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Microbial transformation of citral by *Penicillium* sp.

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Thymol is present in the essential oils from herbs and spices, such as thyme. It is produced by these plant species as a chemical defense against phytopathogenic microorganisms. Therefore, this compound has attracted great attention in food industry, i.e., it has been used as a natural preservative in foods such as cheese to prevent fungal growth. Previous studies concerning the biotransformation of nerol by *Penicillium* sp. and microbial transformation of citral by sporulated surface cultures method (SSCM) of *Penicillium digitatum* have been reported. The objective of this research was to study the pathway involved during biotransformation of citral by *Penicillium* sp. using two methods. The culture preparation was done using different microbial methods and incubation periods to obtain *Penicillium* for citral biotransformation. The biotransformation products were identified by gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS). A comparison of the two methods showed that SSCM was more effective, its major products were thymol (21.5%), geranial (18.6%) and nerol (13.7%). LM produced only one compound — thymol — with a low efficiency.

Keywords: biotransformation, bioconversion, *Penicillium* sp., fungi, thymol, geranial

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INTRODUCTION

Biotransformation by cultured plant cells is an important method to convert cheap and plentiful organic compounds into more useful ones, according to the ability of plant cell cultures specifically to produce secondary metabolites (Suga & Hirata, 1990; Shimoda, 2006). The reactions involved in the biotransformation of organic compounds by cultured plant cells include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis, and glycosylation (Suga & Hirata, 1990; Ishihara *et al.*, 2003; Shimoda, 2006).

In the course of work related to bioconversion of monoterpene aldehyde by fungi, microbial transformation of citral by *Penicillium* sp. with the sporulated surface cultures method (SSCM) and liquid method (LM) approaches was investigated. The bioconversion of geranyl and neryl acetate ((*Z*)-2,6-octadien-1-ol-3,7-dimethyl acetate) by *Aspergillus niger* has been described (Madyastha *et al.,* 1988a; 1988b). The main reaction found was hydrolysis of terpene acetates to the alcohols, followed by further ω-hydroxylation to the respective 8-hydroxy derivatives. The degradation of geraniol by *A. niger* has also been examined by a Japanese group (Goto, 1967).

In 1964, use of edgeraniol was converted to linalool and partially oxidized to citral using the SSCM procedure (Wood, 1969). Microbial transformation of geraniol and nerol by SSCM of five *A. niger* strains and three *Penicillium* strains were compared with that using of submerged LM (Demyttenaere *et al.,* 2000). It has been mentioned by several workers that different fungi can bioconvert citral (Massada *et al.,* 1976; Ramaswami *et al.,* 1988; Larroche *et al.,* 1989; Adams, 1995; Demyttenaere *et al.,* 1998).

In previous studies we done biotransformation of menthol by *Mucor ramannianus, Penicillium* sp. and *A. niger*. Using SSCM menthol was converted to oxygenated compounds. We found that those microorganisms are able to perform oxidations, reductions, hydrolytic reactions, dehydrations and formation of C–C bonds and several degradation reactions (Scharf *et al*., 1986; Jansseens *et al*., 1991; Esmaeili *et al*., 2009a; 2009b).

In this article, microbial transformation of pure terpene aldehyde citral is carried out by SSCM, and the pathways involved in this microbial transformation are investigated. Microbial transformation of citral by *Penicillium* sp. SSCM grown on 50 ml medium in conical flasks was monitored over three weeks. The suspension was extracted with diethyl ether three times and directly analyzed by GC and GC/MS.

MATERIAL AND METHODS

Microorganisms and cultivation. The *Penicillium* sp. strain was isolated from a soil sample collected in April 2008 in the province of Tehran, Iran. The culture was cultivated and conserved on Malt Extract Agar (MEA) (malt extract, 20 g; peptone, 1 g; dextrose, 20 g; agar, 20 g; distilled water, 1 l) for 10 days at 28 °C ±2 °C.

Experiments with SSCM. Spores were recovered from 3-week-old surface cultures of *Penicillium* sp. grown in Petri dishes on SDA (Merck). Spore suspension was prepared by adding 10 ml of 0.2% sterile Tween 80 solution in distilled water. A total of 50 ml of the spore suspension with 5×10^8 spore/ml was obtained, which was shaken in a 250 ml conical flask.

To this spore suspension 1 ml of a solution of 5% citral in absolute ethanol was added, and the suspension was placed on a shaker at 180 rpm. After 5, 10 and 321 weekdays this suspension was extracted with 3×50 ml diethyl ether, and the products were directly analyzed by GC and GC/MS.

Experiments with LM. A 500 ml Erlenmeyer flask containing 100 ml medium (malt extract, 10 g; pep-

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Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectroscopy; LM, liquid method; t_R, SSCM, sporulated surface cultures method

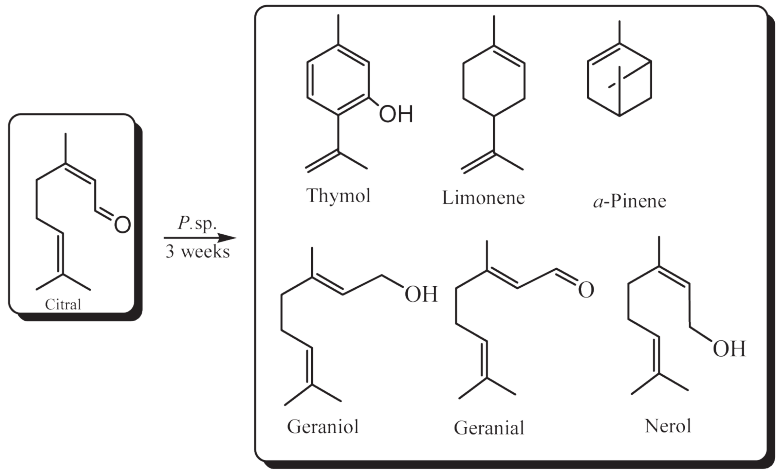

Figure 1. Identified products of bioconversion of citral by *Penicillium* sp.

tone, 1 g; dextrose, 20 g; agar, 20 g; distilled water, 1 l, pH 5.6) was inoculated with a 5 ml suspension of *Penicillium* sp. (5×10^8 spore per ml) and incubated at 28 °C ± 2 °C for 4 days on a rotary shaker operating at 100 rpm. After full growth of the microorganism, solution of citral (100 μg) in ethanol (0.2 ml) was added under sterile conditions to the culture. The incubation was then continued for a further 10 days at 28 °C ± 2 °C. After the completion of the incubation, the culture was extracted with diethyl ether (3 × 100 ml).

Analysis of samples with GC/MS. GC analysis was performed on a Shimadzu 15A gas chromatograph equipped with a DB-5 capillary column (50 m × 0.2 mm, film thickness 0.32 μm). Split/non-split injector and flame ionization detector were heated at 250 °C. Nitrogen was used as carrier gas (1 ml/min). The oven temperature was kept at 60 °C for 3 min and then heated to 220 °C with 5 °C/min rate and kept constant at 220 °C for 5 min. Relative content of compounds was calculated from peak area using a Shimadzu C-R4A chromatopac integrator without correction.

GC/MS analysis was performed using a Hewlett-Packard 5973 device 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 flow rate of helium a carrier gas was 1 ml/min. MS were taken at 70 eV. Identification of the constituents of the oil was made by comparison of their mass spectra and retention indices (RRI) with those given in the literature and are authentic samples (Frank *et al.,* 1977; Chen *et al.,* 1982; Larroche *et al.,* 1989). Mass spectra of thymol (retention time 17.17 min), geranial (retention time 17.21 min), nerol (retention time 16.41 min), geraniol (retention time 16.61 min), α-pinene (retention time 9.89 min), and limonene (retention time 12.18 min), i.e., the six main peaks, are given below:

Thymol: 150[M+]: 135(100), 150(30), 115(17), 91(16), 136(10), 77(10), 65(7), 121(5).

Geranial: 152[M+]: 41(100), 69(62), 84(15), 53(10), 83(7), 94(7), 67(6), 55(5).

Geraniol: 154[M+]: 41(100), 69(75), 81(15), 53(10), 93(7), 123(7), 111(6), 139(5).

α-Pinene: 136[M+]: 93(100), 91(43), 92(39), 77(29), 79(24), 105(17), 121(12), 139(5).

Limonene: 136[M+]: 68(100), 67(86), 93(78), 79(43), 39(36), 53(34), 107(30), 121(30).

Nerol: 154[M+]: 41(100), 69(45), 81(15), 53(10), 93(10), 121(7), 111(3), 139(2).

RESULTS

The microbial transformation of volatile monoterpenoids by fungal SSCM and LM were examined. The results suggested that LM is not suitable for small amount of sample, because this method can only produce one product (thymol) with a very low yield. In the SSCM method we identified six components representing 67.4% in the bioconversion. The main products obtained in the bioconversion of citral were thymol (21.5%), geranial (18.6%), nerol (13.7%), geraniol (6.8%), α-pinene (3.7%) and limonene (3.1%) (Fig. 1).

From the data given in Fig. 1 it can be concluded that citral was mostly converted to thymol, geranial and nerol. This pathway of citral conversion involved formation of a cyclic compound with a single double bond and finally gave an aromatic compound (thymol) (Fig. 2).

Production of geranial (18.6%) and nerol (13.6%) suggests that two isomers of citral were converted — *E* and Z, respectively. Geranial is more stable than nerol because in the *E*-isomer the conjugatated double bond is in the *trans* configuration with the carbonyl bond (Fig. 3).

The time course of the bioconversion was as follows. After 5 days the products obtained in the bioconversion of citral were geraniol (12.22%), geraniol formate (2.215%) and geranial (2.434%).

After 10 days the mass spectra showed 9-octadecenoic acid, hexadecanoic acid and thiazole. These compounds have not been observed in natural products (Adams, 1995).

After 21 days (3 weeks) the main products obtained in the bioconversion of citral were thymol (21.5%), geranial (18.6%), nerol (13.7%), geraniol (6.8%), α-pinene (3.7%) and limonene (3.1%). The highest yield of citral bioconversion was obtained after 18 days (67.4%).

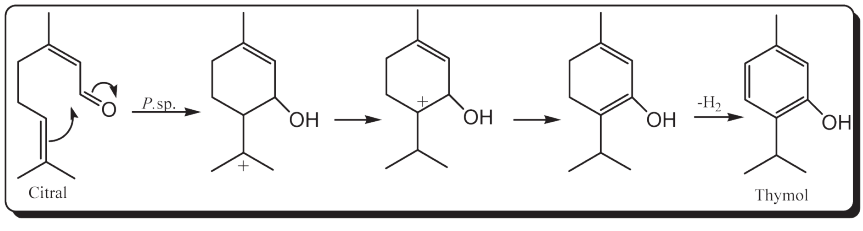

Figure 2. Suggested pathway of thymol formation from citral

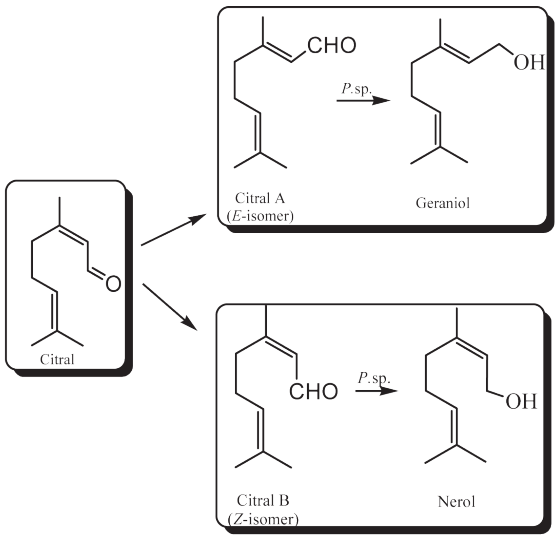

Figure 3. Possible pathway of bioconversion of two isomers of citral to geraniol and nerol

DISCUSSION

In previous studies of bioconversion of citral and nerol by spores of *Penicillium digitatum,* these were transformed into 6-methylhept-5-en-2-one (Demyttenaere *et al.,* 1998). Microbial transformation of geraniol, nerol, and citral by *A. niger* produced linalool and α-terpineol. Bioconversion of nerol with *Penicillium chrysogenum* mainly yielded α-terpineol and some unidentified compounds. With *Penicillium rugulosum* the major bioconversion product from nerol and citral was linalool (Demyttenaere *et al.,* 2000). Microbial transformation of menthol by SSCM of *A. niger* and *Penicillium* sp. produced terpineol and terpineol limonene, *p*-cymene, γ-terpinene, respectively (Esmaeili *et al.,* 2009c). The two main products of microbial transformation of citral were similar to those obtained in the mentioned works. The main bioconversion products of (-)-menthol by sporulated surface cultures *Mucor ramannianus* were *trans-p*-menthan-8-ol, trans-menth-2-en-1-ol, sabinane, *p*-menthane-3,8-diol, isomenthol, and 1,8-cineole (Esmaeili *et al.,* 2009). The main biotransformation products obtained from menthol by surface grown *Penicillium* sp. were α-pinene (18.0 %), terpineol (10.6 %), menthene (5.8 %), sabinene (3.9 %), 1,8-cineole (6.4 %), and limonene (3.2 %) (Esmaeili *et al.,* 2009c). The cited results suggest that microbial transformation of monoterpenes with *Penicillium and Aspergillus* involved an oxidation reaction and resulted in a more stable product. But bioconversion using SSCM and LM of *Penicillium* showed that it was possible to obtain similar product with high yield and selectivity. Thymol can be produced by removal of hydrogen and rearrangement of citral. Thymol is present in the essential oils from herbs and spices. It is produced by these plant species as a chemical defense against phytopathogenic microorganisms. Therefore, this compound has attracted great attention in food industry. It has been used as a natural preservatives in foods such as cheese to prevent fungal growth.

CONCLUSIONS

1. In this research, microbial transformation of citral by *Penicillium* sp. is studied using two types of culture.
2. The major components of the microbial transformation of citral using the SSCM approach appeared to be thymol (21.5 %), geranial (18.6 %) and nerol (13.7 %) with an overall yield of 67.4 %.
3. The results suggested that LM is not suitable for small amount of sample because this method can produce only one product (thymol) with very poor yield (<0.1 %).
4. A proposed pathway of citral bioconversion to thymol is presented in Fig. 2.
5. SSCM can keep its activity over prolonged period of 3 weeks. Spores can also be entrapped, e.g., in Ca-alginate, and used for continuous production of aroma compounds, a system well known for the bioconversion of fatty acids to methyl ketones. Finally, many different terpenoid compounds can be tested for bioconversion reactions with the same batch of fungal spores.

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