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Biotransformation of (+)-carvone and (-)-carvone using human skin fungus: a green method of obtaining fragrances and flavors

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ABSTRACT

The synthesis of optically pure compounds is increasingly in demand by the pharmaceutical, fine chemical and agro-food industries, while the importance of chirality in the activity and biological properties of many compounds has been established. In this context, the aim of the present study was to evaluate the biotransformation capacities of (+)-carvone and (-)-carvone using fungi isolated from human skin. All seven of the fungi genera evaluated were capable of activated alkene hydrogenation, followed by the reduction of chiral ketone to alcohol.
Keywords: biotransformation, fungi skin, carvone, fragrance

INTRODUCTION

Monoterpenes represent one of the largest classes of flavor compounds, comprising more than 400 natural substances, representing a valuable resource for the flavor and fragrance industries (Goretti et al. 2013). Such compounds possess a chiral carbon atom, which allows derivatives with a spatially defined structure to be obtained. This attribute makes them attractive substrates for chemical and biological modifications, allowing products suitable for industrial use to be obtained (Groussin & Antoniott 2012). Desire among consumers for natural flavors and fragrances has encouraged a growing number of the scientific community to study and develop new catalysts for the production of this class of molecule (Brenna et al. 2011). The biocatalysis process meets this requirement, as it involves chemical reactions with structural changes of a substrate using cells (animal or plants) or isolated enzymes, which normally enables stereopure products to be obtained (Balcerzak et al. 2014).

One of these reactions is the emerging asymmetric bioreduction of α,β-unsaturated alkenes catalyzed by enoate reductases dependent flavin mononucleotides (FMN), of the "Old Yellow Enzyme" family (OYE) (Toogood et al. 2011). These enzymes have been studied for their ability to catalyze asymmetric reductions in electronically activated alkenes, such as (4*S*)-(+)-carvone (Silva et al 2013; Aquino et al, 2012; Goretti et al 2009) and (4*R*)-(-)-carvone (Goretti et al 2013; Aquino et al 2012). Although stereoselectivity is normally high, the chemoselectivity of the whole-cell bioreduction (related to competition

between C=C and C=O) is often poor, due to the presence of other enzymes such as carbonyl reductases (CRs) (Goretti et al 2013; Aquino et al 2012).

The objective of this study was to propose a "green" method of obtaining fragrances and flavor through the biocatalysis of (4*S*)-(+)-carvone and (4*R*)-(-)-carvone monoterpenes using human skin fungi.

MATERIALS AND METHODS

Microorganism cultures

Seven filamentous fungi were isolated from human skin and characterized by Silva (2012) at Unicamp. These cultures were maintained under cryopreservation in the Biotechnology Laboratory of the Natural and Synthetic Products Department Pharmacy. The evaluated fungi were *Scolecobasidium* sp. (F37), three lines of *Cladosporium* sp. (F43, F56 and F58), *Phoma* sp. (F45), *Aureobasidium* sp. (F46) and *Epicoccum* sp. (F63). The microorganisms were activated in nutrient agar (NA) and supplemented with beef extract (0.3%), yeast extract (0.3%), peptone (0.5%) and glucose (1%) at a temperature of 28 °C.

Chemicals

The (4*R*)-(-)-carvone and (4*S*)-(+)-carvone substrates were obtained from Sigma-Aldrich. The sodium sulfate anhydrous, NaH_2PO_4, KH_2PO_4 and solvents were purchased from Synth. The culture media was from Kasvi.

Bioconversion Reactions

The method was adapted from Silva (2012), and consists of transferring the fungus from NA plates using 250 mL flasks containing 40 mL of liquid NA medium and incubated at 28 °C under agitation (200 rpm) for 48 h. Next, a new subculture was prepared by transferring the young cells to new flasks under the same conditions. Then 2 g of fresh microbial cells and 10 mg of carvones were transferred to 250 mL flasks containing 50 mL of Sφrensen buffer (NaH_2PO_4 -KH_2PO_4), pH 7. The reaction flasks were maintained under agitation at 200 rpm at 28 °C and monitored through the withdrawal of 2 mL aliquots every 24 h for a period of 4 days. Aliquot samples underwent extraction with 2 mL of ethyl acetate and the organic phase was analyzed by gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC).

GC-MS analysis

Analyzes of the product biocatalysis were performed using a Agilent Technologies 5977-A GC/MS equipped with a HP-5MS column (30 m x 0.25 mm x 0.250 µm), helium carrier gas (flow rate 1.0 mL min^{-1}), split ratio 1:20, a column temperature programmed with a ramp of 60 to 90 °C (3 °C/min) and 90-285 °C (50 °C/min). The volume injected was 2 µL and the injector temperature was 220 °C, with the interface at 250 °C and the mass detector at 300 °C. Identification of the products was made using the Kovats index (Adams 1995) and comparison with the Wiley library 9ª edition NIST 5.

RESULTS AND DISCUSSION

The possible biotransformation pathway of the *α*,*β*-unsaturated (4*S*)-(+)-carvone (**1**) and (4*R*)-(-)-carvone (**2**) ketones catalyzed by human skin fungi can be seen in Figure 1. The pathway observed in the present experiment (Table 1) can be explained by the presence of

enzymes enoate reductase, which performs the hydrogenation of alkenes, and enzymes carbonyl reductase, which is responsible for the reduction of the carbonyl group (Silva et al 2012; Silva et al 2013) and the yielding of chiral alcohols. The F45 and F46 fungi showed the highest conversion capacity, and were always close to full metabolization of the two monoterpenes tested, representing 75% (F45) and 95.7% (F46) of the product (1*R*,2*S*,4*S*) - dihydrocarveol (**3c**) when using the (+)-carvone as a substrate, and 65.8% (F45) and 54% (F46) of the product (1*S*,2*R*,4*R*)-dihydrocarveol (**7b**) when the (-)-carvone was used. This result was similar to the findings of Silva et al. (2013) who obtained up to 80% conversion of substrate **1** by using *Saccharomyces cerevisiae* as a biocatalyst, although the greater diastereoisomer obtained was (1*R*, 2*R*, 4*S*)-dihydrocarveol (**3a**).

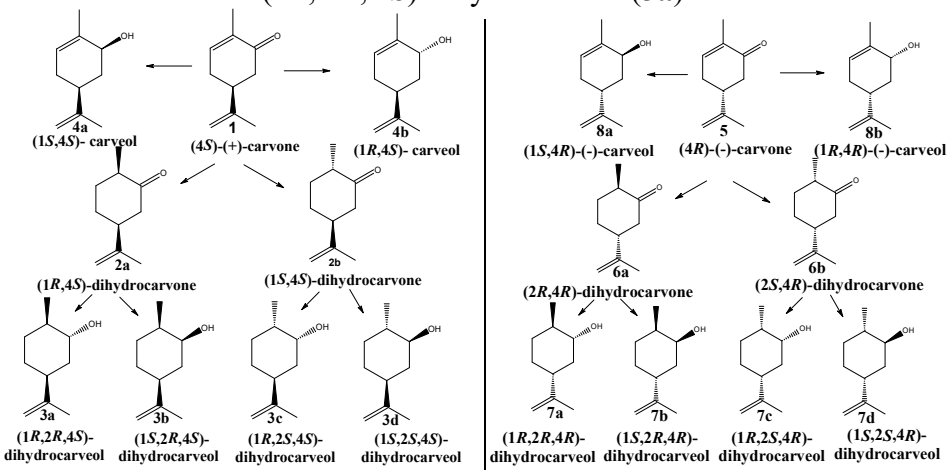

Figure 1: pathway for the bioconversion of **1** and **5** whole cells of fungi.

The F56 and F63 fungi preferentially promoted the reduction of the activated double bond to obtain dihydrocarvones with a significant diastereoisomeric excess (*d.e.*) of 95% **2b** for F56 and 74% for F63. These results were similar to those of Goretti et al. (2009) who used 16 strains of yeast representing 14 species in six genera (e.g. *Candida maltosa*, *Cryptococcus terreus*, *Pichia amylophila* and *Saccharomyces spencerorum*), and where(1*S*,4*S*)-dihydrocarvone was also the major product obtained.

The F43, F63, F37 and F58 fungi should be noted, as these demonstrated an ability to reduce carbonyl only, obtaining carveol, a molecule widely used in fine fragrances, shampoos, decorative cosmetics, soaps and other toiletries, as well as in non-cosmetic products (Bhatia et al., 2008).

Table 1: bioconversion of (4*S*)-(+)-carvone and (4*R*)-(-)-carvone derivatives

Fungi	% Conversion	% of biotransformation products (4*S*)-(+)-carvone								% Conversion	% of biotransformation products (4*R*)-(-)-carvone						
		2a	2b	3a	3b	3c	3d	4a	4b		6a	6b	7b	7c	7d	8a	8b
F45	**97.9**	17.5	4.4	1	0	75	0	0	0	**99.7**	2.9	24.8	65.8	-	6.2	-	-
F43	**19.7**	0.3	2.2	0.2	4.2	0.1	0	12.5	0.04	**9.5**	-	0.7	7.4	-	-	1.4	-
F56	**19.1**	2.2	13.4	0	3.5	0.04	0	0	0	**27.5**	0.7	25.8	0.7	0.03	0.3	-	-
F46	**100**	0	1.8	1.5	1	95.7	0	0	0	**100**	5	31.5	54	1.6	7.9	-	-
F63	**94.5**	14	35.3	3.2	20.9	20	0	1.2	0	**86.4**	6.6	46.2	29.7	0.5	3.3	-	-
F37	**42.8**	0	22	4.4	21.8	0.8	6.2	0.7	0	**89.3**	4.7	22.5	4	58	-	-	-
F58	**85.5**	0	1.4	4.4	51.6	1.1	0	27	0	**85.8**	-	5	75.7	0.5	0.3	0.2	4.1

Conversion determined by GC.

Using F46 as a model fungus and (+)-carvone as a substrate, it can be seen from Figure 2 that while after 24 hours of reaction the major product formed was **3b**, with a 4-day reaction **3c** appeared as the major metabolite. This characteristic can be explained by isomerization of (1*S*,2*R*,4*S*)-dihydrocarveol (3b) to (1*R*,2*S*,4*S*)-dihydrocarveol (3c), and the possible presence of dihydrocarveol isomerase (Figure 2) (Aquino 2012).

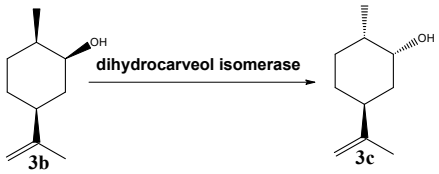

Figure 2: conversion of **3b** and **3c** by dihydrocarveol isomerase

CONCLUSIONS

In conclusion, the present study describes an efficient screening method for obtaining fragrances and flavors from the bioreduction of (4*S*)-(+)-carvone and (4*R*)-(-)-carvone using biocatalyst isolated from human skin as yeast, highlighting *Aureobasidium* sp. (F46) with excellent diastereoisomeric excess (1*R*,2*S*,4*S*)-dihydrocarveol.

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