properties of myrcene and its usage in essential oils of several useful plants, such as lemon grass, hops, bay and verbena were also described [1]. Dihydrolinalool and *cis*-ocimene-δoxo are the most important monocyclic monoterpenes. In the oxygenated terpenes flavour and chemical composition are important due to its lilaceous fragrance [10, 11]. In this research, bioconversion of monoterpene by fungi, the biotransformation of myrcene by *Pseudomonas putida* was investigated. That was shown to successfully metabolize myrcene to oxygenated monoterpenes hydrocarbons. Secondly, alcohol is particularly important among eight products. The aim of the study was to evaluate the pathways involved in biotransformation of myrcene by *Pseudomonas putida*.

MATERIALS AND METHODS

Malt extract, peptone and yeast extract were obtained from Merck & Co., USA. The substrate and the citral product were purchased from Sigma Chemicals Co., USA. Other chemicals of analytical grade were obtained from standard substances (malt extract, yeast, peptone and glucose). Acetate buffer 0.1 M (pH 5.5) [sodium acetate, HCl (1N), distilled water] was prepared.

Microorganisms and cultural conditions

A strain of *Pseudomonas putida* was isolated in our laboratories from soil of Tehran prefecture and was identified by its physiological and morphological characteristics according to the Persian Type Culture Collection (PTCC 1694) at the Iranian Research Organization for Science & Technology, Tehran, Iran.

Culture media

The culture medium contained 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose in distilled water (pH 7.0 for yeast).

Fermentation procedure

Fermentation was carried out in 250-ml Erlenmeyer flasks, medium for cultivation of strain *P. putida* PTCC 1694. The medium were incubated for 30 h, 72 h and 120 h at 30°C.

Agarose entrapment

An agarose solution was prepared by dissolving agarose (15% w/v) in water at 100ºC, then cooled to 40ºC and mixed with separated *P. putida* cells. The mixture was allowed to solidify by leaving at 4ºC. The hard gel was shredded in a Warring blender and non entrapped cells removed by washing with saline [12].

Biotransformation

Biotransformation was started after adding 100 ml of medium containing 4.47 g^{-1}myrcene; 0.1 gl^{-1} of methanol was used as a solubilising agent.

Optimum conditions for biotransformation

P. putida was added into the media at a suitable growth phase and done by employing different phases of growth. An agitation speed of 150 rpm with biotransformation times of 30 h, 72 h and 120 h were employed. The optimum values of pH and temperature for biotransformation were found to be in the area of pH 5.5 and 27ºC for all three incubation periods.

Extraction of products

Extraction of bioconversion products of myrcene after treatment with *P. putida* PTCC 1694 was carried out after removing the bacterial cells by centrifugation, and the supernatant was extracted with diethyl ether (3×25 ml). The combined extract was washed with distilled water (3×10 ml), dried over an-

hydrous sodium sulphate and filtered by using Whatman No.1 filter paper. The solvent was removed under reduced pressure to give pure reaction products. The products were directly analysed by TS, FT-IR, UV, GC, NMR and GC-MS.

Analysis of the samples with FT-IR, UV, GC, NMR and GC-MS

The GC analysis was performed on Shimadzu 15A gas chromatograph equipped with a DB5 capillary column (50 m ´ 0.2 mm, film thickness 0.32 μm). The split/splitless injector and flame ionization detector were heated at 250°C. N_2 was used as the carrier gas (1 ml/min). The oven temperature was kept at 60 °C for 3 min and then heated to 220°C at a 5°C/min rate and kept constant at 220°C for 5 min. Relative percentage amounts were calculated from the peak area by using a Shimadzu C-R4A Chromatopac integrator without correction.

The GC-MS analysis was performed by using a Hewlett-Packard 5973 equipped with an HP 5MS column (30 m ´ 0.25 mm, film thickness 0.25 μm). The oven temperature was kept at 60 °C for 3 min and programmed to 220 °C at a rate of 5 °C/min and kept constant at 220°C for 5 min. The apparatus operated with helium as a carrier gas at a flow rate of 1 ml/min in an electronic impact mode of 70 eV. Identification of the constituents of the oil was made by comparing their mass spectra and retention indices with those given in the literature and the authentic samples [10]. FT-IR mass spectra (6 main peaks) were recorded in $CHCl_3$ on a Perkin-Elmer 457 instrument and data spectra for dihydrolinalool, *cis*-β-dihydroterpineol and *cis*-ocimene-8-oxo:

Dihydrolinalool (No. 236531in NIST library)

156[M^+]:41(100), 69(82), 55(55), 82(30), 67(37), 81(32), 57(10), 95(20). FT-IR (KBr) $\nu_{max}cm^{-1}$: 3460, 1638, 1344, 1034.UV(λmax): 215–220 nm.

***cis*-β-Dihydroterpineol** (No. 104185 in NIST library)

154[M^+]: 41(100), 69(75), 81(15), 53(10), 93(7), 123(7), 111(6), 139(5) (KBr) $\nu_{max}cm^{-1}$: 3200, 1620,1340,1372. UV(λmax): 225 nm.

***cis*-Ocimene-8-oxo** (No. 155690 in NIST library)

150[M^+]: 43(100), 69(45), 41(45), 58(40), 109(30), 71(22), 97(20), 82(20) (KBr) $\nu_{max}cm^{-1}$: 3250, 1670. UV(λmax): 245 nm.

FT-IR analysis in cis-ocimene-8-oxo was $\nu_{max}cm^{-1:}$ 1700 (C=O), 1638 (C=C). Comparing the FT-IR spectra showed a region >3000 cm^{-1} and a peak in the region between 1600 and1700 cm^{-1}. For 120 h it had signals for C=C protons in the 3–5 region, and 30 h had no peak in the 3–5 regions. ^{1}H-NMR and ^{13}C-NMR showed confirmation for 72 h and 120 h.

RESULTS

The biotransformation of myrcene by *P. putida* was studied under constant pH (5.5) and temperature conditions (30°C). TS and FT-IR, UV, GC and GC-MS analyses revealed the following: At first, when myrcene was converted with *P. putida* for 30 h, the main products were dihydrolinalool (59.5%), *cis*-β-dihydroterpineol (25.0%) and hexadecanoic acid (12.5%). Secondly, when myrcene was converted with *P. putida* in 120 h, the main compounds produced were dihydrolinalool (4.1%), *cis*-β-dihydroterpineol (67.6%) and linalool (25.8%). Finally, when myrcene was converted with *P. putida* in 72 h, the main products were dihydrolinalool (16.7%), *cis*-ocimene-8-oxo (61.6%), *trans*-β-dihydroterpineol (8.4%) and β-cadinene (3.5%) (tab. 1).

The culture was at the end of exponential phase and three culture age was used for screening different biotransformation products and our research proposed not for selecting the best culture medium or physical condition. The determination of a suitable culture age during growth of *P. putida* in medium for maximum product formation was done by harvesting cells in various stages of growth and employing them for myrcene biotransformation. The results indicated the optimum cell culture age to be 120 h, with 97.5% product formation; the product formation for 30 h and 72 h was 97.0% and 86.7%, respectively. Here, the cells were at the end of the exponential phase and had attained maximum cell concentration.

Table 1.

Percentage bioconversion of myrcene by *Pseudomonas putida*.

compound	*P. putida*		
	30 h	72 h	120 h
dihydrolinalool	59.5	16.7	4.1
cis-β-Dihydroterpineol	25.0	-	67.6
hexadecanoic acid	12.5	-	-
linalool	-	-	25.8
trans-β-Dihydroterpineol	-	8.4	-
cis-Ocimene-8-oxo	-	61.6	-
α-Terpineol	-	-	-
2,6-Dimethyldecane	-	-	-
total	**97.0**	**86.7**	**97.5**

From the data given in table 1 it can be concluded that myrcene was converted primarily to *cis*-β-dihydroterpineol, *cis*-ocimene-8-oxo, linalool and dihydrolinalool. This formation involved moving electrons and cyclisation and an additional –OH group for *cis*-β-dihydroterpineol. Reduction of the C=C bond and formation of an epoxy bond and finally opening by H^+ dihydrolinalool were produced. Myr-

cene oxidated by *P. putida* formed *cis*-ocimene-8-oxo. Comparing samples of NMR showed no peaks for 30 h in the 3–5 ppm region, while 120 h had signals in the 4–5 region for C=C protons (fig. 1). Investigation of the biosynthesis of myrcene to cis-ocimene-8-oxo, linalool, *cis*-β-dihydroterpineol and dihydrolinalool confirm by TS. Because, in transition state, high energy was showed at the HF levels, they are unstable. When they converted, HF energy has less energy.

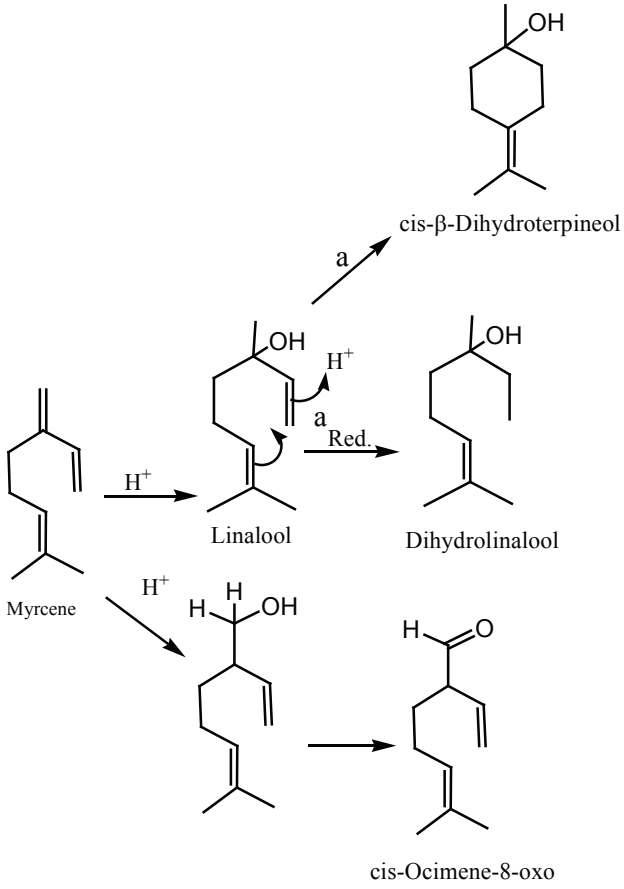

Figure 1. Pathways of dihydrolinalool, cis-ocimene-8-oxo and linalool formation from myrcene

The present result confirms previous investigation finding on biotransformation displayed good activity over a pH range of 4.5–6.0, with the highest activity at pH 5.5.

In a previous study of biotransformation of menthol by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. the main bioconversion product obtained from menthol of *A. niger* was *cis-p*-menthan-7-ol, and the main products ob-

tained by sporulated surface cultures of *Penicillium* sp. were limonene, *p*-cymene and γ-terpinene [13]. Leuenberger reported that product yields could be effectively increased by solubilising/emulsifying immiscible substrates [14]. However, careful selection of the nature and concentration of the solvent are necessary because many miscible solvents are cytotoxic at lower concentrations [15]. Compare up investigation with present study shown oxygenated monoterpenes are main compounds more monoterpenesv in the biotransformation.

Theoretical study of biotransformation

The energy of myrcene compared with other products, especially *cis*-β-dihydroterpineol, dihydrolinalool and *cis*-ocimene-8-oxo, was investigated at the HF levels of theory, using the density functional theory with the Becke3LYP and the 6-311^{++}G basis set. It showed dihydroterpineol, dihydrolinalool and *cis*-ocimene-8-oxo to have less energy (HF=–468.314, 368.321, 420.460 Hartee). The others have high energy, but they are unstable (FT-IR ν_{max}cm^{-1}: 3112, 1560, 1347, 1023). This result showed that FT-IR citronellol in TS and obtained experimentally were similar.

DISCUSSION

In previous studies concerning bioconversion of limonene by *Pseudomonas putida,* these were transformed into perillyl alcohol and *p*-menth-1-ene-6,8-diol (16). In this research, almost all the products were alcohol. This problem showed that biotransformation of myrcene and other compounds was converted by *P. putida* to oxidant reactions.

The study of microbial transformation of one monoterpene by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. produced *cis-p*-menthan-7-ol from *A. niger*; the main products obtained were limonene, *p*-cymene and γ-terpinene [17]. Two main products of microbial transformation of citral were similar to those obtained in former work. The main bioconversion products *of (-)-menthol by Mucor ramannianus* using the sporulated surface cultures method were *trans-p-menthan-8-ol, trans*-menth-2-en-1-ol*, sabinane, p-menthane-3,8-diol, isomenthol and 1,8-cineole (18)*. T*he main biotransformation products obtained from menthol by sporulated surface cultures of Penicillium sp. were α-pinene (18.0%), trans-p*-menthan-1-ol *(10.6%), p*-menth-1-ene *(5.8%), sabinene (3.9%),* 1,8-cineole *(6.4%) and limonene (3.2%) [13]*.

The experimental work (two latest articles) suggested that microbial transformation of monoterpenes with different *Penicillium and Aspergillus* genera caused an oxidation reaction and resulted in a more stable product. Although, bioconversion, with use of *Pseudomonas putida* showed that, it was possible to obtain two or three main products with high percentage and selectivity. Thus, comparison the

reported literature and this research showed that bioconversion of *Pseudomonas, Aspergillus* and *Penicillium have been similarly effective for biotransformation. Cyclization and oxidation are two common characters in this investigation products bioconversion.*

Main products of the biotransformation of limonene obtained by Chatterjee and Bhattacharyya [9] were identified with use of FT–IR and NMR. In present study, main products were identified by several spectroscopy's. There is nothing reported in the literature about TS biotransformation. However, the comparison of main products showed succeed in full metabolism of myrcene to oxygenated monoterpenes hydrocarbons. Among eight bioconversion products and mentioned above, two main products are of 1° and 2° alcohol importance.

The biotransformation products were identified by TS, FT-IR, GC, NMR and GC-MS. C=C and C-OH groups in FT-IR spectra with 30 h, 72 h and 120 h were more clear. In addition, in UV spectra *cis*-ocimene-8-oxo showed C=C and C=O peaks, and for *cis*-β-dihydroterpineol C=C and C-OH peaks were observed. However, because of the high percentage of product, in major products the UV spectra were better displayed. With use of this method, myrcene could be transferred in high percentages to dihydroterpineol, *cis*-ocimene-8-oxo, *cis*-β-dihydroterpineol and linalool.

A volatile terpenoid alcohol of low toxicity – dihydroterpineol – is widely used in many ways in the perfumery industry. It is an important chemical constituent of the essential oil of many plants with widespread applications in folk medicine and in aromatherapy. The NMR showed confirmation of converted samples. For 30 h, there were signals in <3 ppm in ^{1}H-NMR.

CONCLUSION

Many different terpenoid compounds can be tested for bioconversion reactions in the same batch of fungi. The present work confirms the findings of studies, as mentioned above. The purpose of this research is to study the pathways involved in the microbial transformation of myrcene by Pseudomonas putida. The major components of the microbial transformation of the myrcene with P. putida appeared to be dihydrolinalool (59.5%), cis-β-dihydroterpineol (25.0%) and hexadecanoic acid (12.5%), with a yield of 97.0% in a period of 30 h. Using an incubation period of 120 h yielded 97.5% products, the major compounds are dihydrolinalool (4.1%), cis-β-dihydroterpineol (67.6%) and linalool (25.8%). Using an incubation period of 72 h yielded 86.7%, converted to dihydrolinalool (16.7%), cis-ocimene-8-oxo (61.6%), trans-β-dihydroterpineol (8.4%) and β-cadinene (3.5%). Biosynthesis of myrcene to cis-ocimene-8-oxo, linalool, cis-β-dihydroterpineol and dihydrolinalool was investigated. The major bioconversion products were proved by the analysis using TS, FT-IR, UV, GC, NMR and GC-MS. The TS of myrcene compared with other products, especially dihydrolinalool, cis-β-dihydroterpineol, cis-ocimene-8-

oxo and linalool, at HF levels of theory using the density functional theory with the Becke3LYP and 6-311++G basis set showed its major products which generally have less energy and compound stability. There is nothing reported in the literature are about TS biotransformation.

Major bioconversion products have many applications in flavouring, extracts, food and drug manufacturing and as a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in no cosmetic products, such as household cleaners and detergents. Their use worldwide is in the region of greater than 1,000 metric tons per annum.

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BIOTRANSFORMACJA MIRCENU Z UDZIAŁEM BAKTERII *PSEUDOMONAS PUTIDA* PTCC 1694

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Streszczenie:

Terpineol i linalol są związkami zapachowymi należącymi do alkoholi terpenowych. Charakteryzują się wyjątkowo niską toksycznością, dlatego często są wykorzystywane w przemyśle perfumeryjnym, medycynie ludowej oraz aromaterapii. Są głównymi składnikami olejków eterycznych wielu roślin. Poprzednio prowadzone badania dotyczyły biotransformacji limonenu prowadzonej przez *Pseudomonas putida*. Celem niniejszej pracy było zbadanie możliwości biotransformacji mircenu prowadzonej przez bakterie *Pseudomonas putida*. W hodowli kultur wykorzystano różne metody mikrobiologiczne oraz okresy inkubacji w celu uzyskania maksymalnego stężenia komórek bakterii *Pseudomonas putida* zdolnych do biotransformacji mircenu. W trakcie badań stwierdzono wysokie stężenie takich metabolitów biotransformacji mircenu jak: dihydrolinalol, *cis*-β-dihydroterpineol, linalol oraz

cis-ocymen-8-okso. Produkty metabolizmu mircenu zidentyfikowano, wykorzystując dane wynikające z rozważań teoretycznych (TS) oraz wykorzystując techniki spektroskopii w podczerwieni (FTIR), promieniowania ultrafioletowego (UV), chromatografii gazowej (GC), spektroskopii magnetycznego rezonansu jądrowego (NMR) i spektroskopii mas sprzężonej z chromatografią gazową (GC-MS). Na podstawie porównania różnych czasów inkubacji stwierdzono, że 120-godzinna inkubacja jest najbardziej wydajna. Dzięki niej otrzymano dihydrolinalol (16,7%), *cis*-β-dihydroterpineol (67.6%), linalol (25,8%), które stanowiły 97,5% całego substratu. W czasie 72-godzinnej inkubacji otrzymano 86,7% wszystkich związków: dihydrolinalol (16,7%), *cis*-ocymen-8-okso (61,6%), *trans*-β-dihydroterpineol (8,4%) oraz β-kadinen (3,5%). W wyniku inkubacji 30h otrzymano 97% wszystkich związków powstałych w procesie biotransformacji: dihydrolinalol (59,5%), *cis*-β-dihydroterpineol (25,0%) oraz kwas heksadekanowy (12,5%).

Słowa kluczowe: *biotransformacja, biokonwersja, Pseudomonas putida, grzyby, dihydrolinalol*