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Biotransformation of monoterpenoid ketones by yeasts and yeast-like fungi

M.S. van Dyk *, E. van Rensburg, I.P.B. Rensburg, N. Moleleki

Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa

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Abstract

A large number of yeasts were screened for the biotransformation of (−)-piperitone, (+)- and (−)-carvone, (−)-menthone, (+)-pulegone and (−)-verbenone. A relatively small number of yeasts gave hydroxylation products of (−)-piperitone. Products obtained from (−)-piperitone were 7-hydroxy-piperitone, *cis*-6-hydroxy-piperitone, *trans*-6-hydroxy-piperitone and 2-isopropyl-5-methyl-hydroquinone. Yields for the hydroxylation reactions varied between 8% and 60%, corresponding to product concentrations of 0.04 g/l to 0.3 g/l. Not one of the yeasts tested reduced (−)-piperitone. In contrast, almost all the yeasts tested gave reduction of carvone, although the enzyme activity varied. Reduction of (−)-carvone was often much faster than reduction of (+)-carvone. Some yeasts only reduced the carbon=carbon double bond to yield the dihydrocarvone isomers with the stereochemistry at C-1 always *R*, while others also reduced the ketone to give the dihydrocarveols with the stereochemistry at C-2 always *S* for (−)-carvone, but sometimes *S* and sometimes *R* for (+)-carvone. In the case of (−)-carvone yields of up to 90% were obtained within 2 h. Only one organism, a *Hormonema* isolate (UOFS Y-0067), quantitatively reduced (−)-menthone and (+)-pulegone to (+)-neomenthol. This same organism reduced (4*S*)-isopiperitenone to (3*R*,4*S*)-isopiperitenol, a precursor of (−)-menthol.

Keywords: Monoterpenoid; Piperitone; Carvone; Menthone; Pulegone; Verbenone; Menthol; Yeast; Reduction; Hydroxylation

1. Introduction

Monoterpenoids like carvone and menthol are very important to the flavour and fragrance industry. The organoleptic properties of these compounds are determined by their stereochemistry. Stereoselective reduction of these compounds can yield the desired monoterpenoid products, i.e. (−)-menthol from (−)-menthone.

We are interested in the biotransformation of monoterpenes and monoterpenoids by unicellular fungi. These include conventional yeasts, non-conventional yeasts and yeast-like fungi. According to Faber [1] and Holland [2] *Saccharomyces cerevisiae* (baker's yeast) has been extensively studied for the bioreduction of carbonyls and carbon=carbon double bonds. Only monoterpenoids are suitable for bioreductions, since isolated carbon=carbon double bonds without an allylic alcohol, carbonyl or other

* Corresponding author. Department of Microbiology and Biochemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein, 9300, South Africa. Fax: +27-51-4482004; E-mail: smitms@micro.nw.uovs.ac.za

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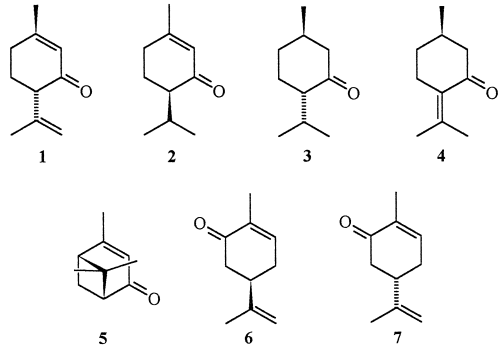


Scheme 1. Monoterpenoid ketones tested for biotransformations: (1) (4*S*)-isopiperitenone, (2) (4*R*)-(−)-piperitone, (3) (1*R*,4*S*)-(−)-menthone, (4) (1*R*)-(+)-pulegone, (5) (−)-verbenone, (6) (4*R*)-(−)-carvone and (7) (4*S*)-(+)-carvone.

'activating' substituent are only rarely reduced by microorganisms [1].

We had previously obtained isopiperitenone (**1**) as a hydroxylation product from (+)limonene [3]. Reduction of this product can yield (−)-menthol. In this study we were thus interested in the reduction of 3-keto monoterpenoids, i.e., (−)-piperitone (**2**), (−)-menthone (**3**), (+)-pulegone (**4**) and (−)-verbenone (**5**) (Scheme 1). The two carvone enantiomers (**6** and **7**; Scheme 1) were included as positive controls, since the reduction of (+)-carvone (**7**) by *Rhodotorula mucilaginosa* had previously been described by Mironowicz et al. [4].

Biotransformations were carried out using two sets of conditions. The first set of conditions involved biotransformation by whole cultures in early stationary phase, while the second set of conditions involved biotransformation by harvested cells in a glucose containing phosphate buffer. The latter conditions were initially regarded as selective for bioreductions.

2. Experimental

2.1. Yeasts

Yeasts tested belonged to the genera *Arxula, Brettanomyces, Bullera, Candida, Debaryomyces, Dekkera, Eremothecium, Exophiala, Geotrichum, Hanseniaspora, Hormonema, Kloeckera, Kluyveromyces, Lipomyces, Metschnikowia, Pachytichospora, Pichia, Rhodotorula, Saccharomyces, Schwanniomyces, Sporodiobolus, Torulaspora, Trichosporon, Yarrowia, Zygozyma.*

2.2. Chemicals

(+)-Carvone, (−)-carvone, (−)-menthone, (+)-pulegone, (−)-verbenone and (−)-menthol were obtained from Fluka. (−)-Piperitone was a generous gift from AECI, South Africa. (4*S*)-Isopiperitenone was prepared from (+)-limonene with the use of *Trichosporon* sp. UOFS Y-2041 [3].

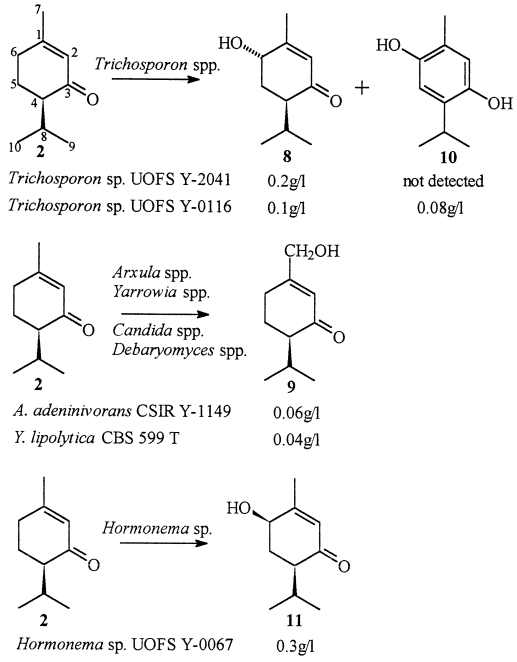
2.3. Screening method A

Yeasts were cultivated in yeast extract/malt extract broth containing 10 g glucose, 20 g malt extract (Difco), 10 g peptone (Biolab) and 3 g yeast extract (Biolab) per liter distilled water with pH unadjusted. Conical flasks (100 ml) containing 20 ml yeast extract/malt extract broth were inoculated from a 48 h old starter culture and propagated on a rotary shaker (150 rpm) at 25°C.

After 48 h, the monoterpenoid substrate was added (0.5 ml/l). Samples were taken after 24 h, 48 h and 120 h.

2.4. Screening method B

Yeasts were cultivated in yeast extract/malt extract broth containing 40 g glucose, 20 g malt extract (Difco), 10 g peptone (Biolab) and 3 g yeast extract (Biolab) per liter distilled water with pH unadjusted. At early stationary phase cells were harvested by centrifugation (5000 rpm, 10 min, 10°C), washed twice and resuspended (20% w/v) in sterile phosphate buffer (10 mM, pH 6.8) containing glucose (7.5% w/v). The resuspended cells were divided in 1



Scheme 2. Hydroxylation products of (−)-piperitone (**2**): (**8**) (4*R*,6*S*)-*trans*-6-hydroxy-piperitone, (**9**) (4*R*)-7-hydroxypiperitone, (**10**) 2-isopropyl-5-methyl hydroquinone, and (**11**) (4*R*,6*R*)-*cis*-6-hydroxy-piperitone.

ml aliquots in 4 ml vials and monoterpenoids (1 μl) added. Reaction mixtures were incubated at 30°C and extracted periodically.

Extraction, analysis and characterization of biotransformation products were done as previously described [3].

3. Results and discussion

(−)-Piperitone was initially included in a general screen for monoterpene biotransformations using method A [3]. This screen involved 100 isolates of yeasts and yeast-like fungi isolated from monoterpene-rich environments as well as 27 classified yeast strains. In a second screen, using method B, we screened specifically for the reduction of (−)-piperitone (**2**), (−)- and (+)-carvone (**6** and **7**), (−)-menthone (**3**), (+)-pulegone (**4**) and (−)-verbenone (**5**). This screen involved yeast strains from 23 genera.

During the initial screen with (−)-piperitone (**2**) only hydroxylation products were observed (Scheme 2). The hydroxylation products (**8**, **9** and **10**) obtained with non-conventional yeasts from the genera *Arxula*, *Candida*, *Yarrowia* and *Trichosporon* have recently been described [3] (Scheme 2). A previously undescribed (−)-piperitone product, *cis*-6-hydroxy-piperitone (**11**) (Scheme 2), was obtained as a major product from a *Hormonema* sp. UOFS Y-0067. The ^{1}H-NMR and mass spectrometry (MS) data for the (−)-piperitone products are given in Tables 1 and 2. The data for the previously described products are also included for comparison. Products **8** and **9** had also been described as biotransformation products of **2** formed by filamentous fungi [5,6].

Table 1
^{1}H-NMR data of identified (−)piperitone biotransformation products

	(a) Chemical shifts (ppm) of specific protons											
	H-2	H-3a	H-3e	H-4	H-5a	H-5e	H-6a	H-6e	H-8	H-7	H-9	H-10
8[a]	5.77	–	–	2.34	2.12	2.01	–	4.33	2.22	2.00	0.86	0.90
9[a]	6.05	–	–	2.06	1.81	2.00	2.20	2.29	2.32	4.20	0.82	0.91
10[a]	6.61	–	–	–	–	6.52	–	–	3.11	2.15	1.19	1.21
11	5.79	–	–	2.1	1.69	2.43	4.43	–	2.48	2.00	0.79	0.92
	(b) Coupling constants (Hz)											
8	$J_{5a-4}=9.0$; $J_{5e-4}=6.0$; $J_{5a-5e}=14.0$; $J_{5a-6e}=4.5$; $J_{5e-6e}=4.5$; $J_{4-8}=5.0$; $J_{8-9}=J_{8-10}=7.0$											
9	$J_{5a-4}=10.8$; $J_{5e-4}=5.0$; $J_{5a-5e}=13.5$; $J_{5a-6a}=8.5$; $J_{5a-6e}=$ $J_{5a-6a}=J_{5e-6e}=$ $J_{4-8}=5.0$; $J_{6a-6e}=17.5$; $J_{4-8}=$ $J_{4-8}=7.0$											
10	$J_{8-9}=J_{8-10}=7.0$											
11	$J_{5a-4}=13.5$; $J_{5e-4}=4.5$; $J_{5a-5e}=11.5$; $J_{5e-6a}=4.5$; $J_{5a-6a}=10.5$; $J_{4-8}=3.0$; $J_{8-9}=J_{8-10}=7.0$											

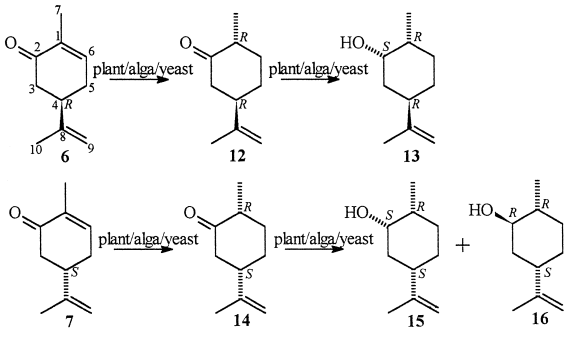
[a1] ^{1}H-NMR data taken from Ref. [3].

Table 2
Mass spectra data of identified biotransformation products

	m/e with relative intensities and assignment in brackets
8[c]	168(1)[M]$^+$;153(2)[M-15]$^+$;150(1)[M-18]$^+$;98(100)[a];126(63)[b];135(5);111(49);69(73)
9[c]	168(18)[M]$^+$;153(13)[M-15]$^+$;97(100)[a];98(99)[a];126(93);140(14);107(24);69(55);67(52)
10[c]	166(28)[M]$^+$;151(100)[M-15]$^+$;123(18);107(17);105(16);95(33);91(27);77(55)
11	168(6)[M]$^+$;153(2)[M-15]$^+$;150(1)[M-18]$^+$;98(100)[a];126(38)[b];135(6);111(28);70(49)69(73)
12	152(13)[M]$^+$;137(10)[M-15]$^+$;109(31);95(71);82(44);81(42);67(100);55(35);41(55)
13	154(1)[M]$^+$;136(56)[M-18]$^+$;121(75)[M-18-15]$^+$;107(100);93(78);79(97);67(62);55(55);41(83)
14	152(30)[M]$^+$;137(12)[M-15]$^+$;109(19);95(89);82(42); 67(100);55(38);41(58)
15	154(3)[M]$^+$;136(27)[M-18]$^+$;121(67)[M-18-15]$^+$;107(77);93(100);79(68);67(67);55(67);41(88)
16	154(8)[M]$^+$;136(53)[M-18]$^+$;121(68)[M-18-15]$^+$;107(99);93(79);82(96);79(96);67(77);55(78);41(100)
17	170(3)[M]$^+$;152(1)[M-18]$^+$;128(5);126(10);99(38);86(17);81(21);71(21);57(13);55(17);43(100);41(43)
18	170(4)[M]$^+$;152(1)[M-18]$^+$;127(3);123(2);109(12);95(6);84(65);71(25);69(26);57(49);43(60);41(100)
19	170(10)[M]$^+$;152(2)[M-18]$^+$;137(3) [M-18-15]$^+$;109(13);84(100);71(27);57(35);43(29);41(47)
20	138(9)[M-18]$^+$;123(9)[M-18-15]$^+$;109(4);95(33);81(30);71(70);67(25);57(33);55(47);43(72);41(100)
21	152(13) [M]$^+$;134(13) [M-18]$^+$;84(100)[a]
22	138(13)[M-18]$^+$;123(28)[M-18-15]$^+$;109(10);95(38);81(74);71(100); 57(29);55(43);43(45);41(80)

[a] Fragments resulting from retro-Diels–Alder type reactions [5].
[b] Fragments resulting from McLafferty rearrangements [5].
[c] Mass spectra data taken from Ref. [3].

During the second screen only reductions of (−)- and (+)-carvone (**6** and **7**) were observed. With the exception of *L. starkeyi* all the yeasts tested had the ability to reduce **6** and **7**, although with some yeasts the yields were quite low. Mironowicz et al. [4] described the reduction of (+)-carvone (**7**) by *R. mucilaginosa* and found that it was reduced exclusively to (−)-neoisodihydrocarveol (**15**) (Scheme 3). Hirata et al. [7] studied the reduction of **6** and **7** by cell suspensions of *Nicotiana tabacum*. They found that (4*R*)-(−)-carvone (**6**) was reduced to (1*R*,2*S*,4*R*)-(+)-neodihydrocarveol (**13**) via (1*R*,4*R*)-(+)-dihydrocarvone (**12**), while (4*S*)-(+)-carvone (**7**) was reduced to (1*R*,4*S*)-(−)-isodihydrocarvone (**14**) and (1*R*,2*S*,4*S*)-(−)-neoisodihydrocarveol (**15**) as main products, and (1*R*,2*R*,4*S*)-(−)-isodihydrocarveol (**16**) as a minor product (Scheme 3). Comparison of mass spectra (Table 2) showed that we obtained the same products from **6** and **7**. Noma and Asakawa [8] investigated the oxidation/reduction of the four carveol isomers by an alga *Euglena gracilis* Z. The alga yielded the same dihydrocarveol products obtained with plant cells and with yeasts. The selectivity and yields obtained with yeasts were, however, remarkable (Fig. 1). With the plant cells yields were in the order of 10–15% after three weeks incubation. Reduction of **6** by yeasts gave single products in yields of 70%–90% after 2 h incubation (Fig. 2), while reduction of **7** gave similar results after 24 h incubation. Some yeasts even gave **16** as a major product (Fig. 1).



Scheme 3. Bioreduction products of (−)- and (+)-carvone (**6** and **7**): (**12**) (1*R*,4*R*)-(+)-dihydrocarvone, (**13**) (1*R*,2*S*,4*R*)-(+)-neodihydrocarveol, (**14**) (1*R*,4*S*)-(−)-sodihydrocarvone, (**15**) (1*R*,2*S*,4*S*)-(−)-neoisodihydro-carveol, (**16**) (1*R*,2*R*,4*S*)-(−)-isodihydrocarveol.

The yeasts that gave hydroxylation of **2** were tested again for the biotransformation of the other monoterpenoid ketones, using whole cul-

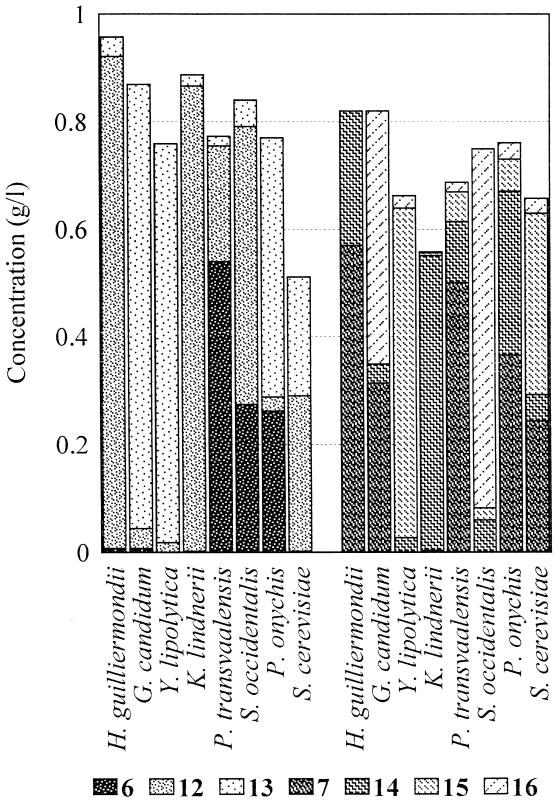


Fig. 1. Reduction of (−)-carvone (**6**) and (+)-carvone (**7**) by different yeasts. Yeast strains: *H. guilliermondii* CSIR Y894 T; *G. candidum* G 400; *Y. lipolytica* KBP 3364; *K. lindnerii* G 392; *P. transvaalensis* CBS 2186 T; *S. occidentalis* KBP 2866; *P. onychis* G 387; *S. cerevisiae* CBS 3093.

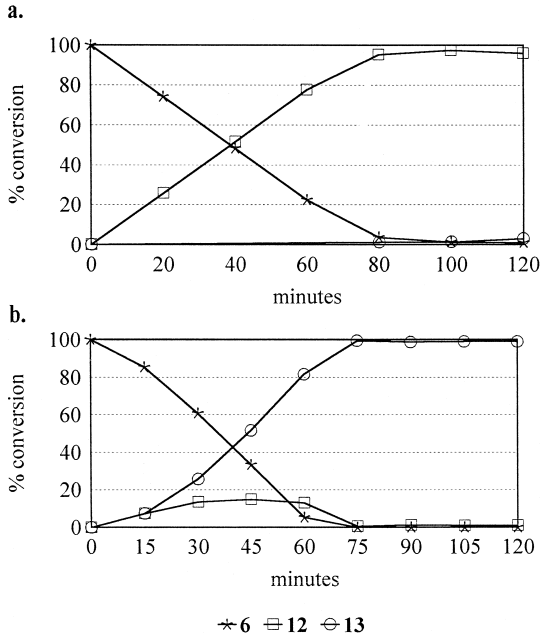
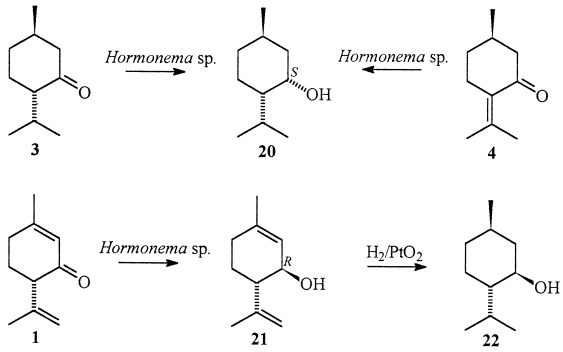


Fig. 2. Time course of the reduction of (−)-carvone (**6**) by: (a) *H. guilliermondii* CSIR Y894 T and (b) *Y. lipolytica* KBP 3364.

tures in early stationary phase (method A). Even under these conditions only reduction products were obtained as major products from **6** and **7**. Under these conditions the *Trichosporon* sp. UOFS Y-2041 formed three products from (−)-menthone (**3**). It was evident from the mass spectra (Table 2, entries **16**, **17** and **18**) that a single oxygen had been incorporated. These products were difficult to separate and the positions of oxygen incorporation were not determined.

Under the above mentioned conditions the *Hormonema* sp. UOFS Y-0067 reduced (−)-menthone (**3**) and (+)-pulegone (**4**) to (+)-neomenthol (**20**) (Scheme 4). This product was identified as **20** because its GC retention time and MS spectrum (Table 2) differed from that of (−)-menthol (**22**) but were the same as that of the second product obtained from the chemical reduction of **3** with $NaBH_4$. Mironowicz and Siewinski [9] found that *R. mucilaginosa* also reduced **3** to **20**. (−)-Menthone (**3**) was reduced to **22** by an NADH dependant hydroxysteroid dehydrogenase from *Cellulomonas turbata* [10].



Scheme 4. Reduction products of (−)-menthone (**3**), (+)-pulegone (**4**) and (4*S*)-isopiperitenone (**1**): (**20**) (1*R*,3*S*,4*S*)-(+)-neomenthol, (**21**) (3*R*,4*S*)-isopiperitenol, (**22**) (1*R*,3*R*,4*S*)-(−)-menthol.

Only the black yeast *Hormonema* sp. UOFS Y-0067 was tested for the biotransformation of (4*S*)-isopiperitenone (**1**), obtained from the hydroxylation of (+)-limonene by the *Trichosporon* sp. UOFS Y-2041 [3]. A single product was formed. Surprisingly, platinum oxide catalyzed hydrogenation of this product (**21**) yielded (−)-menthol (**22**) (Scheme 4). The alcohol (**21**) obtained from **1** thus has the opposite stereochemistry (*R*-configuration) than the alcohol (**20**) obtained from **3** (*S*-configuration), even though the stereochemistries of the adjacent carbons (C-4) are the same.

4. Conclusions

The substrate specificity of the yeast enzymes responsible for the biotransformation of the monoterpenoid ketones is remarkable. It is possible that the positions of the methyl and isopropyl groups relative to the carbonyls determine the orientation and fit of the molecules in the catalytic sites of the monooxygenases, hydrogenases and reductases involved. In fungi, the enzymes responsible for the hydroxylation of alkanes and benzoate were shown to be membrane bound cytochrome *P*-450 monooxygenases [11,12]. Alcohol dehydrogenases are involved in the reduction of carbonyls, while enoate reductases are responsible for the reduction of 'activated' carbon=carbon double bonds [1]. The enzymes responsible for the reduction of the carvone enantiomers are apparently present in many yeasts and fungi, as well as in plants and algae. The dehydrogenases responsible for the formation of the *S*-alcohols from **6** and **7** and from **3** follow Prelog's rule [1]. Dehydrogenases following Prelog's rule are apparently more abundant in nature [1]. The yeast enzymes involved in these biotransformations of the monoterpenoid ketones deserve further characterization.

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