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# New fungi for whole-cell biotransformation of carvone enantiomers. Novel *p*-menthane-2,8,9-triols production

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ABSTRACT

The microbial biotransformation of carvone enantiomers by *Lasiodiplodia theobromae*, *Trichoderma harzianum* and *Mucor circinelloides* was investigated. Biotransformation experiments were conducted using growing or resting fungi cells and the products were analyzed by GC–MS or LC–MS. The isolated compounds were identified by NMR. (*S*)-Carvone yielded only the enone reduction product dihydrocarvone by *M. circinelloides*, while (*R*)-carvone yielded *p*-menthane-2,8,9-triols when biotransformed by *L. theobromae* and *M. circinelloides*. These *p*-menthanetriols are being reported for the first time as biotransformation products. Neodihydrocarveol was the only product from the biotransformation of (*R*)-carvone by *T. harzianum*. These biotransformations can find practical applications, especially in the case of the dihydrocarveol production.



## 1. Introduction

The *p*-menthane-2,8,9-triols **1** (Fig. 1) are monoterpenes that were first isolated from the fruiting body of the basideomycete *Flammulina velutipes* [1] and later from the water-soluble portion of the methanol extract from the caraway fruit [2]. The absolute configurations from six of the sixteen isomers represented by the planar formula **1** were established [1,2], while four compounds had only their relative configuration elucidated [2].

The structures of these triols suggest that these compounds are derived from carvone (**2**) by reduction of the conjugated C=C and C=O bonds and dihydroxylation of the exocyclic double bond. The transformations of both (*R*)-(−)- and (*S*)-(+)-carvone (**2**) by cells of microorganisms [3–9], plants [10–14] and microalgae [15–18] have been described in the literature, and the products (**3**–**9**) are displayed in Fig. 1. These products are formed by reduction of the endocyclic C=C bond (**3**), carbonyl group (**4**) or both the C=C and C=O bonds (**5** and **6**). The products from carvone oxidation (**6**–**9**) have also been reported [3,19]. The stereochemical course of the biotransformation reaction varied according to the investigated organism. Although no product from the dihydroxylation of the exocyclic double bond of carvone have been reported in the literature, this reaction was observed in the microbial biotransformation of limonene [3].

In the course of our ongoing research on the identification of new sources of enzymes based on the Brazilian biodiversity, we investigated the biotransformation of (*R*)-(−)- and (*S*)-(+)-carvone (**2**) by the filamentous fungi *Lasiodiplodia theobromae*, *Trichoderma harzianum* and *Mucor circinelloides*. Herein, we report for the first time compounds (1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol (**1a**) and (1*R*,2*S*,4*R*,8*R*)-*p*-menthane-2,8,9-triol (**1b**) as biotransformation products.

## 2. Experimental

### 2.1. Microorganisms

*L. theobromae* (strain BRF118) was obtained from EMBRAPA Agroindústria Tropical (Fortaleza, CE, Brazil) where the microorganism was isolated in the Laboratory of Phytopathology from infected guava. *M. circinelloides* (strain LaBioMMi #009) was isolated in the LaBioMMi (DQ/UFSCar, São Carlos, SP, Brazil) from the wood of *Pinus taeda* collected on the UFSCar campus. *T. harzianum*

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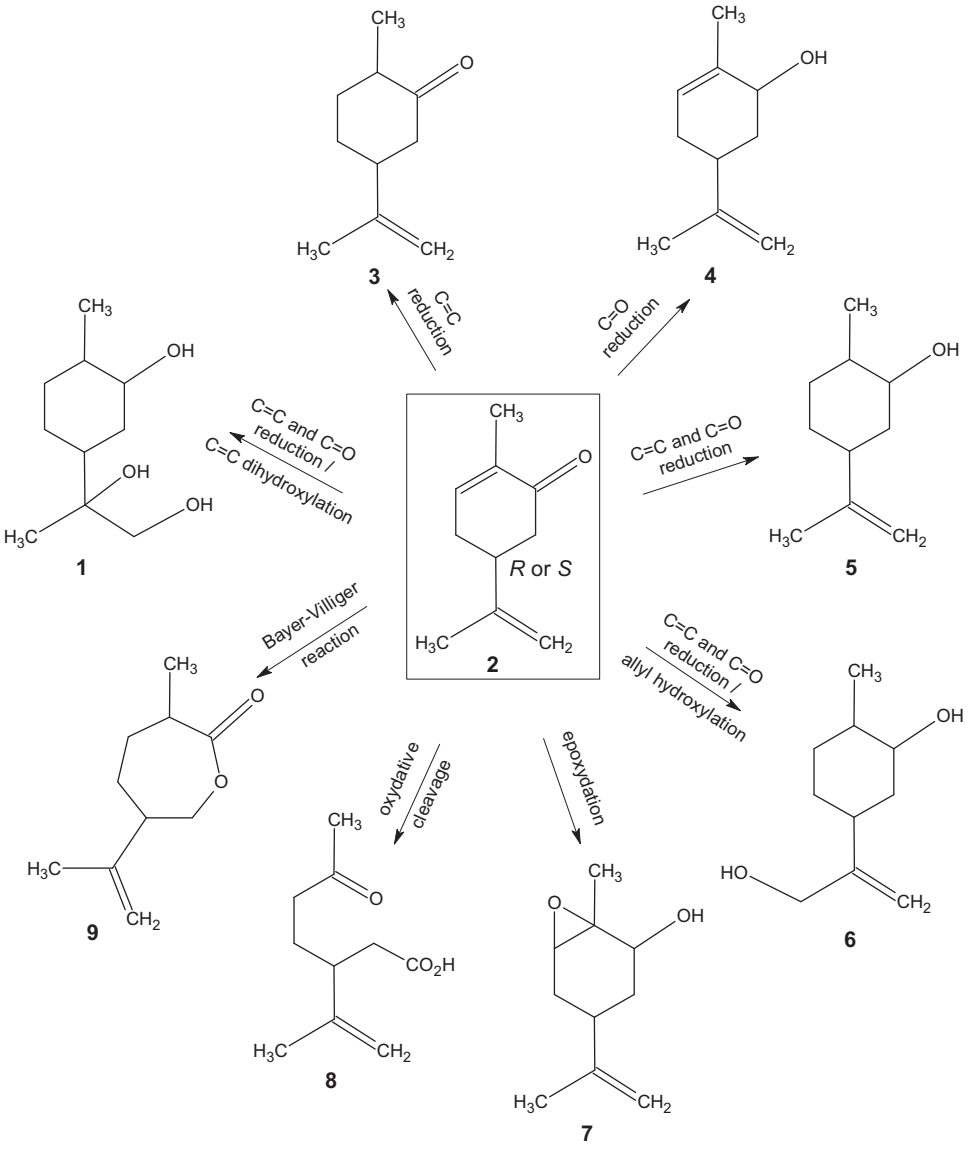


**Fig. 1.** Biotransformation products of carvone (**2**) reported in the literature (compounds **3**–**9**) [3,4,19] and in the present work (compounds **1**, **3**, **5** and **6**).

(strain BRF117) was isolated in the Laboratório de Micologia e Patologia de Sementes (Fortaleza, CE, Brazil) from cattle dung.

### 2.2. Chemical materials

(*R*)- and (*S*)-Carvone (**2**) were purchased from Sigma–Aldrich®. All of the other chemicals ($NaBH_4$, anhydride $Na_2SO_4$, glucose, $NaNO_3$, $KH_2PO_4$, $MgSO_4$, $FeSO_4{\cdot}7H_2O$, $MgSO_4$, KCl and NaCl) were obtained from Sigma–Aldrich® or Fluka®. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Acumedia®. Column chromatography was performed on silica gel 60 (230–240 mesh) from Vetec®. Solvents were purchased from Qhemis®, Sinth® or Vetec®, and HPLC grade solvents were acquired from Mallinckodt® and Tedia®. Analytical TLC analyses were performed on aluminum sheets pre-coated with silica gel 60 F254 (0.2 mm thick) from Merck®.

### 2.3. Chemical reduction of (R)-carvone

To a solution of commercial (*R*)-carvone (1.0 g, 6.67 mmol) in EtOH (10.0 mL), sodium borohydride (8 equiv.) was slowly added at 0 °C, and the reaction was stirred at room temperature for 3 h. The reaction was quenched by the addition of $H_2O$, and the products were extracted with EtOAc ($3 \times 10$ mL). The organic layer was dried over anhydride $Na_2SO_4$ and filtered. After solvent distillation under reduced pressure, the crude products (0.92 mg) were coarsely purified on a silica gel column (silica gel 60, 230–240 mesh; EtOAc/hexane as eluent) and analyzed by gas chromatography–mass spectrometry (GC–MS) recorded on a Shimadzu GCMS-QP5050A apparatus (Column OV5 [30 m × 0.25 mm × 0.25 μm]). A mixture of carveol (**4**) and dihydrocarveol (**5**) was identified as the product of the chemical reduction of (*R*)-carvone (**2**).

### 2.4. Carvone biotransformation by growing cells of L. theobromae

#### 2.4.1. Minimum inhibitory concentration (MIC) of (R)-carvone on L. theobromae growth

Two 3 mm disks of medium containing the microorganism (7 days old in PDA) were inoculated under aseptic conditions in previously autoclaved (121 °C for 15 min) 250 mL Erlenmeyer flasks containing 100 mL of commercial potato dextrose (PD) culture

medium. After 7 days under static conditions, commercial (*R*)-carvone (20, 50, 100, 150 or 250 μL) was added to the flasks and shaken for 3 days at 125 rpm and 28 °C. Mycelium samples from each experiment were replicated on Petri dishes containing PDA culture medium, and the MIC evaluated after 3 and 6 days of growing. All of the experiments were performed in six replicates, and the experiments without carvone were used as a control.

*2.4.2. Analysis and isolation of the biotransformation products*

To the extraction of the biotransformation products from the experiments with 50 μL of (*R*)-carvone, 30.0 g of NaCl was added to each replicate, and after 1 h the mycelium was separated from the liquid medium by filtration. The liquid portion was extracted with EtOAc (3 × 100 mL) followed by drying with anhydride $Na_2SO_4$ and filtration. The solvent was removed under reduced pressure until ca. 5 mL remained. The residual volume was increased, to 10 mL, and an aliquot of the solution from each replicate was analyzed by GC–MS.

The remaining solution from one of the replicates was concentrated at room temperature, and the crude product (43.0 mg) was purified via column chromatography over silica gel [100% EtOAc for **6** (6% yield) and 10% EtOAc/MeOH to yield **1a** (43.5% yield)].

*2.4.3. Confirmation of **1a** as a biotransformation product*

Isolated compound **6** (2.6 mg) was added to a 100 mL Erlenmeyer flask with 50 mL of commercial potato dextrose (PD) culture medium (pH 6.5) and shaken (125 rpm) for 3 days at 28 °C. The material was extracted with EtOAc (3 × 25 mL) followed by drying with anhydride $Na_2SO_4$ and filtration. The solvent was removed under reduced pressure until ca. 5 mL remained, and the residual volume was increased to 10 mL. An aliquot of the solution was analyzed by GC–MS and identified as pure **6**.

*2.4.4. Kinetic monitoring of the biotransformation of (4R)-carvone*

Two 3 mm disks of the microorganism (7 days old in PDA) were inoculated under aseptic conditions in previously autoclaved (121 °C for 15 min) 100 mL Erlenmeyer flasks containing 50 mL of commercial potato dextrose (PD) culture medium. After 7 days under static conditions, commercial (*R*)-carvone (20 μL) was added to the flasks and shaken at 125 rpm and 28 °C for various time periods (12, 24 and 48 h). Sodium chloride (30.0 g) was added to each flask, and after 1 h, mycelium was separated from the liquid medium by filtration. The liquid portion was extracted with EtOAc (3 × 50 mL) followed by drying with anhydride $Na_2SO_4$ and filtration. The solvent was removed under reduced pressure until ca. 5 mL remained. The residual volume was increased to 10 mL, and an aliquot of the solution from each replicate was analyzed by GC–MS. The experiment was performed in duplicate.

*2.5. (R)-carvone biotransformation by the resting cells and culture broth of L. theobromae*

Two 3 mm disks of medium containing the microorganism (7 days old in PDA) were inoculated under aseptic conditions in previously autoclaved (121 °C for 15 min) 100 mL Erlenmeyer flasks containing 50 mL of commercial potato dextrose (PD) culture medium. After 7 days under static conditions, the mycelium was separated from the culture broth by filtration under aseptic conditions and suspended in 50 mL of a buffer phosphate (pH 7) solution. Experiments were performed by the addition of commercial (*R*)-carvone (20 μL) to both the suspended mycelium and the culture broth. The flasks were shaken at 125 rpm and 28 °C for 1, 3 and 6 days. To the experiments with the suspended mycelium, NaCl (30.0 g) was added to each flask, and after 1 h, the mycelium was separated from the liquid medium by filtration. In all of the experiments, the liquid medium was extracted with EtOAc (3 × 50 mL) followed by drying with anhydride $Na_2SO_4$ and filtration. The solvent was removed under reduced pressure until ca. 5 mL remained. The residual volume was increased to 10 mL, and an aliquot of the solution from each replicate was analyzed by GC–MS. The experiments were performed in duplicate.

*2.6. (R)-carvone biotransformation by growing cells of T. harzianum*

A strain of *T. harzianum* was cultivated in PDA for 7 days, and a 3 mm disk of the grown microorganism was inoculated under aseptic conditions in previously autoclaved (121 °C for 15 min) 250 mL Erlenmeyer flasks containing 100 mL of commercial potato dextrose (PD) culture medium. After 7 days under static conditions, 20 μL of commercial (*R*)-carvone was added to the flasks and shaken for 3, 6 and 9 days at 125 rpm and 28 °C. The mycelium was separated from the liquid medium by filtration, and the liquid portion was extracted with EtOAc (3 × 50 mL) followed by drying with anhydride $Na_2SO_4$ and filtration. The solvent was distilled under reduced pressure until ca. 5 mL remained. The residual volume was increased to 10 mL, and an aliquot of the solution from each replicate was analyzed by GC–MS. The experiments were performed in triplicate. Two control experiments were also performed: flasks with the culture broth and the microorganism (control 1) and flasks with the culture broth and carvone (control 2).

*2.7. Carvone biotransformation by growing cells of M. circinelloides*

*2.7.1. Analysis and isolation of the biotransformation products*

Because *M. circinelloides* grows very well in a cleaner basic medium, such as those based on glucose as carbon sources, biotransformation experiments were performed in 47 Erlenmeyer flasks (250 mL) containing 70 mL of a sterilized Czapeck-type liquid medium. This medium contained 20 g of glucose, 1.5 g of $NaNO_3$, 0.5 g of $KH_2PO_4$, 0.25 g of $MgSO_4$, 0.025 g of $FeSO_4{\cdot}7H_2O$, 0.25 g of $MgSO_4$ and 2.5 g of KCl dissolved in 1.0 L of distilled water and 20 mg yeast extract. The flasks were autoclaved and inoculated with pieces of the PDA medium containing mycelium. 6 additional flasks were maintained as sterility controls. All of the flasks were maintained at 28 °C in the dark for one day. Next, 20 μL of (4*S*)-carvone were added to each of the 19 flasks containing the *M. circinelloides* culture, and the other 19 flasks were inoculated with 20 μL of (4*R*)-carvone. The three last flasks, which contained the growing fungus, were maintained to monitor the metabolites produced by the fungus. All of the flasks were maintained at room temperature for 12 days. After this period of time, the mycelium was separated by filtration, and the liquid phase was extracted with EtOAc (3 × 150 mL) and n-butyl alcohol (3 × 150 mL). The organic phases were dried with anhydrous $Na_2SO_4$ and distilled under reduced pressure.

The n-butyl extracts was analyzed by TLC and subjected to a sequence of separation processes. The first separation was performed with a medium pressure chromatography system (Combiflash®Rf Teledyne Isco, equipped with RediSep® C18 275 g ODS column) using linear gradient elution (20–100% methanol/water in 110 min at a flow rate of 20 mL $min^{-1}$). A total of 146 fractions (15 mL each) were collected and analyzed by TLC. Fractions 61–70 contained only substance **1a**, which was analyzed by NMR (Bruker DRX400 operating at 400 MHz for $^1H$ and 100 MHz for $^{13}C$).

Fractions 45–51 eluted from the CombiFlash system were also grouped and chromatographed on a silica gel column. The separation was performed using isocratic elution with 15%

Acetone/Hexane. Twenty-three fractions were collected, and fraction 9 contained (1*R*,2*S*,4*R*,8*R*)-*p*-menthane-2,8,9-triol (**1b**).

*2.7.2. Kinetic monitoring of the biotransformation of (4R)- and (4S)-carvone*

*M. circinelloides* was cultivated in 250 mL Erlenmeyer flasks containing 50 mL of Czapeck medium for 18 h at 28 °C under stirring (125 rpm). Next, 20 μL of the substrate ((4*R*)- or (4*S*)-carvone) was added to each Erlenmeyer flask (48 flasks for each carvone). At 4 h intervals, three flasks were removed, and the mycelium was separated from the liquid medium. Then, the aqueous portion was extracted with 20 mL of dichloromethane and 20 mL of n-butyl alcohol. In the end, 192 samples were obtained (i.e., triplicates of sixteen samples for each carvone extracted with dichloromethane and n-butyl alcohol).

The biotransformation products extracted with dichloromethane were analyzed by GC–MS [Shimadzu CG–MS 2010 plus series equipped with a Restek Rtx® – 5MS capillary column (30 m × 0.25 mm × 0.25 μm)] using helium as the carrier gas. The oven temperature was programmed at 60 °C for 5 min followed by 60–240 °C at 6 °C/min and subsequently isothermal maintained for 5 min. Injector port: 220 °C, interface: 240 °C, split ratio: 1:30. The data were acquired using an ionization voltage of 70 eV. The identification of the compounds was based on mass spectra by comparison with the NIST Library.*n*-Butyl alcohol extracts were analyzed by HPLC–MS/MS. The equipment was composed of an Alliance 2695 HPLC (Waters, Manchester, UK) coupled to a Quattro-LC ESI triple-quadrupole mass spectrometer (Waters, Manchester, UK). The chromatographic separation was performed on a 4.6 mm × 250 mm Phenomenex Synergy C-18 column (Phenomenex®) with a flow rate of 1 mL $min^{-1}$. The chromatographic method employed an isocratic condition with a mobile phase composed of acetonitrile:water (70:30, v/v) with 0.1% of trifluoroacetic acid (JT Baker). The mass spectrometer was operated in the ESI positive mode. During these analyses, a selected ion monitoring experiment (SIM), scanning $[M+Na]^+$ in both quadrupoles (*m*/*z* 211 → *m*/*z* 211), was used to monitor the accumulation of the biotransformation products.

Dihydrocarveol (**5**): Colorless oil. MS (EI, 70 eV) *m*/*z* (%): 154 (3); 136 (72); 121 (84); 107 (97); 93 (100); 79 (84); 67 (63); 55 (59); 41 (75).

8(9)-*p*-menthen-2,10-diol (**6**): colorless oil. IR $\nu_{max}$ ($cm^{-1}$): 477; 1034; 1240; 1372; 1451; 1553; 1653; 1723; 2844; 2923; 3399. MS (EI, 70 eV) *m*/*z* (%): 152 (6), 134 (34), 119 (38), 105 (29), 95 (100), 81 (59), 67 (68), 55 (79), 41 (82). $^1$H NMR (500 MHz, $CDCl_3$) $\delta$: 0.98 (d, *J* = 6.7 Hz, H-7); 1.25 (s, H-5′); 1.47 (m, H-6); 1.47 (ddl, H-3′); 1.56 (m, H-1); 1.82 (ddd, *J* = 12.3; 5.7; 3.7 Hz, H-5″); 1.98 (ddd, *J* = 13.7; 5.7; 3.2 Hz, H-3″); 2.38 (ddd, *J* = 12.4; 5.3; 2.7 Hz, H-4); 3.91 (dl, *J* = 2.5 Hz, H-2); 4.15 (s, H-10); 4.90 (s, H-9′); 5.05 (s, H-9″). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 18.5 (C-7); 28.4 (C-6); 32.1 (C-5); 33.9 (C-4); 36.2 (C-1); 39.2 (C-3); 65.4 (C-10); 71.1 (C-2); 108.2 (C-9); 153.7 (C-8).

(1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol (**1a**): Yellow amorphous solid. IR $\nu_{max}$ ($cm^{-1}$): 754; 840; 954; 994; 1037; 1108; 1143; 1299; 1376; 1453; 1663; 2868; 2928; 3392. ESI–MS: *m*/*z* 211 ($[M+Na]^+$, $C_{10}H_{20}O_3$). MS (EI, 70 eV) *m*/*z* (%): 173 (1); 157 (28); 139 (78); 121 (41); 109 (3); 95 (21); 81(51); 43 (100). $^1$H NMR (500 MHz, $CDCl_3$) $\delta$: 0.95 (d, *J* = 6.4 Hz, H-7); 0.99 (d, *J* = 12.3 Hz, H-5′); 1.01 (s, H-10); 1.32 (ddd, *J* = 13.5; 13.4; 2.0 Hz, H-3′); 1.39 (dd, *J* = 12.5; 3.2 Hz, H-6′); 1.44 (m, H-4); 1.64 (dl, *J* = 12.3 Hz, H-5″); 1.99 (brq, *J* = 12,1 Hz, H-1); 2.07 (dd, *J* = 13.4; 2.0 Hz, H-3″); 3.43 (d, *J* = 11.3 Hz, H-9′); 3.55 (d, *J* = 11.3; H-9″). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 18.6 (C-7); 19.2 (C-10); 27.8 (C-5); 28.4 (C-6); 33.5 (C-3); 36.3 (C-1); 36.5 (C-4); 68.8 (C-9); 71.2 (C-2); 75.0 (C-8). $^{13}$C NMR ($CD_3OD$, 100 MHz) $\delta$: 19.0(C-7), 20.7(C-10), 27.3(C-5), 29.5(C-6), 35.9(C-3), 37.9(C-1), 38.0(C-4), 69.2(C-9), 71.8(C-2), 75.4(C-8).

(1*R*,2*S*,4*R*,8*R*)-*p*-menthane-2,8,9-triol (**1b**): Yellow amorphous solid. IR $\nu_{max}$ ($cm^{-1}$): 754; 840; 954; 994; 1037; 1108; 1143; 1299; 1376; 1453; 1663; 2868; 2928; 3392. ESI–MS: *m*/*z* 211 ($[M+Na]^+$, $C_{10}H_{20}O_3$). $^{13}$C NMR ($CD_3OD$, 100 MHz) $\delta$: 19.0(C-7), 21.1(C-10), 28.3(C-5), 29.5(C-6), 34.9(C-3), 37.9(C-1), 38.0(C-4), 69.1(C-9), 71.9(C-2), 75.3(C-8).

## 3. Results and discussion

### 3.1. Biotransformation of (R)-carvone by L. theobromae

The study on the biotransformation of carvone was initiated using whole-cells of the phytopathogen fungus *L. theobromae*. The minimum inhibitory concentration (MIC) of (*R*)-carvone (**2**) on the fungal growth was evaluated, and concentrations of 0.2 and 0.5 μL/mL of this monoterpene exhibited no inhibition of the microorganism's growth. At higher concentrations, (*R*)-carvone (**2**) exhibited either fungistatic (1.0 or 1.5 μL/mL) or fungicide (2.5 μL/mL) effects.

The biotransformation products from the culture broth of the MIC experiments were analyzed by GC–MS. In the experiments performed with monoterpene concentrations of 0.2, 0.5 and 1.0 μL/mL, three products, BP-1 (10.6 min), BP-2 (14.4 min) and BP-3 (15.8 min), were observed besides the substrate **2** (11.3 min). Their ratio varied in each experiment, and the highest substrate bioconversion was achieved in the experiments performed with 0.2 and 0.5 μL/mL of **2**. In the experiments with higher monoterpene concentrations (1.5 and 2.5 μL/mL), only **2** (major compound) and the product with a retention time of 10.6 min (BP-1) were observed. Thus, concentrations between 0.2 and 0.5 μL/mL of **2** were considered optimal for the biotransformation of this monoterpene by *L. theobromae*.

Column chromatography of the crude products from the experiment with 0.5 μL/mL of **2** yielded isolated compounds BP-2 (6% yield) and BP-3 (43.5% yield). Compound BP-1 was not isolated due to its high volatility.

The main products from the bioreduction of (*R*)-carvone (**2**) are reported in the literature as dihydrocarvone (**3**) [6,13], carveol (**4**) [9] and dihydrocarveol/neodihydrocarveol (**5**) [6,13]. Therefore, we decided to chemically reduce (*R*)-carvone (**2**) and compare the products with those obtained from the biotransformation experiment. Analysis of the reaction products by GC–MS allowed the identification of a mixture of carveol (**4**) and neodihydrocarveol (**5**) with respective retention times of 11.1 and 10.6 min.

A comparison of both the retention time and mass spectra of neodihydrocarveol (**5**) with those from the biotransformation products allowed the identification of BP-1 as **5**.

The biotransformation product BP-2 was identified as a diastereoisomeric mixture (de 39.6%) of 8(9)-*p*-menthen-2,10-diol (**6**) using one- and two-dimensional NMR spectra as well as comparison with the literature data reported for this monoterpene [19]. The $^1$H–$^{13}$C heteronuclear multiple bond correlation (HMBC) spectrum of **6** allowed the unambiguous assignment of C-5 ($\delta$ 32.1) and C-6 ($\delta$ 28.4), which were reversely assigned in a previous study [19].

Compound BP-3 was identified as (1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol (**1a**) by comparison of its NMR data with those reported in the literature [2].

The hypothesis of (1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol (**1a**) being an artifact product formed by the addition of water to the exocyclic C=C bond in 10-hydroxyneodihydrocarveol (**6**) was considered. Therefore, biotransformation product **6** was submitted to the same conditions (culture broth, pH, temperature and time) used in the biotransformation experiments in the absence of microorganisms. In this case, compound **6** was completely recovered from the medium confirming **1a** as a biotransformation product.

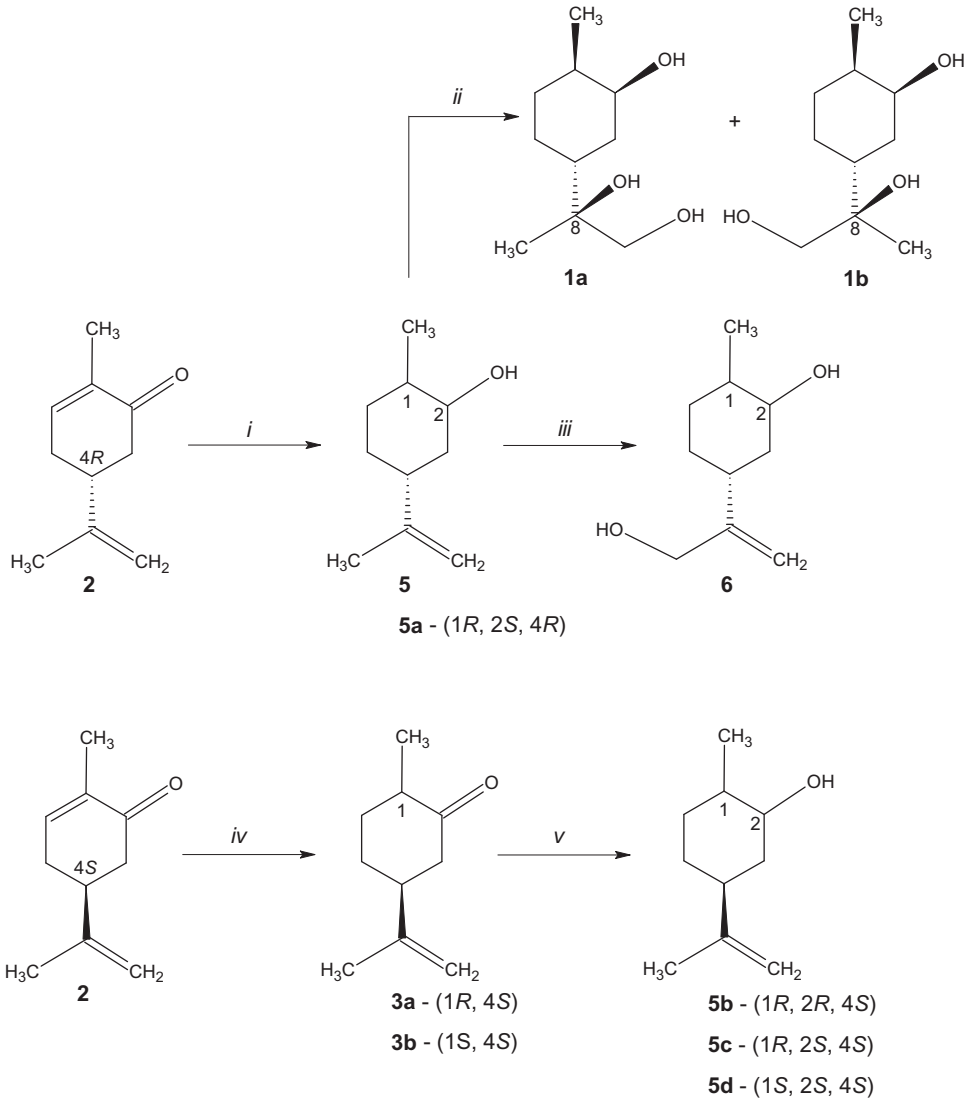


**Fig. 2.** Biotransformation pathway of 4*R*- and 4*S*-carvone (**2**): (i) both C=C and C=O bonds reduction of (4*R*)-**2**; (ii) C=C dihydroxylation of **5**; (iii) allylic hydroxylation of **5**; (iv) C=C bond reduction of (4*S*)-**2**; (v) C=O bonds reduction of **3a** and **3b**.

To establish the biotransformation pathway of **2** by *L. theobromae*, a kinetic experiment (12, 24 and 48 h) was performed under the same conditions described previously.

In the first 12 h of the experiment, only the starting material (**2**) and biotransformation product neodihydrocarveol (**5**) were identified in a relative proportion of 28.6 and 71.4, respectively. This result reveals **5** as the first product formed in the biotransformation of carvone by *L. theobromae*. A similar result was also reported in the literature for the chemical conversion of this monoterpene by other microorganisms [19].

After 24 h of reaction, both neodihydrocarveol (**5**) and (1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol (**1a**) were the only products (ratio 1:1), and no (*R*)-carvone (**2**) was detected. A comparison of the results at 24 h with those obtained at 12 h revealed that the percentage of **5** decreased while the percentage of **1a** increased suggesting that compound **1a** was formed by the dihydroxylation of **5**. It is important to note that microbial dihydroxylation of the exocyclic double bond of a monoterpene has previously been reported from the biotransformation of limonene [3].

The results obtained at 48 h of reaction indicated the presence of biotransformation products **5**, **6** and **1a** (respective ratio 6.5:1.0:2.5). In this experiment, the relative composition of the products revealed that the concentration of **5** decreased while the concentration of both **1a** and **6** increased. These results suggested that **6** is formed by allylic hydroxylation of **5**. However, the formation of **6** from the dehydration of **1a** is also possible. Therefore, a tentative biotransformation pathway of (*R*)-carvone (**2**) by the growing cells of *L. theobromae* is shown in Fig. 2.

The biotransformation of (*R*)-carvone by the enzymes from the culture broth of *L. theobromae* previously grown for 7 days on BD medium was also investigated. In this case, the products were analyzed after 1, 3 and 6 days of reaction. In all experiments, only neodihydrocarveol (**5**) was detected as a biotransformation product with total conversion of (*R*)-carvone (**2**) in the sixth day of reaction.

Because (1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol (**1a**) was not detected as a biotransformation product in this experiment, the enzymes responsible for the conversion of **2** into **1a** were expected to be present in the mycelium of the fungus. To verify this hypothesis, a new experiment was performed using the resting cells of *L. theobromae*, which were previously grown for 7 days on BD medium, suspended in a phosphate buffer at pH 7. The biotransformation products were analyzed after 6 days of reaction, and only neodihydrocarveol (**5**) was detected. Therefore, the formation of **1a** occurs only when growing cells of the fungus are used.

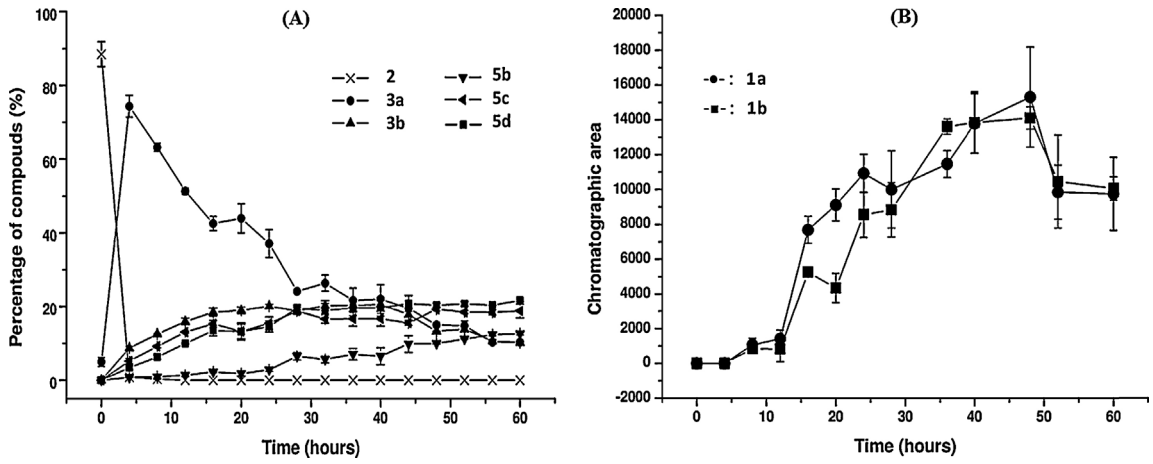


**Fig. 3.** (A) Estimated consumption of the (4*S*)-carvone (**2**) and accumulation of its reduction products (**5b**, **5c**, and **5d**) over 60 h of fermentation; (B) kinetic profile of production of menthanetriols **1a** and **1b** by *M. circinelloides*. The error bars correspond to standard deviation of biological replicates (triplicates).

### 3.2. Biotransformation of (*R*)-carvone by *T. harzianum*

The growing cells of *T. harzianum*, an antagonist fungus from soil used in the control of some phytopathogens [20], were also investigated as source of enzymes for the biotransformation of (*R*)-carvone (**2**).

In contrast to the results with the *L. theobromae* whole-cells, only biotransformation product **5** was identified in the experiments with *T. harzianum*. Since the third day of the reaction, **5** was the major product (84.2%), and its concentration continued to increase until the 9th day of reaction where it reached 98.6%.

### 3.3. Biotransformation of (*R*)- and (*S*)-carvone by *M. circinelloides*

The use of cells from *M. circinelloides* for the biotransformation of terpenes has been reported in the literature [20]. A strain of this fungus was isolated from the wood of *Pinus taeda*, a plant that is a good producer of monoterpenoids including carvone. Therefore, its potential for the biotransformation of (*R*)- and (*S*)-carvone was also investigated. During the analyses of n-butyl alcohol extract by TLC, some biotransformation products from (*R*)-carvone (**2**) were observed. Then, after a sequence of chromatographic procedures, two substances were isolated and analyzed by mass and NMR spectroscopy. By comparing their spectrometric data with those published in the literature [1,2], it was possible to identify compound **1a** as (1*R*,2*S*,4*R*,8*S*)-*p*-menthane-2,8,9-triol and **1b** as (1*R*,2*S*,4*R*,8*R*)-*p*-menthane-2,8,9-triol (Fig. 2).

The ethyl acetate extracts from the (*R*)-carvone biotransformation experiment were analyzed by GC–MS to identify the biotransformation products by comparison with the NIST library. After 4 h of fermentation, the extract from the (*R*)-carvone biotransformation was primarily composed of one product that was identified as neodihydrocarveol (**5a**, 94%), which is presumed to be the precursor of menthanetriols **1a** and **1b** (Fig. 2).

A similar investigation was also performed in parallel using (*S*)-carvone. Analyses of the n-butanol extract using LC–MS/MS showed that no menthanetriol was produced from this carvone enantiomer by *M. circinelloides*. The GC–MS analysis of the ethyl acetate extracts indicated that (*S*)-carvone is almost completely consumed after 4 h of fermentation yielding (1*R*,4*S*)-dihydrocarvone as the main product (**3a**, 75%, Retention Index: 1209). The other dihydrocarvone diastereoisomer (1*S*,4*S*; **3b**) is also produced along with minor compounds that were identified as (4*S*)-dihydrocarveol (**5b**, RI: 1217) and (4*S*)-neo-dihydrocarveol (**5c**, RI: 1196), which formed from **3a** (Fig. 2).

These results indicated that the biotransformation of (*R*)- and (*S*)-carvone by *M. circinelloids* follows different pathways. For (*R*)-carvone, the first biotransformation step is the stereoselective reduction of the double bond and carbonyl groups by enone reductase and carbonyl reductase, respectively [1,15]. These reactions convert (*R*)-carvone (**2**) into (1*R*,2*S*,4*R*)-dihydrocarveol (**5a**). The last step on the (*R*)-carvone biotransformation pathway should be the dihydroxylation of the remaining double bond resulting in trihydroxylated menthanetriols (**1a** and **1b**). Because dihydrocarveol (**5a**) is the biosynthetic precursor of menthanetriols (Fig. 2) in the proposed biotransformation pathway, the stereogenic centers at C-1 and C-2 in **5** are not involved in the biotransformation process. Therefore, both menthanetriols (**1a** and **1b**) and dihydrocarveol (**5a**) must have the same absolute configuration at these stereocenters.

For (*S*)-carvone (**2**), the biotransformation pathway is different, and the first biotransformation products include the (1*R*,4*S*)-dihydrocarvone (**3a**) and (1*S*,4*S*)-dihydrocarvone (**3b**) epimers in a 9:1 ratio after 4 h of fermentation. These compounds result from the regioselective reduction of the endocyclic double bond by enone reductase. During the next biotransformation step, **3a** is converted into a nearly 1:1 mixture of the two (4*S*)-dihydrocarveol stereoisomers (**5b** and **5c**) by carbonyl reductase.

#### 3.3.1. Kinetic monitoring of (*R*)- and (*S*)-carvone consumption and menthanetriol production

After 4 h of biotransformation reaction, the concentration of both *R*- and *S*-carvone drops to a nearly undetectable amount. The products formed were identified as shown above. These experiments were extended to cover 60 h of fermentation to investigate the interconversion of the intermediates to products. The studies were conducted in triplicate, and samples were collected every four days. The dichloromethane extracts were analyzed by GC–MS, while the n-butyl extracts were analyzed by LC–MS/MS.

After 4–24 h of fermentation, the four peaks in the GC–MS chromatogram correspond to products from (*S*)-carvone (**3a** and its reduced products **5b** and **5c**; and **3b**). During this period, **3a** is transformed to **5b** and **5c**, which is observed by a drastic decrease in its peak area and enhancement of the **5b** and **5c** peaks. After 24 h, a shoulder appears in the **3b** peak (R.T. 15.00 min) and is completed formed as a chromatographic peak (R.T. 14.92 min) after 60 h. This peak corresponds to **5d**. These transformations are illustrated in Fig. 3A. Overall, a high percentage of (1*R*,4*S*)-dihydrocarvone (**3a**)

is obtained during the first few hours, and the carveols tend to accumulate with no diastereoselectivity after 60 h.

The biotransformation of (*R*)-carvone over 60 h primarily produces dihydrocarveol (**5a**). The conversion of (*R*)-carvone into this compound occurs with 94% conversion in the first 4 h. During the next few hours, the concentration of **5a** slightly decreased, and after 8 h of culture, the production of both stereoisomers of menthanetriols (8*R* and 8*S*) begins and continues until 48 h when it starts to decrease by an unknown process (Fig. 3B).

The transformation of (*R*)-carvone (**2**) into (1*R*, 2*S*, 4*R*)-dihydrocarveol (**5a**) in only 4 h and 94% yield may be of industrial interest since dihydrocarveol is used as flavoring food additive and it is worth five times more than carvone (**2**) [21]. Additionally, the production of **5** (mixture of isomers) from carvone has been carried out by chemical reagents, and environmentally friendly method of its production would be desirable.

These results indicate the potential of *M. circinelloides* for use in the biotransformation of both (*R*)-carvone and (*S*)-carvone. These compounds are completely consumed by *M. circinelloides* in less than 4 h. This quick conversion is a significant advantage compared to other studies where complete carvone consumption might occur over 84 h to 10 days [7,10]. In addition, good enantiodistinction was observed. In summary, for (4*R*)-carvone, the main biotransformation products are (1*R*,2*S*,4*R*)-dihydrocarveol (**5a**), (1*R*,2*S*,4*R*,8*R*)-p-menthane-2,8,9-triol (**1a**) and (1*R*,2*S*,4*R*,8*S*)-p-menthane-2,8,9-triol (**1b**). The biotransformation of (*S*)-carvone produced two diastereoisomers, (1*R*,4*S*)-dihydrocarvone (**3a**) and (1*S*,4*S*)-dihydrocarvone (**3b**), besides dihydrocarveol diastereoisomers **5b**, **5c** and **5d**. All of these compounds may find applications as intermediaries in organic synthesis of more complex molecules.

## 4. Conclusion

In summary, the biotransformation of carvone (*R* and *S*) by *L. theobromae*, *T. harzianum* and *M. circinelloides* appears to be an important tool for the production of interesting compounds for the food and cosmetic industries. In addition, this is the first report of the production of menthanetriols as biotransformation products. Their accumulation in caraway fruits may be resulted from the participation of endophytic microorganisms.

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