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RESEARCH ARTICLE

Microbial transformation of *p*-cymene

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Abstract

More than 30 bacteria, fungi and yeast were evaluated for the biotransformation ability of p-cymene to obtain new metabolites. In the course of 14 days microbial biotransformation period, 16 different metabolites were detected by TLC and GC/MS methods. *p*-cymen-8-ol and cumin alcohol were characterized as the initial metabolites by *Phanerochaete chrysosporium.*
Metabolites from *Penicillium claviforme* were identified as 3-methyl phenol or 2-methyl phenol, phenylethyl alcohol, 3-hydroxybenzaldehyde or 4-hydroxybenzaldehyde, 3-hydroxybenzyl alcohol.

Keywords: Biotransformation, *p*-cymene, GC/MS

Introduction

There is an increasing trend in research of natural products in drug discovery and food industry. One of the main drawbacks in the area is that usually compounds of interest are available in minute amounts (Balunas & Kinghorn, 2005). Conventional synthetic methods may often require extreme conditions in terms of pH, temperature and pressure, which possess high risks for personnel and the environment (Hegazy et al, 2015). Thus, it is crucial to develop methods which comply with green chemistry strategy.
Microbial transformation is one of such methods gaining popularity among natural product researchers. Various advantages of the microbial transformation include operation at near neutral pH, ambient temperature and atmospheric pressure, high reaction-, enantiomer- and regio-specificity with low cost substrates (Holland, 1998). This provides ample opportunities to utilize microbes to transform inactive or mildly active compounds into compounds with properties valuable in drug or food industries.
p-Cymene is abundant in nature and is a component of numerous essential oils such as the ones obtained from oregano or black cumin (*Nigella sativa*) (Bostancıoğlu, Kürkçüoğlu, Başer & Koparal, 2012; Benkaci-Ali, Baaliouamer, Wathelet & Marlier, 2010). Several microbial transformation studies with *p*-cymene as a substrate were performed previously using *E.coli* and *Pseudomonas* spp. Several metabolites include cumyl alcohol, cumic acid, 3-hydroxycumic acid, 2,3-dihydroxycumic acid, 2-oxo-4-methylpentanoic acid, 9-hydroxy-p-cymene, and p-cymen-9-oic acid (DeFrank and Ribbons, 1977; Wigmore and Ribbons, 1980; de Smet, Friedman and Gunsalus, 1989; Eaton, 1997, Nishio, Patel, Wang and Lau, 2001; Noma & Asakawa, 2010).
In the present study, more than 30 microorganisms were screened for new metabolites obtained from p-cymene transformation. Initially liquid media was used. Due to *p*-cymene's high volatility and lack of metabolites it was decided to impregnate β-cyclodextrin with *p*-cymene and then add it to the liquid and agar media. Sampling was performed periodically and evaluated for presence of new metabolites using TLC and GC/MS techniques.

Materials and Methods

Microorganisms

Microorganism	***Strain No***
Alternaria alternata	NRRL 20593
Aspergillus alliaceus	NRRL 317
Aspergillus niger	ATCC 10549
Aspergillus niger	isolate 1 - Anadolu Uni., Fac. of Pharmacy
Aspergillus niger	isolate 2 - Anadolu Uni. Fac. of Pharmacy
Aspergillus niger	NRRL 326
Botrytis cinerea	AHU 9424
Cellulomonas flavigena	ATCC 482
Corynespora casseiicola	DSM 62474
Corynespora casseiicola	DSM 62475
Fusarium culmorum	isolate Anadolu Uni., Fac. of Science, Biology
Fusarium heterosporum	DSM 62719
Fusarium moniliforme	NRRL 2374
Fusarium solani	ATCC 1284
Hansenula anomala	ATCC 20170
Kluyveromyces lactis	NRRL Y-8279
Mucor rammannianus	ATCC 1839
Neurospora crassa	N24 – Regensburg University, Faculty of Sci.
Neurospora crassa	Wild type – Regensburg University, Faculty of Sci.
Penicillium adametzii	NRRL 737
Penicillium chrysogenum	NRRL 792
Penicillium claviforme	MR376
Penicillium expansum	Balikesir Univ. Dep. of Biology.
Penicillium sp.	Tokushima Univ. Fac. of Pharmacy.
Phanerochaete chrysosporium	isolate -Balikesir Univ. Dep. of Biology.
Phanerochaete chrysosporium	BKM-F-1767
Pseudomonas putida	NRRL B-4067
Pseudomonas syringae pv. *tomato*	isolate Agriculture Research Institute (Ankara)
Saccharomyces cerevisiae	ATCC 9763
Sporobolomyces pararoseus	ATCC 11385
Thamnidium elegans	ATCC 18191
Torulaspora delbrueckii	NRRL Y-866
Trichoderma harzianum	isolate Anadolu Uni., Fac.of Science, Biology.
Yarrowia lipolytica	ATCC 8661
Yarrowia lipolytica	NRRL YB-423

ATCC-American Type Culture Collection (Amerika BD); NRRL-Northern Regional Research Lab. Agricultural Res. Service C.C. (USDA); DSM- Deutsche Sammlung von Mikroorganismen und Zellkulturen (Almanya)

The microorganisms were stored at -85°C in sterile 15% glycerol solutions. To refresh cultures Saboraud glucose agar (SGA) plates were used followed by inoculating in liquid media (glucose, peptone, yeast extract, NaCl and Na_2HPO_4, pH 7.0) or fresh SGA plates. Liquid media flasks were placed in an orbital shaker (New Brunswick Scientific, USA) operating at 200 rpm and 26°C for 14 days.

Extraction of metabolites

Periodically sampling of media was performed using liquid-liquid or liquid-solid extraction. Aliquot of liquid or part of agar media was transferred into a tube containing EtOAc and vortexed. Organic layer was collected separately. This procedure was repeated 3 times. Later organic phase was evaporated using centrifugal vacuum concentrator at 39 °C (Labconco, USA). Metabolite presence was evaluated using TLC or GC/MS techniques.

Thin Layer Chromatography (TLC)

Aluminum TLC plates (0.2/0.25 mm, silica gel, 40/60 GF_{254} , 20x20 cm) were used for qualitative analysis of metabolites. Mobile phases hexane:EtOAc (8:2), hexane:EtOAc:acetone (7:3:1) were used. After elution of samples, TLC plates were examined under UV light at 254/364 nm wavelength. To detect compounds not having UV absorbance, plates were sprayed with anisaldehite/H2SO4 reagent and heated at 120°C for 3-5 min.

Gas Chromatography and Gas Chromatography-Mass Spectrometry (GC/FID, GC/MS)

The GC analysis was carried out using an Agilent 5975 GC-MSD system. Polar HP-Innowax column (60m x 0.25mm, 0.25μm film thickness) and helium as carrier gas (0.8 ml/min) were used. The temperature program (A) was as follows: 60°C hold for 10 min, ramp at 4°C/min to 220°C, 220°C hold for 10 min, ramp at 1°C/min to 240°C. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450. Also, Thermo PolarisQ GC-MS system, with nonpolar Teknokroma TRB-5MS column (30mx0.32mm, 0.25μm film thickness) was used. The temperature program (B) was as follows: 50 ˚C hold 0 min, ramp at 20 ˚C/min to 100 ˚C, 100 ˚C hold for 1 min, ramp at 30 ˚C/min to 145 ˚C, 145 ˚C hold for 2 min, ramp at 40 ˚C/min to 220 ˚C, 220 ˚C hold for 1 min, ramp at 30 ˚C/min to 300 ˚C, 300 ˚C hold for 5 min.

Compound Identification

To identify compounds computer matching against commercial libraries (Wiley GC/MS Library, Adams Library, MassFinder 3.1 Library) and in-house “Başer Library of Essential Oil Constituents” built up by genuine compounds and components of known oils were used.

Results and Discussion

p-Cymene was subjected to transformation using 35 microorganisms. Presence of metabolites was evaluated using TLC and GC/MS methods. Microorganisms which yielded detectable metabolites were *Aspergillus niger* NRRL 326 (1 metabolite), *Aspergillus alliaceus* NRRL 317 (2 metabolites), *Phanerochaete chrysosporium* BKM-F-1767 (7 metabolites) and *Penicillium claviforme* MR376 (5 metabolites).

Metabolites from *Aspergillus niger* NRRL 326 ve *Aspergillus alliaceus* NRRL 317

Although the metabolites were detected using TLC, it was not possible to detect them using different GC/MS systems with polar and nonpolar columns. It is proposed that the metabolites are not volatile. We suggest using other chromatographic methods for their detection.

Metabolites from *Phanerochaete chrysosporium* BKM-F-1767

It was possible to detect 7 metabolites using GC/MS system with polar column and temperature program A. While 5 (M2-M7) metabolites were unidentified, 2 metabolites were identified as p-cymen-8-ol and cumin alcohol (Figure 1).

Metabolites from *Penicillium claviforme* MR376

Metabolites of the biotransformation were detected using TLC method. Although it was not possible to detect them using GC/MS system with polar column, the metabolites were detected by GC/MS system with nonpolar column and using temperature program B. Search of Wiley and NIST libraries matched compounds below showing relevant mass spectrums: 3-methyl phenol or 2-methyl phenol (M8), phenylethyl alcohol (M9), 3-hydroxybenzaldehyde or 4-hydroxybenzaldehyde (M10), 3-hydroxybenzyl alcohol (11) (Figure 2).

Figure 1. Biotransformation of *p*-cymene using *Phanerochaere chrysosporium* BKM-F-1767

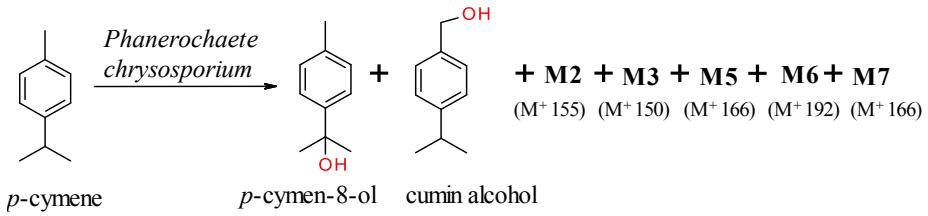
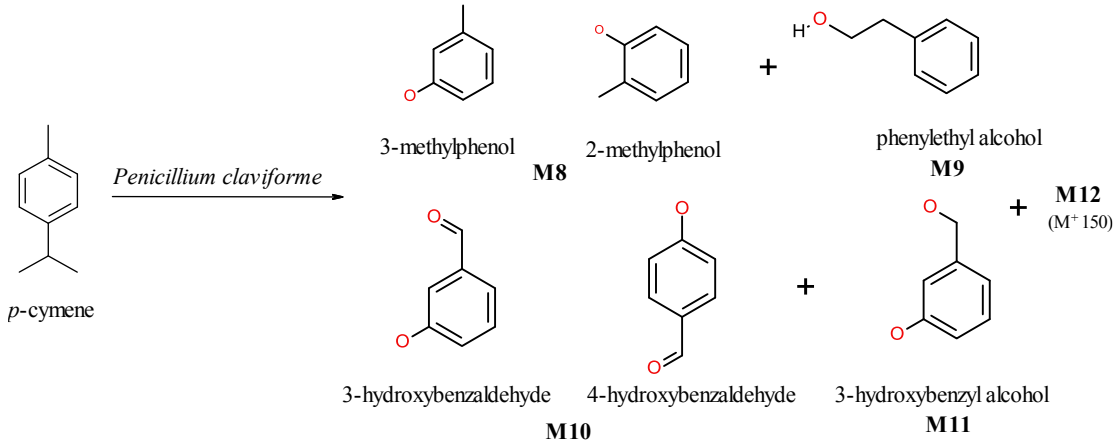


Figure 2. Biotransformation of *p*-cymene using *Penicillium claviforme* MR376



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