Biotransformation of menthol by sporulated surface cultures of *Penicillium* sp. and study of the pathways involved

AKBAR ESMAEILI[1*], AZITA HOSEINY ZAREA[2], SHIRIN SHARAFIAN[1], SHILLA SAFAIYAN[1], ABDOLHOSSEIN RUSTAIYAN[3]

[1]Department of Chemistry
North Tehran Branch
Islamic Azad University
PO Box 19585-936, Tehran, Iran
[2]Department of Pharmacy
Pharmaceutical Sciences Branch
Islamic Azad University
Tehran, Iran
[3]Department of Chemistry
Science & Research Campus,
Islamic Azad University,
PO Box 14515-775, Tehran, Iran

[*]corresponding author: phone: +98 0912 1484813, fax:+98 2188 787204,
e-mail: akbaresmaeili@yahoo.com

Summary

A simple and efficient method of carrying out biotransformation reactions on terpenoid compounds was developed. For these experiments, a sporulated surface culture of *Penicillium* sp. was inoculated on solid media in conical flasks. After a short incubation the spores germinated and a mycelia culture was formed. After a week the cultures had completely sporulated and a bioconversion reaction started. For this purpose, a known volume of menthol was added onto the sporulated surface of culture. After 7 days, a period during which transformation took place, menthol was extracted with Et_2O three times. After evaporation the recognition by GC and GC/MS was followed. The main bioconverted products obtained from menthol by surface *Penicillium* sp. with the use of sporulated surface culture were α-pinene (18.0%), sabinene (11.6%), trans-p-menthan-1-ol (10.6%), p-menth-1-ene (5.8%), 1,8-cineole (6.4%) and limonene (3.2%). The pathways of biotransformation of menthol by *Penicillium* sp. to main products are also discussed.

Key words: *biotransformation*, *bioconversion*, *Penicillium sp*., *fungi*, *limonene*, α-*pinene*

INTRODUCTION

The bioconversion of monoterpene alcohols by *Penicillium* sp. was investigated. In recent years, the biotechnological production of natural aromatic chemical (NACS) has stimulated consumers' demand for natural and healthy products [1]. This interest in natural flavors instead of synthetic ones has led an increasing research focused on the microbial production of so-called "Biflavor" [2-4].Nearly 80% of flavors and fragrances used worldwide are produced chemically [5].

The bioconversion of geranyl and neryl acetate by *A. niger* has been described [6, 7]. The main found reaction was a hydrolysis of terpene acetates to corresponding alcohols followed by further hydroxylation experiments for which liquid cultures of *A. niger* were used. Monoterpene (citral) transformation to menthols by liquid phase over Ni supported by H-MCM-41 and H-Y has also been examined by a Finnish scientists [8]. Biotransformation of some monoterpene by *A. niger* was studied. Linalool and α-terpineol and limonene were the main products obtained from nerol and citral by sporulated surface culture [9]. Using a surface culture of the evidence was found to suggest that geraniol converted to linalool and partially oxidized to citral [10]. In this paper, the biotransformation of a monoterpene (menthol) by sporulated surface cultures of *Penicillium* sp.strain is studied.

MATERIALS AND METHODS

Microorganisms

A strain of *Penicillium* sp. was isolated from the soil in our laboratories in Tehran prefecture and identified according to physiological and morphological characteristics. *Penicillium* sp. (PTCC5074) was identified according to its Persian Type Culture-e Collection, Iranian Research Organization for Science & Technology, Tehran, Iran. A spore suspension of *Penicillium* sp. was prepared in Nutrient Broth solution for inoculating fungi in Petri dishes.

Growth medium and conditions

For the isolation, growth and conservation of the fungi in Petri dishes, the same solid medium was used: Sabouraud Dextrose Agar (SDA) medium contained mycological peptone 1.0%, glucose 4.0% and agar 1.5%.

In the solid agars medium inoculated with spores of *Penicillium* sp., first germination of the spores and mycelial growth took place. Afterwards, the growth medium was stored at room temperature. After a week, the surfaces of Petri dishes were covered with spores and biotransformation reaction had started.

Experiments with spore suspension

Both spores recovered from one-week-old surface cultures of *Penicillium* sp. were grown in Petri dishes on SDA by adding 10 ml of sterile Tween 80 solution (0.2% Tween 80) in distilled water to each culture, bringing spores into suspension. A total spore suspension of 50 ml was obtained and shaken in a 250 ml conical flask [11].

To this suspension of spores 1 ml of a solution of 5% menthol in ethanol was added, and the suspension was placed on a shaker at 180 rpm. Then sample was taken out and extracted with 3×50 ml Et_2O, and the products were directly analyzed by GC ad GC/MS.

GC/MS sample analysis

The analysis was performed using a Hewlett-Packard 6890 with a DB-5 capillary column (30 m $\times$0.25 mm; film thickness 0.25 μm) in 60°C for 5 min. up to 220°C at a rate of 4°C/min. The flow rate of helium as a carrier gas with 2 mL/min. MS was taken at 70 eV. The retention indices of C9-C28 n-alkanes were matched with the Wiley 275 Library. Their mass spectrums were compared with those of authentic samples or with data are available in the literature [12-14].

RESULTS AND DISCUSSION

In this experiment, the biotransformation of menthol by sporulated surface cultures of *Penicillium* sp. (PTCC5074) grown on the same medium culture flasks was monitored similarly for two weeks. Cultures were grown in Petri dishes on solid medium Sabouraud Dextrose Agar (SDA) containing menthol. After incubation, SDA culture was extracted (see experimental section). It was noticed that after 7 days, the cultures with 0.05 menthol were fully grown and sporulation had occurred. The cultures with 0.1% menthol covered only a part of the surface. The suspension was extracted with Et_2O three consecutive times and directly analyzed by GC and GC/MS.

In these analyses, various chemicals were obtained. The main products obtained in the bioconversion of *Penicillium* sp. of menthol were α-pinene(18%), sabinene (11.6%), *trans*-p-menthan-1-ol (10.6%), *p*-menth-1-ene (5.8%), 1,8-cineole(6.4%) and limonene(3.2%), respectively. From the data in the figure 1 it can be concluded that menthol converted much more rapidly and conveniently than α-pinene. Biosynthesis of menthol to sabinene is showed in the figure 2. Synthesis of α-pinene and *trans*-1-methyl-4 (1-methylethyl) cyclohexanol from menthol showed mass spectra of α-pinene, sabinene, *p*-menth-1-ene, 1,8-cineole, limonene, and *trans-p*-menthan-1-ol: 6 main peaks.

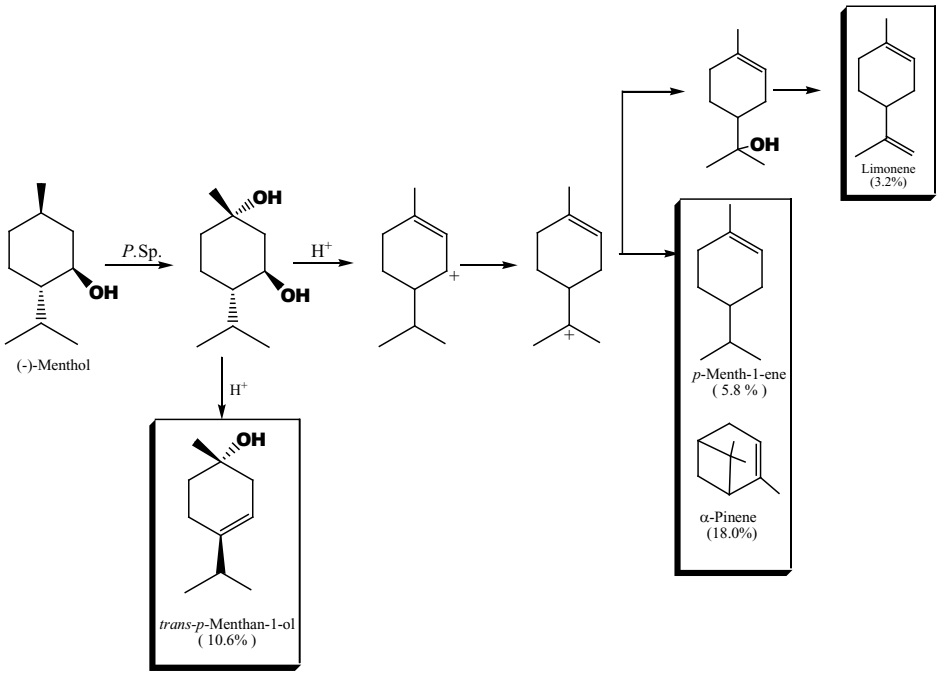

Figure 1. Possible pathway of bioconversion of menthol by *Penicillium* sp.

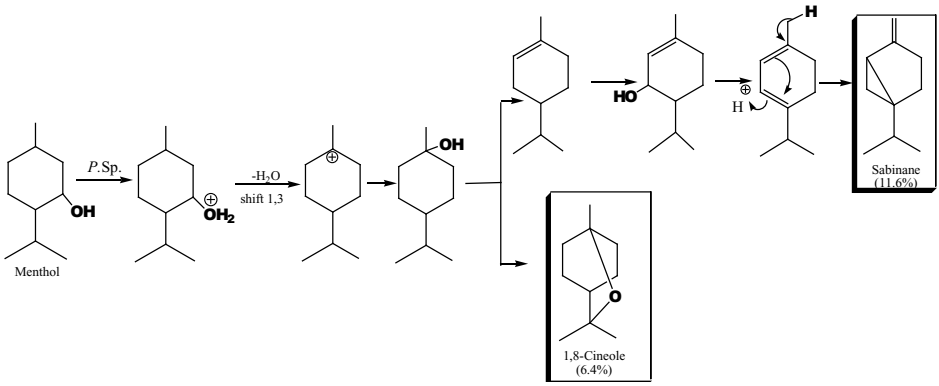

Figure 2. Biosynthesis of menthol to sabinene and 1,8-cineole by *Penicillium* sp.

α–pinene:	136[M$^+$]: 93(100),92(37),91(27),41(20),77(24),79(17),39(12),139(5)
sabinene:	136[M$^+$] :93(100),77(24),91(20),79(17),41(20),137(10),94(10),69(10).
p-menth-1-ene:	138[M$^+$]: 68(100),67(50),138(30),80(27),41(20),55(20),96(15(,123(10)
1,8-cineole:	154[M$^+$]:43(100),81(80),71(50),108(47),84(41),154(40),70(35),111(10).
limonene:	136[M$^+$]: 68(100),67(86),93(78),79(43),39(36),53(34),107(30),121(30).
trans-p-menthan-1-ol:	156[M$^+$], 71(100),43(60),94(50),113(23),41(20),98(15),55(13),79(10).

CONCLUSION

1. In this experiment, the biotransformation of menthol by sporulated surface cultures of *Penicillium* sp. grown on a medium culture.
2. The main components of the essential oil were α-pinene (18.0%), sabinene (11.6%), *trans*-*p*-menthan-1-ol (10.6%), *p*-menth-1-ene (5.8%), 1,8-cineole (6.4%) and limonene (3.2%).
3. Biosynthesis of menthol to sabinene and α-pinene and *trans*-1-methyl-4(1-methylethyl)cyclohexanol from menthol showed.

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BIOTRANSFORMACJA MENTOLU ZA POMOCĄ KULTUR ZARODNIKOWYCH Z RODZAJU *PENICILLIUM* ORAZ BADANIA SZLAKÓW METABOLICZNYCH

AKBAR ESMAEILI[1*], AZITA HOSEINY ZAREA[2], SHIRIN SHARAFIAN[1], SHILLA SAFAIYAN[1], ABDOLHOSSEIN RUSTAIYAN[3]

[1]Wydział Chemii
Filia Teheran Północny
Uniwersytet Islamski
PO Box 19585-936, Tehran, Iran

[2]Wydział Farmacji
Filia Nauk Ścisłych
Uniwersytet Islamski
Tehran, Iran

[3]Wydział Chemii
Kampus Naukowo-Badawczy
Uniwersytet Islamski
PO Box 14515-775, Tehran, Iran

[*]autor, do którego należy kierować korespondencję: tel.: +98 0912 1484813, faks:+98 2188 787204, e-mail: akbaresmaeili@yahoo.com

S t r e s z c z e n i e

Opisano prostą i skuteczną metodę przeprowadzania reakcji biotransformacji związków terpenoidowych. W tym celu na stałej pożywce umieszczonej w kolbach zaszczepiono kulturę zarodnikową *Penicillium*. Po krótkim okresie inkubacji zarodniki wykiełkowały i utworzyły grzybnię. Po tygodniu kultura była całkowicie pokryta zarodnikami i rozpoczęły się reakcje biokonwersji. W tym celu na powierzchnię grzybni w kolbach dodano określoną ilość mentolu. Po 7 dniach, czyli po czasie, w którym odbywała się transformacja, za pomocą Et_2O trzy razy wyekstrahowano mentol i po odparowaniu badano go za pomocą GC/MS. Podstawowymi produktami uzyskanymi z mentolu za pomocą *Penicillium* były *α*-pinen (18,0%), sabinen, trans-p-mentan-1-ol (10,6%), p-ment-1-en (5,8%), 1,8-cineol (6,4%) i limonen (3,2%) uzyskane z powierzchniowej kultury zarodnikowej. Omówiono także sposoby biotransformacji mentolu przez Penicillium do głównych produktów.

***Słowa kluczowe**: biotransformacja, biokonwersja, Penicillium, limonen, α-pinen*