Biotransformation of α -Pinene to Terpineol by Resting Cell Suspension of *Absidia corulea*

Busi Siddhardha · M. Vijay Kumar ·
U. S. N. Murty · G. S. Ramanjaneyulu ·
S. Prabhakar

Received: 9 December 2009 / Accepted: 11 May 2010 / Published online: 28 January 2011
© Association of Microbiologists of India 2011

Abstract Microbial biotransformation of monoterpenes results in the formation of many valuable compounds. Many microorganisms can be used to carry out extremely specific conversions using substrates of low commercial value. *Absidia corulea* MTCC 1335 was examined for its ability to transform α -Pinene enantiomers. The substrates (–)- α -Pinene and (+)- α -Pinene converted to α -terpineol and isoterpineol, were detected in gas chromatographic analysis. The Biotransformation kinetics of the oxidized products were analysed using GC–MS. With both the substrates the products formed were similar and not much difference in the rate of transformation was observed, suggesting no enantioselectivity of organism towards the substrate.

Keywords Biotransformation · *Absidia corulea* · (–)- α -Pinene · (+)- α -Pinene · α -Terpineol · GC–MS

Terpenes occur widely in nature. Monoterpenes as substrates of microbial transformations have led to a great variety of oxyfunctionalized compounds [1]. Allylic hydroxylations of monoterpene hydrocarbons are interesting reactions because of the multiple bioactivities of many of the resulting aroma compounds [2].

The biotechnological generation of natural aroma compounds is rapidly expanding. Production of desired

compounds by single-step biotransformation using microorganisms is natural, effective and economical [3, 4]. Hydroxylation of monoterpene substrates has been exclusively achieved by the use of filamentous fungi [5]. Terpenes and their oxygenated derivatives are extensively used by the flavour and fragrance industries. The important attribute of the biotransformation is the formation of monoterpene precursors or intermediates into their more aggregated value products for the flavour and fragrance industries [6, 7].

Terpene such as α -pinene is inexpensively available in large quantities. Biotransformation of (–)- α -Pinene and (+)- α -Pinene results in the formation of α -Terpineol. The product, α -Terpineol ($C_{10}H_{18}O$) is the most important monocyclic monoterpene alcohol and used as flavour and fragrance chemical because of its lilac odour [8].

Absidia coerulea is known to transform terpenes and steroid hormones. Testosterone, androstenedione, progesterone and testosterone derivatives were selectively hydroxylated using *A. coerulea* [9]. Oxygenation of taxadiene derivatives and hydroxylation of α -santonins was achieved using *A. coerulea* [10, 11]. Based on the literature the current study was focused on the biotransformation of α -Pinene using *A. coerulea*.

(–)- α -Pinene, (+)- α -Pinene and α -Terpineol were purchased from the Sigma-Aldrich. All the chemicals and solvents were of the best available commercial grade. The fungal culture *A. corulea* MTCC 1335 was procured from microbial type culture collection (MTCC), IMTECH, Chandigarh, India and the culture was maintained on potato dextrose agar. Biotransformation of (–)- α -Pinene and (+)- α -Pinene were carried out by *A. corulea* cultures. Before each experiment spore suspension was transferred to a 250 ml conical flask containing 100 ml of freshly prepared potato dextrose broth. Mycelium was then grown for

B. Siddhardha · M. Vijay Kumar · U. S. N. Murty (✉)
Biology Division, Indian Institute of Chemical Technology,
Uppal Road, Hyderabad 500 007, India
e-mail: usnmurty@iictnet.org; murty_usn@yahoo.com

G. S. Ramanjaneyulu · S. Prabhakar
National Centre for Mass Spectrometry, Indian Institute
of Chemical Technology, Hyderabad 500 007, India

1 week at $27 \pm 2^\circ\text{C}$. After stipulated time of incubation, 1.0 ml of a methanolic solution (60 mg/ml) of substrate, without prior sterilization, was added to the mycelial suspensions, and the cultures were incubated for 3 days. All experiments were conducted in triplicate.

The flasks were closed with a glass stopper in order to avoid substrate and product evaporation. At the end of the experiments the product recovery was performed by liquid–liquid extraction with of ethyl acetate (v:v). The final solution was dried over anhydrous sodium sulphate. For kinetic resolution samples were extracted on 24, 48 and 72 h. The residue obtained was made up to 1 ml with hexane and 3 ml of the solution was subjected to gas chromatography–mass spectrometry (GC–MS). GC–MS analysis was performed with a agilent 6890 GC coupled with a agilent 5973 MSD (mass selective detector) and a inventory CP-Sil 8CB ($30 \mu\text{m} \times 250 \mu\text{m} \times 0.30 \mu\text{m}$) was used. The column temperature was programmed at 50°C for 3 min, increased to $12^\circ\text{C}/\text{min}$ at 130°C , and then increased at $15^\circ\text{C}/\text{min}$ at 220°C by 5 min. Helium was the carrier gas, and the injection and detector temperatures were 250 and 300°C , respectively. One microliter of the dried solution was injected into the GC/MS system. The apparatus operated with a flow rate of 1 ml/min in an electronic impact mode of 70 eV and in split mode (split ratio of 10:1). Compounds identification was based on a comparison of retention indexes (determined relatively to the retention times of a series of *n*-alkanes) and mass spectra with those of authentic standard purchased from Sigma-Aldrich. The amount of pinene, terpineol and isoterpineol present in the samples was calculated with respect to the area of standard compound and characterised by means of the GC–MS fragmentation pattern.

The chromatographic analyses of the extracts indicated that the biotransformation products obtained using (–)- α -Pinene and (+)- α -Pinene are basically the same. To the

Fig. 1 (+)- α -Pinene Biotransformation efficacy of *A. corulea*

confirmatory studies, the crude extract was purified by column chromatography on silica gel (70–230 mesh—Aldrich) using hexane:EtOAc (90:10 and 80:20), collecting 10 ml fractions. The optical rotation value of purified α -Terpineol ($[\alpha]_D^{20} = -258^\circ$) was measured with a JASCO DIP-360 polarimeter.

Biotransformation of (–)- α -Pinene and (+)- α -Pinene were carried out in order to achieve terpineol formation, by the use of *A. corulea*. The results showed that under the evaluated conditions, *A. corulea* was able to transform both the enantiomers. The time courses analysis of bioconversion of (–)- α -Pinene and (+)- α -Pinene by *A. corulea* shown in Fig. 1. Depicted values in Fig. 1 correspond to the overall mean concentrations obtained in three independent experiments. *A. corulea* afforded the best results, achieving 54.69% conversion (relative integrated area GC–MS) of (+)- α -Pinene to α -Terpineol after 72 h

Table 1 Biotransformation of (+)- α -Pinene and (–)- α -Pinene by *A. corulea*

Time (h)	Substrate consumed ^a (mg) $\pm$ SD	Product formed ^a (mg) $\pm$ SD	
	(+)- α -Pinene)	α -Terpineol	Isoterpineol
24	38.796 $\pm$ 1.248	13.506 $\pm$ 2.262	7.698 $\pm$ 1.05
48	28.86 $\pm$ 1.584	23.262 $\pm$ 0.66	7.878 $\pm$ 1.56
72	16.356 $\pm$ 1.95	34.116 $\pm$ 3.708	9.528 $\pm$ 1.35
	(–)- α -Pinene)	α -Terpineol	Isoterpineol
24	48.756 $\pm$ 1.968	7.89 $\pm$ 5.01	3.354 $\pm$ 1.626
48	34.806 $\pm$ 0.756	15.888 $\pm$ 3.006	9.306 $\pm$ 2.76
72	22.08 $\pm$ 2.262	28.146 $\pm$ 0.654	9.774 $\pm$ 1.446

The values are calculated from the relative integrated area of GC–MS chromatogram

SD standard deviation

^a Mean of three replicates

incubation (Fig. 1), whereas *A. corulea* was less efficient on (–)- α -pinene for the production of α -Terpineol (45.47% conversion in 3 day incubation: Fig. 1). Isoterpineol was formed as minor product in both the substrates transformation with a percentage of 14.07 and 14.87% respectively for (+)- α -Pinene and (–)- α -Pinene after 72 h incubation. From the GC–MS data the amount (mg) of α -Terpineol and isoterpineol formed from 60 mg of (+)- α -Pinene was calculated to be 34.116 ± 3.70 and 9.528 ± 1.35 mg respectively after 72 h of incubation (Table 1). Where as biotransformation of (–)- α -Pinene produced 28.146 ± 0.65 mg of α -Terpineol and 9.774 ± 1.44 mg of isoterpineol at the end of 72 h.

The results obtained in this work indicate that the enzymes responsible for the biotransformation producing the metabolites found in all the reaction systems studied are probably not having stereoselectivity, since the bioconversions of both the substrates resulted in the production of same compounds. *Aspergillus niger* selectively transformed α -pinene to verbenone, verbenol and α -terpineol whereas β -pinene to only α -terpineol [12, 13]. The biotransformation of (–)- α -Pinene and (+)- α -Pinene results in the formation of oxidized products through the insertion of oxygen into the substrate molecule. Alpha-terpineol was identified as the biotransformation products of (–)- α -Pinene and (+)- α -Pinene. The product (α -Terpineol) has wide applications in the pharmaceutical industry as an intermediate in the chemical synthesis and in the aroma and fragrance industry.

Acknowledgments Authors are thankful to the Director, Indian Institute of Chemical Technology, Hyderabad for providing necessary facilities and encouragement for carrying out of this work.

References

1. Trudgill PW (1994) Microbial metabolism and transformation of selected monoterpenes. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer, Dordrecht, pp 33–61
2. Onken J, Berger RG (1999) Effects of *R*-(+)-limonene on submerged cultures of the terpene transforming basidiomycete *Pleurotus sapidus*. J Biotechnol 69:163–168
3. Abraham BG, Berger RG (1994) Higher fungi for generating aroma components through novel biotechnologies. J Agric Food Chem 42:2344–2348
4. Hagedorn S, Kaphammer B (1994) Microbial catalysis in the generation of flavour and fragrance chemicals. Annu Rev Microbiol 48:773–800
5. Van Rensburg E, Moleleki N, van der Walt JP, Botes PJ, van Dyk MS (1997) Biotransformation of (+)limonene and (–)piperitone by yeasts and yeast-like fungi. Biotechnol Lett 19:779–782
6. Adams, Demyttenaere JCR, De Kimpe N (2003) Biotransformation of (*R*)-(+)- and (*S*)-(–)-limonene to α -terpineol by *Penicillium digitatum*—investigation of the culture conditions. Food Chem 80:525–534
7. Tan Q, Day DF (1998) Bioconversion of limonene to α -terpineol by immobilized *Penicillium digitatum*. Appl Microbiol Biotechnol 49:96–101
8. Demyttenaere JCR, Van Belleghem K, De Kimpe N (2001) Biotransformation of (*R*)-(+)- and (*S*)-(–)-limonene by fungi and the use of solid phase microextraction for screening. Phytochemistry 57:199–208
9. Brzezowska E, Dmochowska Gladysz J, Kolek T (1996) Biotransformation XXXIX. Metabolism of testosterone, androstenedione, progesterone and testosterone derivatives in *Absidia coerulea* culture. J Steroid Biochem Mol 57:357–362
10. Sun DA, Sauriol F, Mamer O, Zamir Lolita O (2001) Reanalysis of the biotransformation of 4(20), 11(12)-taxadiene derivatives. Can J Chem 79(9):1381–1393
11. Yang Lin, Dai Jungui (2008) Biotransformation of tetrahydro-alpha-santonins by *Absidia coerulea*. Nat Prod Res 22(6): 499–506
12. Toniazzo G, Oliveira D, Dariva C, Oestreicher EG, Antunes OAC (2005) Biotransformation of (1S)-(–)alpha-pinene, (–)beta-pinene and (+)limonene by *Aspergillus niger* ATCC 9642. Biotechnol Appl Biochem 123:837–844
13. Rozenbaum HF, Patitucci ML, Antunes OAC, Pereira N Jr (2006) Production of aromas and fragrances through microbial oxidation of monoterpenes. Braz J Chem Eng 23(3):273–279