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Article

Studies on Whole Cell Fluorescence-Based Screening for Epoxide Hydrolases and Baeyer-Villiger Monoxygenases

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Reações de biocatálise foram realizadas em microplacas (200 μL) visando a utilização de substratos fluorogênicos (100 μmol L^{-1}) para prospecção rápida de epóxido hidrolases (EHs) e Baeyer-Villiger monoxigenases (BVMOs) em microrganismos (células inteiras). Um protocolo final foi alcançado para EHs, com a detecção de 3 novas fontes enzimáticas (*Agrobacterium tumefaciens*, *Pichia stipitis*, *Trichosporom cutaneum*). O ensaio fluorogênico para BVMO não ocorreu como esperado. A abordagem de algumas variáveis envolvidas (aeração; pH) proporcionou a detecção inédita da atividade enzimática de BVM em *T. cutaneum*.

Biocatalysis reactions were performed on microtiter plates (200 μL) aiming at the utilization of fluorogenic substrates (100 μmol L^{-1}) for rapid whole cell screening for epoxide hydrolases (EHs) and Baeyer-Villiger monoxygenases (BVMOs). A final protocol was achieved for EHs, with 3 new enzymatic sources being detected (*Agrobacterium tumefaciens*, *Pichia stipitis*, *Trichosporom cutaneum*). The fluorogenic assay for BVMO did not work as expected. However, an approach to possible variables involved (aeration; pH) provided the first detection of a BVMO activity in *T. cutaneum*.

Keywords: microorganisms, Baeyer-Villiger monoxygenase, epoxide hydrolase

Introduction

A challenge that chemists are presently facing is the development of more efficient and cleaner routes to make old and new products. It is here that catalysts such as enzymes are unique making the best of raw material saving energy and replacing toxic reagents. Notwithstanding these qualities enzymes can display high stereoselectivities and that is why biotechnological processes are developing rapidly in industrial organic synthesis. It is also true that efficient biocatalysts are nowadays limited because industrial substrates are usually artificial and suitable enzymes are often unknown. Therefore, special biocatalysts must be found by screening microorganisms or any other biological source rich in enzymes and this have been a reason for several achievements in catalysis assays applied to screening enzyme activity in high throughput format with liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis, and assays based on fluorogenic or chromogenic substrates.[1-5]

The presence of useful biocatalysts in microorganisms of two Brazilian Culture Collections (CCT – Coleção de Culturas Tropical, Fundação Tropical de Pesquisas André Tosello; CBMAI – Coleção Brasileira de Microrganismos de Ambiente e Indústria, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, UNICAMP) have been investigated.[6-8] However, the time-consuming procedures typical of classical methodologies for screening whole cell enzymatic activity have been a critical drawback. Although high throughput screening (HTS) assays are being rapidly innovated,[2,9-11] whole cell HTS assays have been almost neglected, excepting those based on fluorogenic substrates which have been applied to metagenomic libraries (in function-driven analysis) but seldom to natural culture collections. In order to address this problem, this paper reports the application of a 96-well microplate fluorogenic assay, originally designed for purified hydrolytic enzymes,[12,13] to screen microbial whole cells for epoxide hydrolases (EHs) and Baeyer-Villiger monooxygenases (BVMOs).

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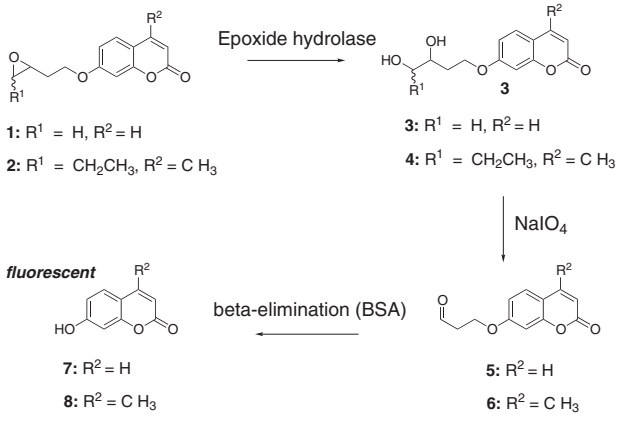
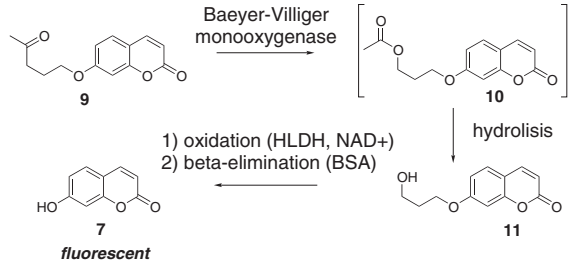
Results and Discussion

The experimental approach herein described was adapted from the high throughput screening assay methodology introduced by Reymond and Klein[12] (Scheme 1) and by Furstoss and co-workers[14] (Scheme 2). Epoxides, probe-substrates for EH, were known to endure the assay conditions.[4,12] The same was not true for ketone **9**, therefore its stability in borate buffer pH 8.8 had to be evaluated in the presence and in the absence of the assay components (Scheme 2), namely horse liver alcohol dehydrogenase (HLADH), NAD^+ and BSA. No time-dependent increase in fluorescence was observed for any combination of reagents within 48 h, discarding the formation of **11** and release of **7** in the absence of a specific Baeyer-Villiger monooxygenase.



Scheme 1. Fluorogenic epoxide hydrolase assay with epoxides **1** and **2**.



Scheme 2. Fluorogenic Baeyer-Villiger monooxygenase assay with ketone **9**.

In order to validate the oxidation of **11** by HLADH/NAD^+ and subsequent β-elimination step (Scheme 2), the fluorescence of a borate buffer solution (pH 8.8) of **11** and BSA with or without HLADH/NAD^+ was evaluated. A significant time-dependent increase in fluorescence intensity was only observed in the presence of HLADH/NAD^+, thus indicating the release of **7** due to the oxidation of **11**.

To adjust the enzymatic assay to the cell format, fixed amounts of diol **3**, metaperiodate ($NaIO_4$), and BSA (see Scheme 1) were set to react in the presence of increasing concentrations of *Rhodotorula glutinis* CCT 2182. As shown in Figure 1, the time-dependent increase in fluorescence intensity due to **7** released as final product was inversely proportional to cell concentration. The presence of 0.1 mg mL^{-1} cells in the solution was not detrimental to the lecture, as estimated from the control reaction. Thus, this limit was established as the maximum cell concentration to run the screening assays. The microorganisms used (Table 1) were cultivated for 3 days on appropriate agar media, at room temperature. The colonies were collected and suspended in borate buffer pH 8.8 just prior to the reactions, which were performed without cell ´ substrate pre-incubation.

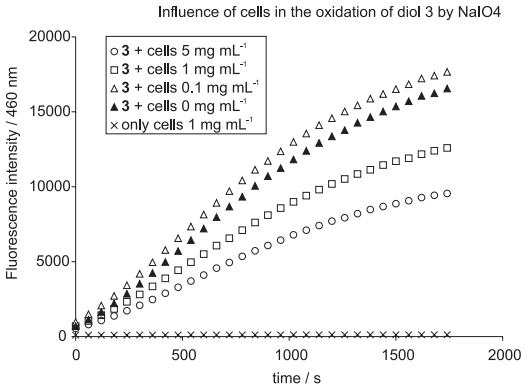


Figure 1. Fluorescence (460 nm) in borate buffer (20 mmol L^{-1}, pH 8.8, 30 °C) due to the conversion **3** → **7** by $NaIO_4$/BSA in the presence of increasing concentrations of *R. glutinis* CCT 2182. Diol **3** (100 μmol L^{-1}), $NaIO_4$ (1 mmol L^{-1}), BSA (2 mg mL^{-1}), cells (dry weigh, 0-5 mg mL^{-1}).

Table 1. Microorganisms screened for EH and BVMO activities

Microorganisms	Original environment
Geotrichum candidum CCT 1205	Metal contaminated industrial fluid. Preston. United Kindon
Pichia kluyeri CCT 3365	Fruit of *Peurouma guianensis*. Altlantic Forest (SP). Brazil
Pichia stipitis CCT 2617	Insect larvae. France
Rhodotorula glutinis CCT 2182	Leaves of Guará tree
Rhodotorula minuta CCT 1751	Cerrado soil. Corumbataí (SP). Brazil
Trichosporon cutaneum CCT 1903	Petroleum refinery sulfate. Japan
Agrobacterium tumefaciens CCT 6515	Not specified
Citrobacter amalonaticus CCT 4059	Poultry slaughtherhouse. Brazil
Pseudomonas aeruginosa CCT 1987	Outer ear infection
Pseudomonas aeruginosa CCT 2738	Animal room water bottle
Serratia rubidea CCT 5732	Coconut fruit. Brazil

CCT: Coleção de Culturas Tropical.

Screening for EH

R. glutinis was included among the 11 microrganisms as a positive control due to its known EH activity.[6,15,16] The reactions with **1** and **2** (Scheme 1) were monitored for 10 h. The time-dependent increase in fluorescence intensity revealed three different EH biocatalysts. The EH activity revealed by *T. cutaneum* CCT 1903 was selective for **1** while that of *A. tumefaciens* CCT 6515 or *P. stipitis* CCT 2617 was selective for **2**. On the other hand, the EH activity of *R. glutinis* CCT 2182 did not discriminate between **1** and **2** (Figures 2 and 3). *T. cutaneum*, *A. tumefaciens* or *P. stipitis* have never been mentioned before as EH sources, and we are now investigating the site and stereoselectivity of the detected biocatalysts.

Screening for BVMO

Following the same strategy described for the EH, fungus *G. candidum* CCT 1205 was included among the investigated microorganisms, as it was known to convert methyl-cyclohexanones into methyl-ε-caprolactones.[17] However, monitoring BVMO activity applying the fluorogenic HTS protocol (Scheme 2) was not as straightforward as expected. No significant time-dependent increase in fluorescence was detected, and even after 48 h of incubation only a very discrete fluorescence increment was registered for the reaction with *T. cutaneum* CCT 1903. Assigning these results to a putative low oxygenation level of the microtiter plate, traditional resting cell biocatalysis of ketone **9** into ester **10** or alcohol **11** (Scheme 2) by G. *candidum* CCT 1205 or *T. cutaneum* CCT 1903 was monitored by HPLC. In these reactions the release of **7** was not expected as no HLADH/NAD^+ and BSA were added to the medium. However, in accordance with the previous results, no time-dependent significant increase in compounds **10** or **11** was detected (Table 2).

As the BVMO of *G. candidum* CCT 1205 was previously detected in aqueous phosphate (pH 6.5),[17] the buffer influence on the BVMO activity was investigated by performing the reaction in this condition. However, the conversion of **9** into **11** by *G. candidium* CCT 1205 in

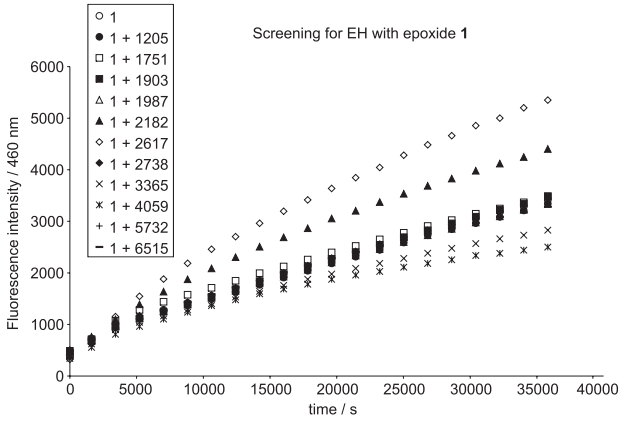


Figure 2. Development of fluorescence intensity (460 nm) in borate buffer (20 mmol L^{-1}, pH 8.8, 30 °C) due to reactions of epoxide **1** (100 μmol L^{-1}) with microbial cells (0.1 mg mL^{-1}) in the presence of $NaIO_4$ (1 mmol L^{-1}) and BSA (2 mg mL^{-1}). To correlate legend numbers to microorganisms see Table 1.

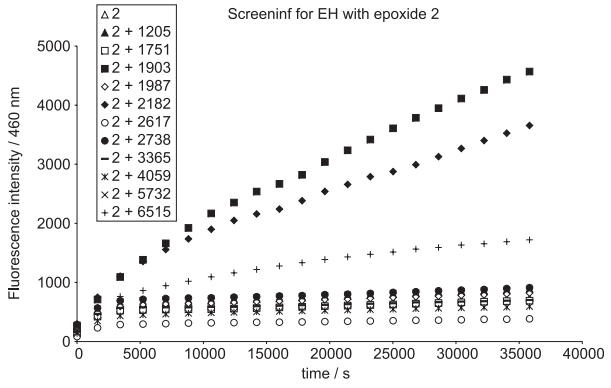


Figure 3. Fluorescence (460 nm) in borate buffer (20 mmol L^{-1}, pH 8.8, 30 °C) due to reactions of epoxide **2** (100 μmol L^{-1}) with microbial cells (0.1 mg mL^{-1}) in the presence of $NaIO_4$ (1 mmol L^{-1}) and BSA (2 mg mL^{-1}). To correlate legend numbers to microorganisms see Table 1.

Table 2. Biocatalysis of **9** in borate buffer pH 8.8 monitored by HPLC

	G. candidum CCT 1205 A%[b]				*T. cutaneum* CCT 1903 A%				C[a] A%			
t[c]	**7**	**9**	**10**	**11**	**7**	**9**	**10**	**11**	**7**	**9**	**10**	**11**
24	0.1	98.1	-	0,4	0,6	96,9	-	1,1	nd	nd	nd	nd
48	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
72	0.5	98.3	-	0,1	0,7	96,5	-	2,0	nd	nd	nd	nd
96	0.6	98.2	-	0,1	0,7	96,1	-	2,2	nd	nd	nd	nd
144	nd	nd	nd	nd	0,6	95,2	-	2,2	nd	nd	nd	nd

[a]Control (reactional mixture without microbial cells). [b]Percentual chromatographic area. [c]Reaction time (hours). nd: not detemined. -: not detected.

Table 3. Biocatalysis of **9** in phosphate buffer pH 6.5 monitored by HPLC

	G. candidum CCT 1205 A%[b]				*T. cutaneum* CCT 1903 A%				C[a] A%			
t[c]	**7**	**9**	**10**	**11**	**7**	**9**	**10**	**11**	**7**	**9**	**10**	**11**
24	0.6	97.6	-	1.3	0.6	86.5	-	12.3	0.6	99.2	-	-
48	0.6	96.1	-	2.8	0.9	70.4	-	27.7	0.6	99.2	-	-
72	nd	nd	nd	nd	1.8	52.4	-	45.0	0.6	99.2	-	-

[a]Control (reactional mixture without microbial cells). [b]Percentual chromatographic area. [c]Reaction time (hours). nd: not detemined. -: not detected.

aqueous phosphate (pH 6.5) did not even reach 3% within 48 h. On the other, the lower pH was found essential to activate the BVMO of *T. cutaneum* CCT 1903, as almost 30% of the starting **9** was converted into **11** within 48 h. In both cases the intermediate **10** was never detected, probably due to the action of hydrolytic enzymes such as lipases or esterases, as previously mentioned.[14]

As reviewed recently, BVMOs are classified according to substrate specificity,[18] therefore BVMOs of *G. candidum* CCT 1205 and *T. cutaneum* CCT 1903 belong to different groups. Therefore, probe-substrates for enzyme activity must be carefully selected as "you get what you screen for". Taking this in consideration we have tested both microorganisms in whole cell biocatalysis experiments using several ketones confirming the presence of CHMO in *G. candidum* and BVMO for special alkanones in *T. cutaneum*, these results will be published elsewhere.

The oxidation of **11** by HLADH/NAD^+ in phosphate buffer pH 6.5 did not work satisfactorily, preventing the application of the Scheme 2 to screening for BVMO activity.

Experimental

*7-(2'-oxyranyl-ethoxy)-2H-1-benzopyran-2-one, (**1**)*

A mixture of 7-hydroxy-2*H*-1-benzopyran-2-one (0.91 g, 5.6 mmol), NaH (60% suspension in oil; 0.61 g, 15 mmol) and 3-butenyl *p*-toluenosulfonate (1.14 g, 5 mmol) were stirred in DMF (20 mL) for 24 h. The mixture was then diluted with AcOEt and washed successively with H_2O and 1 mol L^{-1} aqueous NaOH. Evaporation of the organic phase and flash chromatography (FC; hexane/AcOEt gradient) gave the olefinic precursor of **1**, which (0.10 g, 0.46 mmol) was dissolved in CH_2Cl_2 (3 mL) containing MCPBA (0.10 g, 0.55 mmol). After 24 h at ambient temperature, the mixture was diluted with CH_2Cl_2 and washed successively with 10% aqueous Na_2SO_3 and 0.1 mol L^{-1} aqueous sodium bicarbonate. Evaporation of the organic phase and FC (hexane/EtOAc gradient) gave **1** (0.85 g, 0.37 mmol, 80%) as colorless crystals; mp 67.8 – 69.3°C (EtOAc). lit.[12] 60 - 64 °C. ^{1}H NMR (300.067 MHz, $CDCl_3$, δ_{TMS} 0.00 ppm) δ 7.45 (1 H, d, ^3J 9.2), 7.38 (1 H, d, ^3J 8.4), 6.85 (1 H, dd, ^3J 8.4; 2.5), 6.83 (1 H, d, ^3J 2.5), 6.26 (1 H, d, ^3J 9.2), 4.19 (2 H, m), 3.17 (1 H, m), 2.86 (1 H, t, ^3J 4.7), 2.60 (1 H, dd, ^3J 4.7; 2.7), 2.19 (1 H, m), 1.95 (1 H, m); ^{13}C NMR (75.45 MHz, $CDCl_3$, δ_{CDCl_3} 77.0 ppm) δ 161.7 (C), 161.0 (C), 155.7 (C), 143.2 (CH), 128.7 (CH), 113.1 (CH), 112.7 (CH), 112.6 (C), 101.4 (CH), 65.3 (CH_2), 49.5 (CH), 47.2 (CH_2), 32.3 (CH_2); EIMS ,*m/z* 232 (M^+, 86%), 162 (42), 134 (100), 89 (29), 71 (34).

A similar procedure starting with *cis*-3-hexenyl *p*-toluenosulfonate (1.66 g, 6.5 mmol) and 7-hydroxy-4-methyl-2*H*-1-benzopyran-2-one (1.26 g, 7.1 mmol) gave a diasteroisomeric mixture of *cis*/*trans* (1.5:1.0) **2** as a pale oil (1.26 g, 73%). ^{1}H NMR (499.883 MHz, $CDCl_3$, δ_{TMS} 0.00 ppm), *cis*-isomer, δ 7.50 (1 H, d, ^3J 8.7), 6.87 (1 H, dd, ^3J 8.7; 2.5), 6.83 (1 H, d, ^3J 2.5), 6.15 (1 H, s), 4.20 (2 H, m), 3.19 (1H, ddd, ^3J 7.3, 4.6, 4.6), 2.99 (1H, ddd, ^3J 6.4, 6.4, 4.3), 2.15 (1 H, m), 1.96 (1 H, m), 1.09 (3H, t, ^3J 7.6); *trans*-isomer, δ 7.50 (1 H, d, ^3J 8.7), 6.87 (1 H, dd, ^3J 8.7; 2.5), 6.83 (1 H, d, ^3J 2.5), 6.15 (1 H, s), 4.16 (2 H, m), 2.94 (1H, ddd, ^3J 6.2, 4.6, 2.1), 2.79 (1H, ddd, ^3J 5.5, 5.5, 2.1), 1.61 (2 H, m), 1.01 (3H, t, ^3J 7.6); ^{13}C NMR (75.45 MHz, $CDCl_3$, δ_{CDCl_3} 77.0 ppm) *cis*-isomer, δ 161.8 (C), 161.2 (C), 155.3 (C), 152.4 (C), 125.6 (CH), 113.7 (C), 112.4 (CH), 112.0 (CH), 101.7 (CH), 65.8 (CH_2), 58.3 (CH), 54.3 (CH), 27.7 (CH_2), 21.6 (CH_2), 18.6 (CH_3), 10.4 (CH_3); *trans*-isomer, δ 161.8 (C), 161.2 (C), 155.3 (C), 152.4 (C), 125.6 (CH), 113.7 (C), 112.4 (CH), 112.0 (CH), 101.7 (CH), 65.2 (CH_2), 60.1 (CH), 55.4 (CH), 31.8 (CH_2), 24.9 (CH_2), 18.6 (CH_3), 9.8 (CH_3); *cis*-isomer, EIMS: *m/z* 274 (M^+, 26%), 256 (7), 188 (30), 176 (58), 148 (100); *trans*-isomer, EIMS: *m/z* 274 (M^+, 100%), 256 (12), 188 (18), 176 (44), 148 (46).

*7-(4'-oxo-pentyl)-2H-1-benzopyran-2-one, (**9**)*

A mixture of 7-hydroxy-2*H*-1-benzopyran-2-one (0.57 g, 3.5 mmol), NaH (60% suspension in oil; 0.4 g, 10 mmol) and 4-pentenyl *p*-toluenosulfonate (0.8.4 g, 3.5 mmol) were stirred in DMF (15 mL) for 24 h. The mixture was then diluted with EtOAc and washed successively with H_2O and

1 mol L^{-1} aqueous NaOH. Evaporation of the organic phase and FC (hexane/AcOEt gradient) gave the olefinic precursor of **9**, which (0.16 g, 0.69 mmol) was dissolved in a mixture of $PdCl_2$ (12.1 mg, 0.07 mmol) and CuCl (68.3 mg, 0.69 mmol) in DMF-H_2O (5 mL, 7:1) under O_2 atmosphere. After 24 h at ambient temperature the mixture was diluted with 5% aqueous HCl and washed with EtOAc. Evaporation of the organic phase and FC (hexane/AcOEt gradient) gave **9** (0.11 g, 64%) as colorless crystals; mp 74.4 – 75.8 °C (EtOAc). ^{1}H NMR (300.067 MHz, $CDCl_3$, δ_{TMS} 0.00 ppm) δ 7.62 (d, 1 H, ^3J 9.5), 7.35 (d, 1 H, ^3J 8.2), 6.80 (dd, 1 H, ^3J 8.2; 2.5), 6.76 (d, 1 H, ^3J 1.8), 6.22 (d, 1 H, ^3J 9.5), 4.02 (t, 2 H, ^3J 6.0), 2.66 (t, 2 H, ^3J 7.1), 2.17 (s, 3 H), 2.08 (2 H, qt, ^3J 6.6); ^{13}C NMR (75.45 MHz, $CDCl_3$, δ_{CDCl_3} 77.0 ppm) δ 207.5 (C), 161.8 (C), 160.9 (C), 155.5 (C), 143.2 (CH), 128.6 (CH), 112.9 (CH), 112.5 (CH), 112.4 (C), 101.3 (CH), 67.4 (CH_2), 39.6 (CH_2), 30.1 (CH_2), 23.0 (CH_3); EIMS: *m/z* 246 (M^+, 5%), 162 (7), 134 (31), 85 (100), 43 (75).

*7-(3'-acetoxy-propyl)-2H-1-benzopyran-2-one, (**10**)*

Acetyl chloride (0.69 g, 8.5 mmol) was added to 3-propanol *p*-toluenosulfonate (1.15 g, 5 mmol) and pyridine (1.5 mL, 17.5 mmol) in CH_2Cl_2 (6 mL). After 30 min the mixture was diluted with EtOAc (50 mL) and washed successively with 0.1 mol L^{-1} aqueous $NaHCO_3$, 10% aqueous $CuSO_4$ and H_2O. Evaporation of the organic phase and FC (hexane/AcOEt gradient) gave the *p*-toluenosulfonil precursor of **10**, which (1.06 g, 3.9 mmol) was stirred with 7-hydroxy-2*H*-1-benzopyran-2-one (0.66 g, 4.1 mmol) and NaH (60% suspension in oil; 0.62 g, 12 mmol) in DMF (35 mL) for 24 h. The mixture was then diluted with EtOAc and washed successively with H_2O and 1 mol L^{-1} aqueous NaOH. Evaporation of the organic phase and FC (hexane/EtOAc gradient) gave **10** (0.26 g, 26%) as pale crystals; mp 67.9 – 68.9 °C (EtOAc). ^{1}H NMR (300.067 MHz, $CDCl_3$, δ_{TMS} 0.00 ppm) δ 7.64 (d, 1 H, ^3J 9.3), 7.37 (d, 1 H, ^3J 8.4), 6.84 (dd, 1 H, ^3J 8.4; 2.4), 6.80 (d, 1 H, ^3J 2.4), 6.25 (d, 1 H, ^3J 9.3), 4.27 (t, 2 H, ^3J 6.2), 4,11 (t, 2 H, ^3J 6.2), 2.16 (qt, 2 H, ^3J 6.2), 2.07 (s, 3 H); ^{13}C NMR (75.45 MHz, $CDCl_3$, δ_{CDCl_3} 77.0 ppm) δ 170.7 (C), 161.7 (C), 160.9 (C), 155.6 (C), 143.2 (CH), 128.6 (CH), 113.0 (CH), 112.7 (CH), 112.5 (C), 101.2 (CH), 64.9 (CH_2), 60.9 (CH_2), 28.4 (CH_2), 21.0 (CH_3);EIMS: *m/z* 262 (M^+, 100), 219 (7), 162 (29), 134 (72), 101 (83), 43 (89). The synthesis of compounds **3** and **11** were described elsewhere.[13]

Microorganisms

Yeasts and bacteria were cultured for 3 days, at room temperature, in 1.5 mL *Eppendorf* flasks respectively containing yeast-malt-agar medium (3 g L^{-1} yeast extract *Merck*, 3 g L^{-1} malt extract *Merck*, 5 g L^{-1} peptone *Difco*, 10 g L^{-1} glucose, 20 g L^{-1} agar *Merck*, H_2O) and Müller Hinton-agar medium (20 g L^{-1} Müller Hinton Merck, 20 g L^{-1} agar Merck, H_2O). Just prior to the tests the grown colonies were weighed into 1.5 mL *Eppendorf* flasks and suspended in aqueous buffer.

Fluorescence measurements

All buffers and solutions were prepared using *MilliQ*-deionized H_2O. Microbial cells were diluted from 0.2 mg mL^{-1} suspentions in 20 mol L^{-1} borate buffer (pH 8.8). Substrates were diluted from 20 mmol L^{-1} stock solutions in 50% aqueous MeCN. BSA was diluted from a 40 mg.mL^{-1} stock solution in 20 mmol L^{-1} borate (pH 8.8). For the EH assay, $NaIO_4$ was diluted from a 4.3 mg mL^{-1} aqueous solution. For BVMO assay, HLADH was diluted from a 10.6 mg mL^{-1} aqueous stock solution and NAD^+ was diluted from a 13 mg mL^{-1} aqueous stock solution. For screening EH activity, reactions were initiated by addition of $NaIO_4$ to a solution containing substrate and BSA. For screening BVMO activity, reactions were initiated by addition of cells to a solution containing substrate, HLADH, NAD^+ and BSA. The 200 μL assays were followed in individual wells of flat-bottom polypropylene 96-well microtiter plates (*Costar*) with a *Cytofluor-II* fluorescence plate reader (*Perseptive Biosystems*, filters λ_{ex} 360 ± 20 nm, λ_{em} 460 ± 20 nm).

Biocatalysis reactions

Microbial cells weighed into 100 mL conic flasks (*Costar*) were suspended at 2 mg mL^{-1} in aqueous buffer (20 mmol L^{-1} borate pH 8.8 or 20 mmo L^{-1} phosphate pH 6.5, 25 mL). Then, a solution of ketone **3** (50 μL, 100 mg mL^{-1} in MeCN) was added to provide 0.2 mg mL^{-1} substrate solutions and the resulting mixtures were agitated on a plate stirrer at room temperature. To follow the reactions, 20 μL aliquots were taken and analyzed by HPLC on a *Chromolith Speed Rod* column (RP-18e, 5.0 × 10 cm, *Merck*, flow 3.0 mL min^{-1}, 100% A to 100%B in 10 min, A = 100% H_2O + 0,1% TFA, B = 60% MeCN + 40% H_2O + 0,1% TFA): t_R 4.1 (**7**), 5.1 (**11**), 6.3 (**9**), 6.8 (**10**) min. The integral of the peaks recorded by UV at 320 nm was used to calculate the conversion of released products.

Conclusions

An enzymatic HTS fluorogenic assay was smoothly adapted to a HTS protocol for EH of whole cells, and its

application revealed 3 new microbial sources of EH (*A. tumefaciens*, *P. stipitis*, and *T. cutaneum*). Considering the practical application of EH in organic synthesis, the utilization of the assay presented here for gathering selective biocatalysts is strongly recommended. On the other hand, the HTS fluorogenic assay based on Furstoss's procedure,[14] used for present evaluation of monooxigenase, proved to be inadequate for cyclohexanone monooxigenase. Thus, a series of modifications are being evaluated. Notwithstanding this shortcoming the experiments revealed 2 sources of BVMO biocatalysts (*G. candidum* and *T. cutaneum*).[19]

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19. After submission of the present work we became aware of Furstoss's publication on the optimized method: Gutiérrez, M.C.; Sleegers, A.; Simpson, H. D.; Alphand, V.; Furstoss; *Org. Biomol. Chem*. **2003**, *1*, 3500 and the BVMO in *G.candidum*: Carballeira, J.D.; Alvarez, E.; Sinisterra. J.V.; *J. Mol. Catalysis B, Enzymatic.* **2004**, *28*, 25.

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