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Biocatalytic reduction of (+)- and (–)-carvone by bacteria

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Abstract

The biotransformation of the two enantiomers of carvone, (–)-(**1a**) and (+)-(**1b**), by two bacteria (*Pseudomonas putida* and *Acinetobacter lwoffi*) isolated from different environments is reported. The metabolites from the biotransformations carried out in liquid cultures using the sterile TS-2 mineral medium were screened by headspace solid phase microextraction/GC–MS. The results show that these bacteria are efficient tools in the diastereoselective bioreduction of (+)- and (–)-carvone. ***To cite this article: M.R. Cramarossa et al., C. R. Chimie 8 (2005).***

Résumé

La biotransformation des deux énantiomères de la carvone, (–)-(**1a**) et (+)-(**1b**), par les deux bactéries *Pseudomonas putida* et *Acinetobacter lwoffi* est présentée. Les métabolites formés lors de la biotransformation dans des cultures en phase liquide utilisant le milieu stérile TS-2 sont analysés par headspace solid phase microextraction/GC–MS. Les résultats montrent que ces bactéries sont des outils efficaces pour la bioréduction diastéréosélectives de la (+) et la (–)-carvone. ***Pour citer cet article : M.R. Cramarossa et al., C. R. Chimie 8 (2005).***

Keywords: Bacteria; Bioconversion; Carvone; Headspace-SPME/GC–MS; Oxidoreductase

Mots clés : Bacteries ; Bioconversion ; Carvone ; Headspace-SPME/GC–MS ; Oxidoreductase

1. Introduction

The flavor substances that can be classified as 'natural' according to European Community regulations [1] are those that can be obtained using physical, or enzymatic or microbial methods. Monoterpenes are commonly associated with flavors and fragrances and are the main constituents of many essential oils of spices, herbs and conifer species.

Volatility, the property that make monoterpenes so interesting as flavor and fragrance compounds, causes

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many problems during their large-scale bio-production; furthermore, they may be toxic for the whole cells [2].

The relatively small variety of commercially terpenoids and other natural products accumulated by plants together with the consumer request for biological and natural flavors have encouraged a considerable interest in the development of microbial or enzymatic processes as a useful approach for the production of these metabolites [3].

In the case of carvone, a very important component of the flavor and fragrance industry, the organoleptic properties are determined by its stereochemistry. The (–)-(4*R*)-carvone (**1a**) is the main component of spearmint (*Mentha spicata*), whereas (+)-(4*S*)-carvone (**1b**) is the main component in the essential oil of caraway (*Carum carvi*). Both enantiomers are much used for the enantioselective syntheses of natural products [4].

In a continuation of our researches on the biocatalytic transformation of monoterpenes, here we report the regio-selective reduction of (–)-(**1a**) and (+)-(**1b**) by the bacteria *Pseudomonas putida* and *Acinetobacter lwoffi* isolated from various environments. The diastereo- or enantio-selective reduction of C=C double bond of α,β-unsaturated carbonyl compounds is not a plain chemical reaction; we therefore tried this reaction by use of bacteria [5]. The products were investigated with headspace solid phase micro extraction (SPME) and GC/MS analysis.

Biotransformation of (**1a**) and (**1b**) have been previously carried out by marine microalgae [6], yeasts and yeast-fungi [7], bryophytes cell cultures [8], bacteria like *Escherichia coli* [9]. The use of some bacteria, among which a strain of *P. putida*, has been reported by Demirci et al. [10].

2. Results

P. putida and *A. lwoffi* were isolated from olive residues and citrus orchard, respectively. The stock bacteria cultures were maintained on agar slants (Tryptic soy agar medium, TSA) stored at 4 °C, and in frozen form at –80 °C.

Sealed vials (50 ml) containing 15 ml of sterile TS-2 mineral medium [(g l^{-1}): $MgSO_4$ = 0.1, NH_4Cl = 2, $CaCl_2$ = 0.01, $FeSO_4$ = 0.005, KH_2PO_4 = 1.5] were inoculated with calibrated cell suspension (A_{580} = 0.5, corresponding to an average cell concentration of 10^6 ml^{-1}). Fifty microliters of a solution of the substrates in EtOH (30% v/v) was then added and the vials were placed at 28 °C in an orbital shaker (280 rpm).

The biotransformation products were screened by headspace SPME (extraction: 30 min at 25 °C with a 75 μm carboxen/polidimethylsiloxane fiber, Supelco, Bellafonte, PA, USA). GC–MS analysis of the SPME extracts were performed with an Agilent Technologies 5973 Network Mass Selective Detector equipped with a HP-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness); working conditions were: injector 280 °C, transfer line to MS system 280 °C, oven temperature—start 40 °C, hold 2 min, programmed from 40 to 110 °C at 5 °C min^{-1} and from 110 to 280 °C at 10 °C min^{-1}, hold 1 min, carrier gas He 1 ml min^{-1}, split mode (1/10), electron impact ionization 70 eV; acquisition parameters 35–400 *m/z*. The biotransformation products were identified on the basis of their mass fragmentation pattern and by comparison with standard samples spectra. Quantitative determination of the products was carried out using external standard method.

The metabolites of carvone enantiomers transformed by the two bacteria are shown in Figs. 1 and 2. *P. putida* was the most effective bacteria for the bioconversion of (**1a**) and (**1b**); *A. lwoffi* instead gives rise to a slow conversion with low yields. Both bacteria reduce (**1a**) and (**1b**) at the endocyclic double bond. *A. lwoffy* gives rise subsequently to the carbonyl reduction affording a dihydrocarveol.

In Fig. 3 the pathways for the biotransformation of (**1a**) and (**1b**) using the two bacteria are reported.

P. putida reduces regio-selectively (**1a**) and (**1b**) at the endocyclic double bond to give (1*R*,4*R*)-(**2a**) (95%, 84 h) and (1*R*,4*S*)-dihydrocarvone (**2b**) (84%, 82 h), respectively. (**2b**) is transformed in time into (1S,4S)-dihydrocarvone (**2c**) (15%, 84 h), affording a 1:1 mixture after 10 days and a diastereomeric excess (**2c**/**2b** = 4) after 40 days.

A. lwoffy reduces (–)-(4R)-carvone (**1a**) first at the endocyclic double bond to give (**2a**) (20%, 84 h) and then to the carbonyl group affording (1*R*,2*R*,4*R*)-dihydrocarveol (**3**) and (+)-(4*S*)-carvone (**1b**) only at the endocyclic double bond, as *P. putida*, but with lower yields (34%, 84 h).

P. putida was previously used as biocatalyst by Demirci et al. [10] for the reduction of (**1a**) and (**1b**).

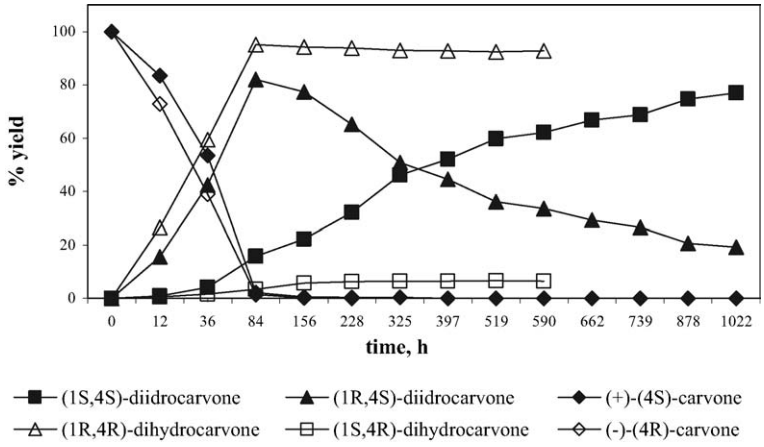

Fig. 1. Bioconversion of (+)- and (–)-carvone versus time using *P. putida*.

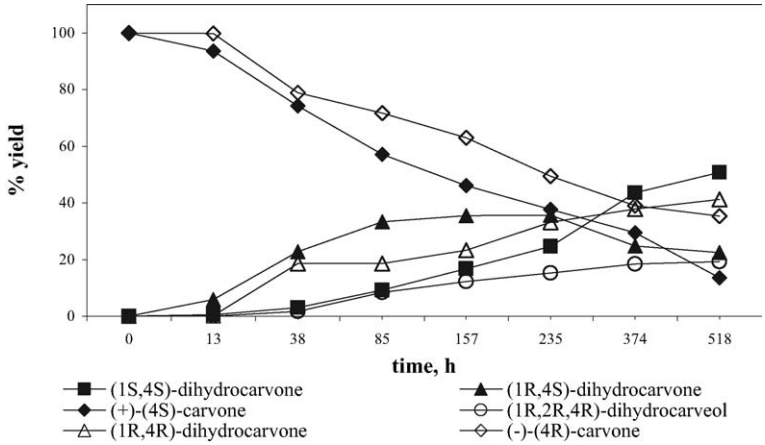

Fig. 2. Bioconversion of (+)- and (–)-carvone versus time using *A. lwoffi*.

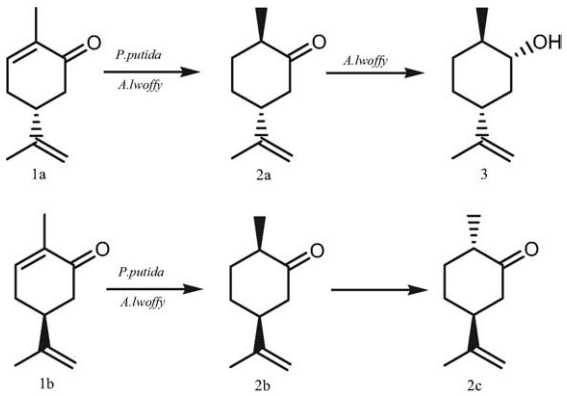

Fig. 3. Biotransformation pathways of (**1a**) and (**1b**).

The major products, screened by SPME, were recognized as (**2a**) and (**2b**), respectively, and the rate of conversion of (**1a**) was less than (**1b**); minor products were reduced ketones. In our case, the rate of conversion of (**1a**) to (**2a**) is significantly higher with respect to that reported by Demirci, whereas that of (**1b**) is comparable; no reduced ketones are found starting from both substrates.

In this paper we confirm the capability of bacteria cultures to reduce (+)- and (–)-carvone. The diastereo- or enantio-selective reduction of C=C bonds of α,β-unsaturated carbonyl compounds, a difficult chemical transformation [5], is successfully carried out by whole cell systems possessing NADH dependent enoate reductase activity [11]. The different steps and the stere-

ochemical outcome of the enzymatic enoate reduction have been elucidated [12]; the trans-addition of hydrogen across the C=C bond [13] yielding dihydrocarvones occurs first and an alcohol dehydrogenase reduction of the saturated carbonyl compounds follow yielding dihydrocarveols.

Our results show that the enoate reductase step of (–)-carvone (**1a**) occurs with high diastereoselectivity to (1*R*,4*R*)-dihydrocarvone (**2a**) by both *P. putida* and *A. lwoffi*; conversion rate is however higher using *P. putida*. Alcohol dehydrogenase works only with *A. lwoffi* and is highly diastereoselective, yielding (1*R*,2*R*,4*R*)-dihydrocarveol (**3**). The reduction of (+)-(4*S*)-carvone (**1b**) occurs with the same selectivity of (–)-(4*R*)-carvone (**1a**). In this case, however, the reduced product (1*R*,4*S*)-dihydrocarvone (**2b**) isomerizes to (1*S*,4*S*)-dihydrocarvone (**2c**): it is at present unknown if this epimerization is due to a dihydrocarvone isomerase [14].

References

[1] The Council of the European Communities: Council Directive 88/388/EEC of 22 June 1988 on the approximation of the laws of the member States relating to flavourings for use in foodstuffs and to source materials for their production.

[2] M.J. van der Werf, J.A.M. De Bont, D.J. Leak, in: R.G. Berger (Ed.), Advances in Biochemical Engineering Biotechnology, 1997, p. 147.

[3] (a) R. Barkovich, J.C. Liao, Metab. Eng. 3 (2001) 27; (b) W.R. Strohl, Metab. Eng. 3 (2001) 4.

[4] T.L. Ho, Enantioselective Synthesis, in: Natural Products from Chiral Terpenes, Wiley, New York, 1992, p. 123.

[5] A. Pfaltz, in: G. Helmchem, R.W. Hoffmann, J. Mulzer, E. Schaumann (Eds.), Houben-Weyl, Stereoselective Synthesis, vol. E 21d, Thieme, Stuttgart, 1995, p. 4343.

[6] I.L. Hook, S. Ryan, H. Sheridan, Phytochem. 63 (2003) 31.

[7] M.S. van Dyk, E. Van Rensburg, I.P.B. Rensburg, N. Moleleki, J. Mol. Catal., B Enzym. 5 (1998) 149.

[8] A. Speicher, H. Roeser, R. Heisel, J. Mol. Catal., B Enzym. 22 (2003) 71.

[9] O.A. Carter, R.J. Peters, R. Croteau, Phytochemistry 64 (2003) 425.

[10] F. Demirci, N, B. Kirimer, Y. Demirci, K.H.C. Noma, Başer, Z. Naturforsch. 56 (2001) 58.

[11] H. Simon, J. Bader, H. Günther, S. Neumann, J. Thanos, Angew. Chem. Int. Ed. Engl. 24 (1985) 539.

[12] M. Desrut, A. Kergomard, M.F. Renard, H. Veschambre, Tetrahedron 37 (1981) 3825.

[13] G. Fronza, C. Fuganti, M. Mendozza, R. Rigoni, S. Servi, G. Zucchi, Pure Appl. Chem. 68 (1996) 2065.

[14] (a) M.J. van der Werfe, A.M. Boot, Microbiology 146 (2000) 1129; (b) Y. Noma, S. Nonomura, H. Sakai, Agric. Biol. Chem. 39 (1975) 937.