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Multibioreaction Methodology for Baeyer-Villiger Monooxygenase Monitoring

*Regina A. C. Gonçalves[1,a], André L. M. Porto[1], Lucimar Pinheiro[1], José R. Cagnon[1], Gilson P. Manfio[2,b] and Anita J. Marsaioli[1]**

[1]Chemistry Institute, State University of Campinas, CP 6154, CEP 13083-970, Campinas (SP), Brazil
[a]Present address: State University of Maringa (UEM), DFF, CEP 87020-900, Maringá (PR), Brazil

[2]Fundação Tropical »André Tosello«, Rua Latino Coelho 1301, CEP 13087-010, Campinas (SP), Brazil
[b]Present address: CPQBA, State University of Campinas, CP 6171, CEP 13083-970, Campinas (SP), Brazil

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Summary

Baeyer-Villiger monooxygenase (BVMO) activity was monitored using traditional biocatalytic methods and also using a multibioreaction approach. The prochiral ketones 4-methyl-cyclohexanone and 3-hexyl-cyclobutanone, among others, were used in screening for BVMO in several microorganisms, leading to the selection of *Geotrichum candidum* CCT 1205, *Aspergillus oryzae* CCT 0975, *Curvularia lunata* CCT 5629, *Aspergillus niger* CCT 5559, *Trichoderma* sp. CCT 5551, *Cunninghamella echinulata* CCT 4424 and *Cunninghamella echinulata* CCT 4259 as good candidates for further BVMO investigations. Additionally, a multibioreaction methodology was used to confirm the presence of BVMO, an activity previously detected by a rapid fluorescence methodology. It was therefore possible to confirm the presence of a BVMO, more precisely a cyclohexanone monooxygenase (CHMO) and also to reveal the presence of an alkene monooxygenase in *Trichosporum cutaneum* CCT 1903.

Key words: Baeyer-Villiger monooxygenase, alkene monooxygenase, multibioreaction

Introduction

Oxidation of cyclic ketones to lactones is known as the Baeyer-Villiger (BV) reaction (Fig. 1). In organic synthesis it is generally carried out using stoichiometric quantities of various oxidants (*1–5*). Modern versions of the BV reaction are catalytic and asymmetric. Most employ metal catalyzed reactions (*6–13*), but these usually display moderate enantiomeric excesses. A »green« enzymatic asymmetric version of the BV reaction can be achieved by using oxygenases, biocatalysts generally referred to as Baeyer-Villiger monooxygenases (BVMO) (*14*). These enzymes are preferentially used as whole cells (*15–20*) due to the advantages (*5,21–26*) of working without the need for independent coenzymes and recycling systems. The increasing interest in the preparation of target molecules in a single enantiomer form has driven the search for BVMO activity towards a wide range of microorganisms. Bioprospecting in culture collections has proven to be a very efficient way of selecting enzymatic activities that can be further optimized by strategies such as mutagenesis, cloning and directed evolution. Our group has been bioprospecting oxygenase and hydrolase activities in microorganisms deposited in Brazilian collections including the CCT, Coleção de Culturas Tosello (*27*) and the CBMAI, Coleção Brasileira de Microrganismos de Ambiente e Indústria (*28,29*), focusing on the oxidation of sulfides (*30,31*) and the hy-

* Corresponding author; E-mail: anita@iqm.unicamp.br

drolysis of epoxides (*32*). We now report our results on the bioprospection of BVMO in 14 microorganisms for the oxidation of cyclohexanones, cyclopentanones and cyclobutanones.

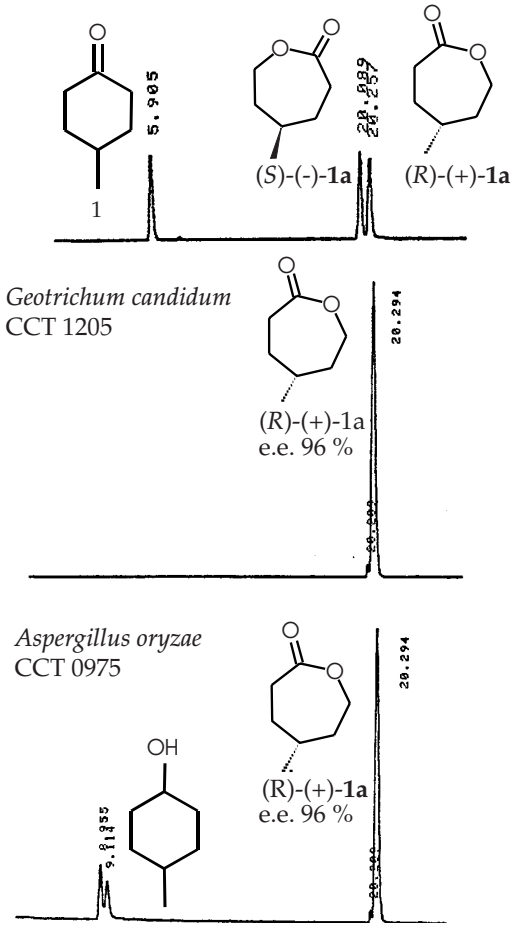


Fig. 1. Chromatograms obtained for Baeyer-Villiger oxidation reactions carried out by different fungi

Materials and Methods

General methods

Chemical oxidation was monitored by silica gel TLC (aluminum foil plates, 60 F_{254}, Merck), with visualization done using *p*-anysaldehyde/sulfuric acid followed by heating (approx. 120 °C). Flash column chromatography was performed using Merck 60 silica (230–400 mesh). Enzymatic reactions were monitored by GC (FID) in HP 5890 chromatograph, using hydrogen as the carrier gas, or by GC/MS using HP 5970-MSD and helium as the carrier gas. The fused silica capillary columns used were either a J & W Scientific DB-5 (30 m x 0.25 mm x 0.25 μm) or a (chiral) heptakis-(2,3-dimethyl-6-penthyl)-β-cyclodextrin (25 m x 0.25 mm x 0.25 μm). ^{1}H NMR spectra were recorded with a Bruker AC 300 (300.13 MHz), an Inova 500 (499.88 MHz) or a Varian Gemini 300 (300.07). $CDCl_3$ was used as solvent, with Me_4Si (TMS) as internal standard. ^{13}C NMR spectra were obtained with a Bruker AC 300 (75.47 MHz), an Inova 500 (125.70 MHz) or a Varian Gemini 300 (75.45).

*Synthesis of lactones **1a–2a***

Lactones (±)-**1a,** (±)-**2a** were obtained by *m*-chloroperbenzoic acid oxidation (*33*) of the corresponding ketones **1–2** in dichloromethane (10 mL) and sodium bicarbonate at 0 °C with shaking for 4 h. Dicloromethane (100 mL) was added to the reaction solution and the organic layer was extracted with saturated aqueous solution of $NaHCO_3$ (5 mL) and with saturated aqueous solution of Na_2SO_3. The organic layer was separated and dried over Na_2SO_4 and then the solvent evaporated. The residue was purified on a silica gel column using hexane and ethyl acetate as eluent (9:1), yielding lactones **1a** (89 %) and **2a** (60 %). *Compound **1a***: **MS** (EI, 70 eV) *m/z* (rel. inten.): 128 ($M^{+\cdot}$, 8), 98 (12), 69 (62), 56 (100), 55 (48), 41 (52). **IR** (film) ν_{max} (cm^{-1}): 2960, 2928, 1733, 1713, 1177. **^{1}H NMR** (300.07 MHz, $CDCl_3$) δ: 0.99 (3H, d, *J* 6.8 Hz, H-8), 1.33 (1H, m, H-6), 1.50 (1H, m, H-6´), 1.77 (1H, m, H-5), 1.90 (2H, m, H-4), 2.64 (2H, m, H-7), 4.17 (1H, dd, *J* 13.0 Hz and *J* 10.5 Hz, H-3), 4.27 (1H, ddd, *J* 13.0 Hz, *J* 6.0 Hz and *J* 2.0 Hz, H-3´). **^{13}C NMR** (75.45, $CDCl_3$) δ: 22.3 (CH_3), 30.8 (CH_2), 33.2 (CH_2), 35.3 (CH), 37.3 (CH_2), 68.1 (CH_2), 172.2 (C_0). *Compound **2a***: **MS** (EI, 70 eV) *m/z* (rel. inten.): 170 ($M^{+\cdot}$, 2), 152 (2), 139 (4), 128 (6), 114 (18), 111 (24), 97 (12), 85 (26), 83 (46), 70 (43), 69 (49), 56 (98), 55 (64), 43 (82), 42 (44), 41 (100). **IR** (film) ν_{max} (cm^{-1}): 2950, 2927, 2857, 1779, 1467, 1420, 1379, 1171, 1021, 837, 726, 686, 553. **^{1}H NMR** (499.88 MHz, $CDCl_3$) δ: 0.89 (3H, t, *J* 6.8 Hz, CH_3-11); 1.25 – 1.33 (8H, m, H-7, H-8, H-9 and H-10), 1.45 – 1.49 (2H, m, H-6), 2.15 – 2.22 (1H, dd, *J* 7.8 Hz and *J* 1.2 Hz, H-5c or H-5d), 2.50 – 2.59 (1H, broad heptet, *J* 7.6 Hz, H-4), 2.60 – 2.66 (1H, ddd, *J* 16.8 Hz, *J* 8.3 Hz and *J* 1.2 Hz, H-5d or H-5c), 3.93 (1H, ddd, *J* 8.8 Hz, *J* 7.3 Hz and *J* 1.2 Hz, H-3a or H-3b), 4.42 (1H, ddd, *J* 8.8, *J* 7.3 and *J* 1.2 Hz, H-3b or H-3a). **^{13}C NMR** (125.70 MHz, $CDCl_3$) δ: 14.04 (C-11), 22.56 (C-10), 27.36, 29.15, 31.64, 33.14 (C-9, C-6, C-7, C-8), 34.57 (C-5), 35.74 (C-4), 73.49 (C-3), 177.38 (C-1).

*Synthesis of ketone **2***

A solution of 1-octene (2.0 g, 17.8 mmol), Zn powder, previously activated (2.9 g, 44.5 mmol) and diethyl ether (60 mL) was sonicated for 2 h while a solution of trichloroacetyl cloride (2.4 mL, 21.4 mmol) was slowly added. The reaction mixture was filtered over a Celite pad and washed with $NaHCO_3$ solution, then dried over Na_2SO_4. Solvent evaporation and purification of the crude residue by flash silica chromatography (eluted with hexane and diethyl acetate 10:1) produced 2.1 g (52 %) of a dichlorocyclobutanone. **MS** (EI, 70 eV) *m/z* (rel. inten.): 223 ($M^{+\cdot}$, absent), 180 (9), 175 (3), 138 (8), 118 (9), 109 (36), 102 (27), 97 (7), 82 (17), 69 (29), 56 (52), 43 (100), 42 (40), 41 (62). **IR** (film) ν_{max} (cm^{-1}): 2957, 2930, 2859, 1870, 1811, 1466, 1393, 1064, 987, 732, 568. **^{1}H NMR** (300.07 MHz, $CDCl_3$) δ: 0.90 (3H, t, *J* 6.9 Hz, CH_3-10), 1.21 – 1.50 (8H, m, H-6, H-7, H-8 and H-9), 1.51 – 1.65 (1H, m, H-5), 1.86 – 2.00 (1H, m, H-5), 2.82 – 2.92 (1H, m, H-3), 2.96 (1H, dd, *J* 17.2 Hz and *J* 9.1 Hz, H-4), 3.36 (1H, dd, *J* 17.2 Hz and *J* 9.2 Hz, H-4). **^{13}C NMR** (75.45

MHz, $CDCl_3$) δ: 13.99 (C-10), 22.51 (C-9), 27.36 (C-8), 29.01 (C-7), 31.36 (C-6), 31.57 (C-5), 45.98 (C-3), 47.76 (C-4), 88.88 (C-2), 193.02 (C-1). Zinc powder (2.6 g, 30.32 mmol) was added at room temperature to a stirred solution of the dichlorocyclobutanone (1.5 g, 6.7 mmol) in acetic acid (10 mL) and water (1 mL). After 2 h the mixture was heated to 50 °C for 1 h, cooled, filtered over Celite and diluted with 100 mL of water and 100 mL AcOEt. The organic layer was treated with saturated $NaHCO_3$ solution and dried over anhydrous $MgSO_4$. Flash chromatography (hexane:AcOEt/10:1) gave 0.870 g (80 %) of pure cyclobutanone **2**. **MS** (EI, 70 eV) *m/z* (rel. inten.): 154 ($M^{+\cdot}$, 1), 136 (3), 126 (6), 111 (7), 98 (100), 84 (37), 83 (27), 70 (39), 69 (35), 56 (49), 55 (67), 43 (66), 42 (45), 41 (77). **IR** (film) ν_{max} (cm^{-1}): 2958, 2926, 2855, 1787, 1466, 1386, 1206, 1168, 1097, 724. **^{1}H NMR** (300.07 MHz, $CDCl_3$) δ: 0.89 (3H, t, *J* 6.9 Hz, CH_3-10), 1.26 (8H, m, H-6, H-7, H-8, H-9), 1.58 (2H, m, H-5), 2.35 (1H, m, H-3), 2.66 (2H, m, H-2b and H-4b or H-2a and H-4a), 3.07 – 3.19 (2H, m, H-2a and H-4a or H-2b and H-4b). **^{13}C NMR** (75.45 MHz, $CDCl_3$) δ: 13.99 (C-10), 22.52 (C-9), 23.77 (C-3), 28.15, 29.03, 31.72 (C-6, C-8, C-7), 36.29 (C-5), 52.44 (C-2 and C-4), 208.75 (C-1).

Microorganisms

Aspergillus oryzae CCT 0975, *Aspergillus terreus* CCT 3320, *Geotrichum candidum* CCT 1205, *Aspergillus niger* CCT 5559, *Aspergillus niger* CCT 4648, *Cunninghamella echinulata* CCT 4259, *Cunninghamella echinulata* CCT 4424, *Curvularia eragrostides* CCT 5634, *Curvularia lunata* CCT 5628, *Curvularia lunata* CCT 5629, *Curvularia pallescens* CCT 5654, *Drechslera halodes* CCT 5636, *Drechslera dematioidea* CCT 5631, *Geotrichum candidum* CCT 1205, *Trichosporum cutaneum* CCT 1903 and *Trichoderma* sp. CCT 5551 were obtained from the CCT culture collection (Fundação André Tosello, Campinas, SP, Brasil; *http://www.cct.fat.org.br*) (*27,29*).

*Reaction of the microorganisms with **1a–2a***

The fungi were grown in 250-mL shaker-flasks (140–170 rpm) using 100 to 130 mL malt extract (8 g/L, Difco or Merck) for 72 to 96 h, at 28–32 °C. Fungal biomass was harvested by filtration and used immediately in the biocatalysis reactions. The experiments were performed under aseptic conditions in a laminar flow cabinet.

Small-scale reactions

A volume of 20 μL of the appropriate ketones, **1–2,** and 0.5–2.0 g of wet fungal biomass was mixed in Erlenmeyer flasks (125 mL) containing 30 mL of buffer phosphate solution (Na_2HPO_4 and KH_2PO_4, pH=7.0). The mixture was stirred on a rotary shaker (28 °C, 140 rpm) until the starting material was completely depleted. The reaction was monitored by chiral GC.

Determination of the enzymatic activity of the fungi

Bioreaction was monitored every 24 h by the analysis of 2 mL samples. These were extracted with ethyl acetate (0.5 mL) and the organic phase was analyzed by GC/FID (1 μL) using a fused silica chiral capillary column. The products of the biocatalyzed reactions were compared with a racemic mixture previously obtained by chemical oxidation. GC conditions used consisted of: *Lactone* **1a**: oven at 70 °C; injector at 180 °C; detector at 200 °C; 3 °C/min ramp; retention time for **1a** (*S* = 20.1 min; *R*= 20.3 min). *Lactone* **2a**: oven at 100 °C; injector at 180 °C; detector at 200 °C; 5 °C/min ramp; retention time for **2a** 14.2 min.

Assignment of the absolute configuration of 1a

Optical rotation values were measured in an LEP--A2 Carl Zeiss polarimeter. The reported data refer to the Na-line value using a 1-dm cuvette. The specific optical rotation for (*R*)-(+)-5-methylheptalactone **1a** was $[\alpha]_D^{20}$+44.3° (*c*=2.33, $CHCl_3$). Therefore the e.e. was 96 %. The absolute configurations were determined by comparison of the sign of the measured optical rotation with literature values (*34,35*).

Multibioreaction

Trichosporum cutaneum CCT 1903 biomass was obtained as previously described, but using yeast and malt extract (8 g/L) medium. Two grams of wet biomass were added to 50.0 mL of phosphate buffer (pH=6.5) containing 10.0 mg of each compound to be tested, including 2-benzyl-2-carbomethoxy-cyclopentanone, *cis*-jasmone, 3-methyl-cyclopentanone, 2-methyl-cyclohexanone and 4-methyl-cyclohexanone. The bioreaction was monitored for 72 h. The samples (1 mL) were saturated with sodium chloride and extracted with AcOEt (3 x 800 μL). The organic solvent evaporated and the residue was diluted in 100.0 μL of pentadecane in diethyl acetate (0.2 mg/mL). GC/MS analysis was conducted as follows: 45 °C/5 min, rising to 225 °C with a 10 °C/min ramp, followed by 30 °C/min until 290 °C, 1 mL/min flux, splitless 50.

Scaling up a bioreaction for *cis*–jasmone (200 mg) and following the same procedure described above resulted in the isolation of 43 mg of the corresponding epoxide, after purification in a silica gel chromatography column eluted with hexane:ethyl acetate 5 %. *Epoxy-jasmone*: **MS** (EI, 70 eV) *m/z* (rel. inten.): 180 ($M^{+\cdot}$, 8) 165 (26), 152 (12), 147 (11), 123 (27) 122 (46), 121 (26), 111 (13), 110 (64), 109 (32), 107 (19), 95 (26), 93 (18), 91 (18) 81 (22), 79 (100) 77 (29) 71 (15) 67 (31) 43 (18) 41 (28). **^{1}H NMR** (499.88 MHz, $CDCl_3$) δ: 1.03 (3H, t, *J* 7.5 Hz, H-1), 1.60 (2H, m, H-2), 2.84 (1H, m, H-3), 3.01 (1H, m, H-4), 2.26 and 2.49 (2H, m, H-5), 2.53 (2H, m, H-8), 2.37 (2H, m, H-9), 2.09 (3H, s, H-11). **^{13}C NMR** (125.70 MHz, $CDCl_3$) δ: 10.5 (C-1), 21.1 (C-2), 58.5 (C-3), 55.6 (C-4), 21.9 (C-5), 136.7 (C-6), 209.1 (C-7), 31.8 (C-8), 34.1 (C-9), 172.5 (C-10), 17.5 (C-11).

Results and Discussion

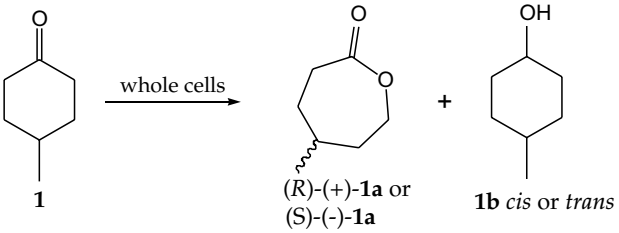
Microbial oxidation of ketone **1** was carried out using wet biomass prepared from 14 microorganisms (Table 1). These microorganisms had different selectivities for the monitored bioreactions. The best results for the cyclohexanone Baeyer-Villiger monooxygenase (BVMO) reaction (Scheme 1) were obtained when whole cells of *G. candidum* CCT 1205 were used. The reaction occurred in a short time, yielding lactone (*R*)-(+)-**1a** with high enan-

Table 1. Microbial oxidation of methyl-cyclohexanone using whole cells

Microorganisms	*t*/h	**1***/%	**1b**/%	**1a**/%	**1a** e.e./%	**1a**
Aspergillus niger CCT 4648	72	25	25	–	–	–
Aspergillus niger CCT 5559	96	24	23	1.0	–	–
Aspergillus oryzae CCT 0975	72	77	23	54	96	*R*
Aspergillus terreus CCT 3320	72	95	93	2.0	–	–
Cunninghamella echinulata CCT 4259	72	81	81	–	–	–
Cunninghamella echinulata CCT 4424	96	–	–	–	–	–
Curvularia eragrostides CCT 5634	96	77	75	2	–	–
Curvularia lunata CCT 5628	24	51	51	–	–	–
Curvularia lunata CCT 5629	96	97	94	3	–	–
Curvularia pallescens CCT 5654	96	98	97	1	–	–
Drechslera dematioidea CCT 5631	96	–	–	–	–	–
Drechslera halodes CCT 5636	72	99	99	–	–	–
Geotrichum candidum CCT 1205	72	100	–	100	96	*R*
Trichoderma sp. CCT 5551	72	89	88	1.0	–	–

*conversion determined by GC; e.e.: enantiomeric excess

tiomeric excesses (e.e.=96 %) and conversion (almost 100 %). BVMO activity of this organism was also recently reported by Carballeira *et al.* (*36*). The second best biocatalyst, also previously reported (*37*), was *A. oryzae* CCT 0975, which produced lactone (*R*)-(+)-**1a** in high enantiomeric excess (e.e.=96 %). However, the production of the *cis* and *trans* isomers of 4-methyl-cyclohexanol with 23 % yield in a 1:1 ratio did not encourage further studies (Table 1). The (*R*)-configuration was determined by combining chiral chromatography, optical rotation of the purified samples and data from the literature for the (*R*)-lactone, **1a**.



Scheme 1. BVMO reaction of 4-methyl-cyclohexanone

Based on the mechanism proposed by Kelly (*38*), the preferential formation of the *R*-lactone **1a** can be rationalized (Fig. 2A) via a 4-hydroxy-1,2,5-trioxane adduct formed from the ketone and flavin hydroperoxide, with all orbitals perfectly oriented for the Baeyer-Villiger rearrangement. Rationalizing our result according to Fig. 2, the ion peroxide must be linked onto the *Si* face of the flavin, thus producing the lactone with absolute configuration *R*, following the migration pathway as indicated in Fig. 2 (B).

The 14 selected fungal strains were also tested for cyclobutanone BVMO activity by using the prochiral compound 3-hexyl-cyclobutanone **2,** a good probe for the production of enantiomerically pure γ-butyrolactones, which are important building blocks for various natural products (Table 2, Scheme 2).

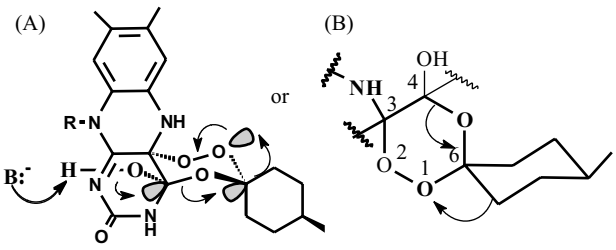


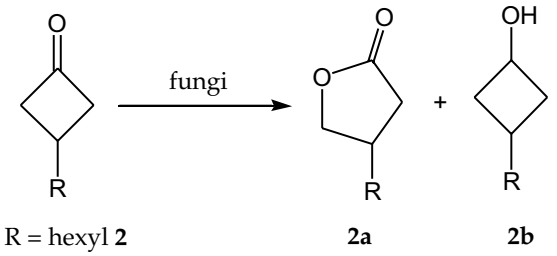
Fig. 2. The BVMO mechanism proposed by Kelly (*38*). (A) Formation of 4-hydroxy-1,2-5-trioxane adduct for BV rearrangement, (B) enantioselective group migration on flavin

Table 2. Microbial oxidation of 3-hexyl-cyclobutanone

Microorganisms	*t*/h	**2***/%	2a/%	**2b**/%
Aspergillus niger CCT 4648	90	–	–	–
Aspergillus niger CCT 5559	48	85	80	–
Aspergillus oryzae CCT 0975	90	–	–	–
Aspergillus terreus CCT 3320	90	–	–	–
Cunninghamella echinulata CCT 4259	48	80	80	–
Cunninghamella echinulata CCT 4424	48	80	80	–
Curvularia eragrostides CCT 5634	90	85	20	10
Curvularia lunata CCT 5628	90	70	44	26
Curvularia lunata CCT 5629	48	100	80	–
Curvularia pallescens CCT 5654	48	100	54	–
Drechslera dematioidea CCT 5631	90	–	–	–
Drechslera halodes CCT 5636	90	80	26	44
Geotrichum candidum CCT 1205	90	–	–	–
Trichoderma sp. CCT 5551	60	70	70	–

*conversion determined by GC

The best results for the bioxidation of cyclobutanone **2** were obtained using *Aspergillus niger* CCT 5559, *Cunninghamella echinulata* CCT 4224 and CCT 4259, *Curvu-*



Scheme 2. Cyclobutanone BVMO reaction

laria lunata CCT 5629 and *Trichoderma* sp. CCT 5551. These microorganisms produced lactone **2a** in high yields after a 48-h reaction time. The BVMO activities of *Curvularia eragrostidis* CCT 5634, *Curvularia lunata* CCT 5628 and *Drechslera halodes* CCT 5636 were not outstanding, and ketone reduction was observed instead (Table 2).

Multibioreaction in BVMO screening

So far, »new« Baeyer-Villigerase activities have been sought after through conventional screening, which is time-consuming. Thus, rapid methods for BVMO bioprospection have been developed. One such method, based on fluorescence, has recently been reported by Furstoss *et al.* (*39*). Our own efforts for the optimization of a rapid fluorescence-based method have been carried out and *Trichosporum cutaneum* CCT 1903 was selected for its BVMO activity for open chain ketones (*40*). Searching for a rapid screening method, we visualized the application of a »multibioreaction« (*41*), with the simultaneous monitoring of several BVMO activities. For this purpose we selected commercially available ketones, including 4-methyl-cyclohexanone (**1**), 3-methyl-cyclopentanone (**3**), 2-methyl-cyclohexanone (**4**), 2-allyl-2-carbomethoxy-cyclopentanone (*42,43*) (**5**) and *cis*-jasmone (**6**) (Fig. 3).

The multibioreaction allowed simultaneous monitoring of the 5 ketones (**1**,**3–6**). We have also observed that the biomass/substrate ratio of the mixture can be approximately the same as that used in the single substrate assays, *i.e.* 2 g of wet cells per 10 mg of substrate. The biocatalytic reactions were monitored by GC/MS, with the addition of an internal standard for quantification purposes (*i.e.* pentadecane, 0.2 mg/mL). Epoxidation of the *cis*-jasmone was detected in these assays, suggesting the presence of an alkene monooxygenase, instead of the expected cyclohexanone or cyclopentanone BVMO (Fig. 4). The oxidation of the *cis*-jasmone was confirmed by ^{1}H and ^{13}C NMR. Oxidoreductase activity was also observed with the reduction of ketones **4** and **5**.

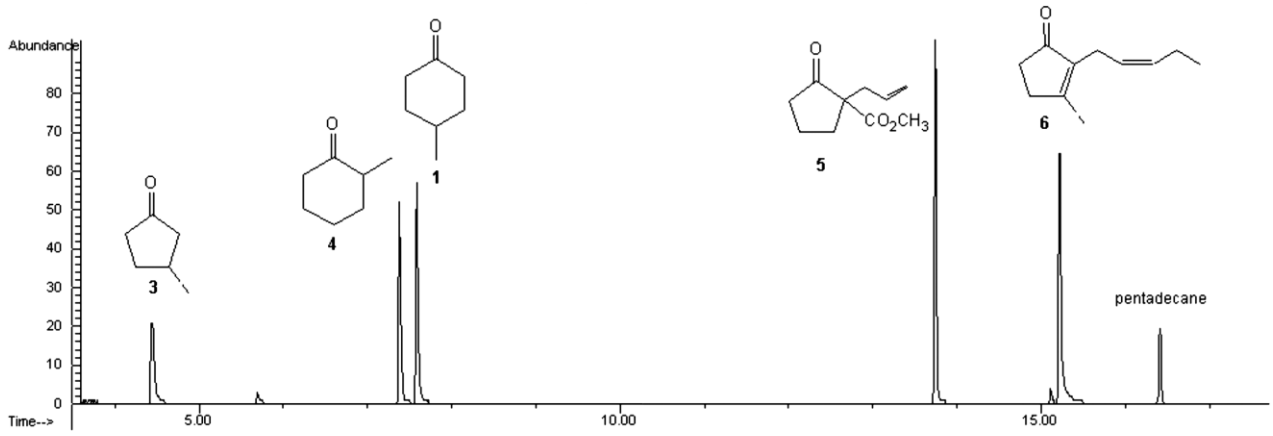


Fig. 3. Total ion chromatograms (GC/MS) of cycloalkanones used for monitoring cyclohexanone, cyclopentanone and alkene monooxygenases in *T. cutaneum* CCT 1903

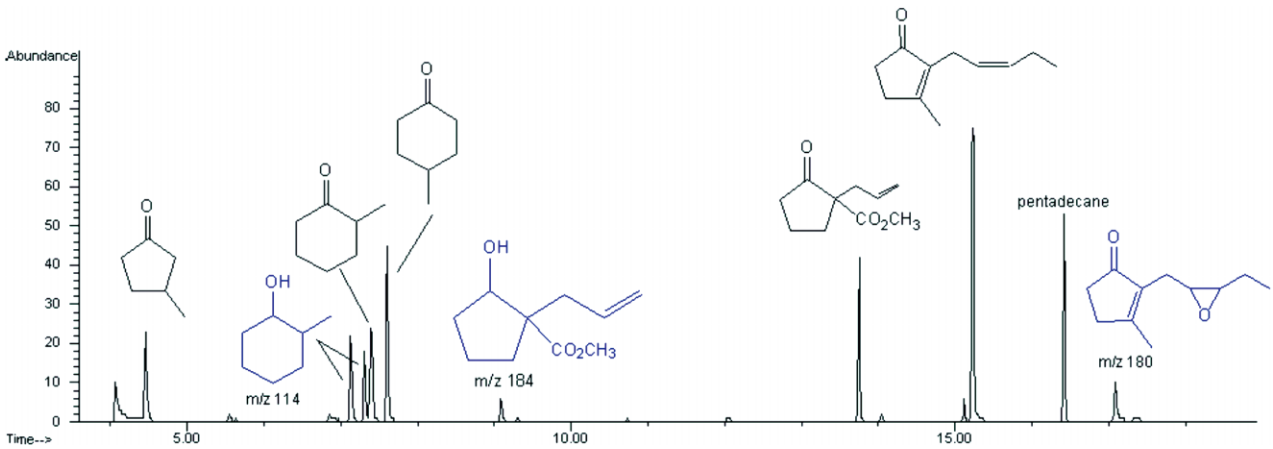


Fig. 4. Total ion chromatogram of the *T. cutaneum* CCT 1903 multibioreaction with different ketones after 12 h of incubation

Conclusion

Bioprospection of BVMO in 14 fungi resulted in the detection of cyclohexanone BVMO in *Aspergillus oryzae* and *Geotrichum candidum*. Cyclobutanone BVMO was detected in *Aspergillus niger* CCT 5559, *Cunninghamella echinulata* CCT 4424 and CCT 4259, *Curvularia lunata* CCT 5629, and *Trichoderma* sp. CCT 5551. A multibioreaction methodology was also efficient in the detection of an alkene monooxygenase in *Trichoderma cutaneum* CCT 1903.

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Multibioreakcijska metodologija za kontrolu Baeyer-Villiger monooksigenaze

Sažetak

Aktivnost Baeyer-Villiger monooksigenaze (BVMO) kontrolirana je koristeći uobičajene biokatalitičke postupke, te multibioreakcijski pristup. Prokiralni ketoni 4-metilcikloheksanon i 3-heksilciklobutanon korišteni su među ostalima pri odabiru nekoliko mikroorganizama koji sadržavaju BVMO. Tako su odabrani sojevi *Geotrichum candidum* CCT 1205, *Aspergillus oryzae* CCT 0975, *Curvularia lunata* CCT 5629, *Aspergillus niger* CCT 5559, *Trichoderma* sp. CCT 5551, *Cunninghamella echinulata* CCT 4424 i *Cunninghamella echinulata* CCT 4259 kao dobri izvori za daljnja istraživanja BVMO. Nadalje, multibioreakcijska metodologija primijenjena je da bi se potvrdila prisutnost BVMO, a aktivnost je prethodno utvrđena brzom fluorescentnom metodologijom. Stoga je bilo moguće utvrditi prisutnost BVMO, tj. cikloheksanon-monooksigenaze (CHMO) u *Trichosporon cutaneum* CCT 1903 te otkriti prisutnost još jedne alken-monooksigenaze u tom mikroorganizmu.