Chemistry of Natural Compounds, Vol. 47, No. 6, January, 2012 [Russian original No. 6, November–December, 2011]

BIOTRANSFORMATION OF THYMOL BY *Aspergillus niger*

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UDC 547.563.162.1

Biotransformation by cultured plant cells is an important method to convert many organic compounds into more useful ones, according to the ability of plant cell cultures specifically to produce secondary metabolites [1]. The biotransformation of menthol by SSCM of *Aspergillus niger* and *Penicillum* sp. has been described [2, 3]. The reactions involved in the biotransformation of organic compounds and monoterpene hydrocarbons by culture plant cells include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis, and glycosylation [4, 5].

The biotransformation of thymol, carvacrol, and eugenol by cultured cells of *Eucalyptus perriniana* has been studied previously. The cultured cells of *E. perriniana* are able to convert the aromatic compounds of spices into glycosides, which are accumulated in the cells [4]. Biotranformation of geraniol, nevol, and citral by *A. niger* produced linalool and α-terpineol and some unidentified compounds. With *Penicillium regulosum*, the major bioconversion product from nerol and citral was linalool [6].

The biotransformation of menthol by SSCM of *A. niger* and *Penicillium* sp. produced menthol from *A. niger*; the main products obtained were limonene, *p*-cymene, and γ-terpinene [2]. The main products of biotransformation of thymol were similar to those obtained in the mentioned work. The bioconversion of (–)-menthol by SSCM of *Mucor ramannianus* produced *trans*-*p*-menthan-8-ol, *trans*-menth-2-en-1-ol, sabinane, sabinene, *p*-menthane-3, 8-diol isomenthol, and 1,8-cineole [7].

The main biotransformation products from menthol by SSCM grown *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%) [3].

Using the surface of the organism and adding a methanolic solution converted geraniol to linalool and partially oxidized it to citral [8].

Fungal isolation was studied and growth conditions of the fungal strain identified in our laboratories in April 2008 in the province of Tehran, Iran, according to its physiological and morphological characters [9] and according to the physiological type culture cellection PTCC 5011 of the Iranian Research Organization for Science & Technology, Tehran, Iran.

A spore suspension of *A. niger* was prepared in nutrient broth solution for inoculation in Petri dishes. The strain was isolated from a soil sample collected in malt extract agar (MEA) (malt extract agar peptone, 1 g; dextrose, 20 g; agar, 20 g; distilled water, 1 L) and yeast extract sucrose agar (YES) (Yeast extract, 20 g; sucrose, 150 g; agar, 20 g; distilled water, 1 L) media. These were used for isolation, cultivation, and identification of the fungal isolate. The same media (without agar) were used for production and determination of secondary metabolites. *A. niger* was cultured on yeast extract sucrose or malt extract media for 10 days at 28 ± 2°C.

The microbial transformation of volatile oil monoterpene by fungal SSCM was examined. Fifteen components representing 80.1% of the biotransformation of thymol for 10 days were identified, of which toluene (43.4%), 3-methylcyclohexanol (9.0%), *p*-xylene (6.3%), and menthol (4.8%) were found to be the major constituents. Thus the bioconversion of thymol yields six aromatic hydrocarbons (57.5%), seven oxygenated monoterpenes (12.6%), and two oxygenated hydrocarbons (9.8%). Two components were identified in the 5-day transformation of thymol, making up 36.7% of the total composition. 3-Methylcyclohexanol and pentanol were the major components in this bioconversion. Thus, 3-methylcyclohexanol (89.0%) was identified in the biotransformation (10 days). In this transformation we found one compound. The main component in 5, 10, and 15 days was 3-methylcyclohexanol (2.9%, 9.0%, and 89.0%). As can be seen from the above information, the bioconversion of thymol was an oxidation reaction (Table 1).

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 0009-3130/12/4706-0966

TABLE 1. Percentage Bioconversion of Thymol by *A. niger*

Compound	5 days	10 days	15 days	Compound	5 days	10 days	15 days
Toluene	–	43.4	–	1-Ethyl-3-methylbenzene	–	2.12	–
Cyclohexanol 3-methyl	2.92	8.96	89.05	1,2,3-Trimethylbenzene	–	1.92	–
p-Xylene	–	6.29	–	1-Methyl 4-(1-methyl ethyl)benzene	–	2.7	–
Pentanol	33.84	–	–	Total	36.76	67.64	89.05
Ethyl benzene	–	2.25	–				

The determination of a suitable culture age during growth of *A. niger* in a medium for maximum product formation was done by harvesting cells in various stages of growth and employing them for thymol biotransformation. The results indicated the optimum cell culture age to be 10 and 15 days, with 80.1% and 89.0% product formation, respectively. Therefore, when thymol was converted with *A. niger* for 10 days, the main product was toluene. Second, when thymol was converted for 15 and 5 days, the main producs were 3-methylcyclohexanol and pentanol. The optimum initial medium pH and temperature values for biotransformation by SSCM were similar to those found in the literature for the biotransformation of another optical isomer, *d*-citronellal [8]. From the data in Table 1, it can be concluded that thymol was converted to toluene and 3-methylcyclohexanol. This formation involved oxidation of methyl groups to toluene. Reduction of benzene, formation of cyclohexane, and oxidation of methyl groups produced 3-methylcyclohexanol.

Theoretical Study of Biotransformation. The energy of thymol as compared with other products, especially toluene and 3-methylcyclohexanol, was investigated at the HF level of the theory using the density functional theory with the Becke 3LYP and the 6-311** G basis set. This showed the main products to have less energy (HF = –271.6 and –348.0 Hartree), but they are stable (FTIR ν_{max}, cm^{-1}): 1344 (-CH_3 or -CH_2), 1600 (C=C aromatic), and the main peak was observed at > 3000 cm^{-1} for the OH group. This result showed that the FTIR of 3-methylcyclohexanol in TS and experiment was similar.

The cited results suggest that the microbial transformation of monoterpenes with *Penicillium* and *Aspergillus* involves an oxidation reaction and results in a more stable product. So bioconversion using SSCM and LM of *Penicillium* showed that it was possible to obtain similar products with high yields and selectivities to thymol [10]. Therefore, this compound has attracted great attention in the food industry. It has been used as a natural preservative in foods to prevent fungal growth.

Growth Medium Conditions. For isolation, growth of the fungi in Petri dishes or Sabouraud dextrose agar (SDA) medium containing mycological 1.0% peptone, 4.0% glucose, and 1.5% agar was described in [11]. After 3 weeks, the surfaces of the Petri dishes were completely covered with spores, and the microbial transformation reaction started.

Experiment with SSCM. Spores were recovered from 3-week-old-surface cultures of *A. niger* grown in Petri dishes on SDA. Spore suspension was prepared by adding 10 mL 20% sterile Tween 80 solution in distilled water. A total at 50 mL of the spore suspension was obtained, which was shaken in a 250 mL conical flask. To this spore suspension 1 mL of a solution of 5% thymol in absolute ethanol was added, and the suspension was placed on a shaker at 180 rpm. After 3 weeks this suspension was extract with 3 × 50 mL diethyl ether, and the products were directly analyzed by GC, TS, UV, NMR, and GC/MS.

Analysis of Samples with GC/MS. GC analysis was performed on a Shimadzu 15A gas chromatograph equipped with a DB-J capillary column (50 m × 0.2 mm, film thickness 0.32 μm) 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 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 mass spectrometer with an HP-5MS column (30 × 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 carrier gas was 1mL/min. MS were taken at 70 eV. Identification of the constituents of the oil was by comparison of their mass spectra and retention indices (RRI) with those given in the literature and authentic samples [12–14]. The main peaks in the mass spectra of toluene (retention time 4.61 min), 3-methylcyclohexanol (retention time 9.29 min), and pentanol (retention time 5.17 min) are as follows: toluene 97 [M^+]: 97 (100); 51 (12); 30 (10); 63 (75); 51 (64); 93 (54); 50 (41). 3-Methyl cyclohexanol 114 [M^+]: 71 (100); 82 (80); 42 (52); 44 (50); 43 (40); 27 (32). Pentanol 88 [M^+]: 42 (100); 55 (70); 70 (60); 45 (50); 31 (43); 30 (38).

The FT-IR spectra (KBr, ν_{max}, cm^{-1}) showed 1344 (CH_3 or CH_2) and 1600 (C=C aromatic) for 10 days, a 3-methylcyclohexanol (15 days) peak in the region between 1344 and 2945. A main peak was observed at > 3000 for the OH group.

FT-IR (KBr) analysis in pentanol showed a peak at > 3000 cm^{-1} and a peak in the region between 1344–1382 cm^{-1}.

The UV spectra for 5, 10, and 15 days showed λ_{max} 163, 270, and 173 nm.

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