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Journal of Molecular Catalysis B: Enzymatic 52–53 (2008) 173–177
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Whole cell quick *E* for epoxide hydrolase screening using fluorescent probes

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Available online 23 December 2007

Abstract

We describe herein a modified quick *E* to access the enantioselectivity (*E*) of epoxide hydrolases using chiral fluorogenic probes. Accessing the true *E* values of the same biocatalytic reaction with chiral HPLC validated this methodology.

Keywords: Quick *E*; Epoxide hydrolases; Enantioselectivity; Whole cells; High throughput screening

1. Introduction

Enzymatic production of enantioenriched or enantiopure compounds is obtained by kinetic or dynamic kinetic resolution of racemates [1]. The enzymatic resolution quantification is either referred as the enantiomeric ratio or enantioselectivity (*E*) and is expressed by the fast enantiomer over the slow enantiomer ratios of the turn-over number k_{cat} [2] over the Michaelis constant K_M [3,4], k_{cat}/K_M (Eq. (1)) [5].

$$\text{enantiomeric ratio} = E = \frac{(k_{cat}/K_M)_{\text{fast enantiomer}}}{(k_{cat}/K_M)_{\text{slow enantiomer}}} \quad (1)$$

The most popular methodology to measure enantioselectivity was developed by Sih's group [5], to which other methods are compared. The *E* values are obtained by measuring the enantiomeric excess of the residual substrate (ee_S) or the corresponding product (ee_P) at a certain degree of conversion (*c*) (Eqs. (2) and (3)).

$$E_{\text{product}} = \frac{\ln[(1-c)(1+ee_P)]}{\ln[(1-c)(1-ee_P)]} \quad (2)$$

$$E_{\text{substrate}} = \frac{\ln[(1-c)(1-ee_S)]}{\ln[(1-c)(1+ee_S)]} \quad (3)$$

for $c = (A+B)/(A_0+B_0)$, *A* and *B* are the concentration of fast and slow-reacting enantiomers and A_0 and B_0 the initial concentrations of both.

However, measuring enantiomeric excess and conversion are time consuming and inappropriate for screening large collections of commercial enzymes, microorganisms or metagenomic clones [6]. Therefore, Reymond and co-workers [7] proposed calculation of *E* from the initial enantiomeric reaction rates of both enantiomerically pure fluorescent probes, measured in individual wells of the same microtiter plate and correlated to the umbelliferone concentration (Scheme 1). Though practical, Reymond's methodology provides estimated *E* values that can be far from the true values.

The main difference between experiments with the separate enantiomerically pure fluorescent probes in a high throughput format and the true *E* values calculated by Sih's methodology is the absence of enantiomeric competition for the same enzymatically active site.

This limitation can be overcome by reintroducing competition as in the quick *E* assay proposed by Janes and Kazlauskaz [8] to measure hydrolases enantioselectivity using chiral chromogenic substrates. These authors reintroduced competition by adding the same reference compound of similar reactivity to each enantiomer, which was monitored at a different wavelength during hydrolyses.

Searching for epoxide hydrolases and monooxygenases in natural microorganisms and metagenomic libraries using fluorogenic probes and essays in a 96-well microtiter plates format [9,10,11] we felt the urgent need to access true *E* applying the same high throughput screening methodology to whole cells with enantiomerically pure fluorogenic probes as proposed by Reymond et al. while introducing competition as proposed by Kazlauskaz and Janes for isolated enzymes.

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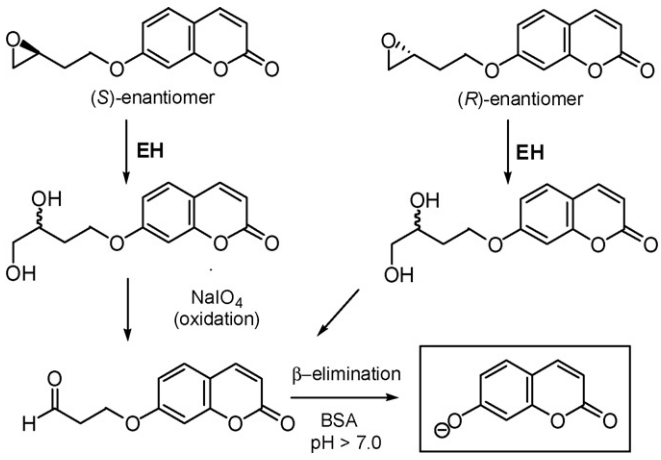
doi:10.1016/j.molcatb.2007.12.013



Scheme 1. High throughput assay for epoxide hydrolases (EH) using fluorogenic probes in separate vessels.

2. Experimental

2.1. General methods

^{1}H NMR (300.07 MHz, $CDCl_3$) spectra were recorded in $CDCl_3$ on a Gemini 300P—Varian spectrometer. Chemical shifts are expressed in parts per million using $(CH_3)_4Si$ as internal reference (δ 0.0), and coupling constants J are given in hertz. GC–MS analyses were performed with an Agilent 6890 Series GC System and with a Hewlett-Packard 5973 Mass Selective Detector (70 eV), equipped with a HP-5MS (crosslinked 5% phenyl methyl siloxane, 30 m × 0.25 mm i.d. × 0.25 μm film thickness) fused silica capillary column and helium as carrier gas (1 mL min^{-1}). Chiral HPLC was performed with a Shimadzu Prominence System equipped with a DAD detector, using hexane/isopropanol and a Chirasel OJ-H (Daicel 4.6 × 250 mm × 10 μm) chiral column. Merck 60 silica gel (230–400 mesh ASTM) was used for flash chromatography. The fluorescence was measured with an Analytic Jena spectrophotometer, Analytik Jena AG Flashscan 530, equipped with λ_{ex} 360 nm and λ_{em} 460 nm filters.

2.2. Microorganisms and culture conditions for fluorescence measurements

Microorganisms were grown in slants containing culture medium as follow: filamentous fungi were grown for 48 h at 30 °C in 20 g L^{-1} malt extract (pH 7.6); bacteria were grown in nutrient broth for 24 h at 30 °C and yeast were grown in 1% yeast extract, 2% peptone and 10% D-glucose (200 g L^{-1}) for 24 h at 30 °C.

2.3. Fluorescence assays

All solutions were prepared using Milli-Q deionized water. The microbial cells were suspended in 20 mol L^{-1} phosphate buffer (pH 7.2) or borate buffer (pH 8.0), at 0.2 mg mL^{-1} for bacteria and yeasts, 0.5 mg mL^{-1} for filamentous fungi. Substrates were diluted with MeCN/water 1:1 to a 2 mmol L^{-1} or 1 mmol L^{-1} concentration. All the assays and controls were measured in duplicates and performed in 200 μL polypropylene 96-well microtiter plates used as described below.

2.3.1. Selection of epoxide hydrolases in natural microorganism whole cells

2.3.1.1. Enzymatic assay. Racemic epoxides **1** and **2** (10 μL, 2 mmol L^{-1}), BSA (80 μL, 75 pmol L^{-1} or 5.0 mg mL^{-1}), cell suspension in borate buffer (pH 8.0, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.1.2. Positive control. Diol probes (10 μL, 2 mmol L^{-1}), BSA (80 μL, 75 pmol L^{-1} or 5.0 mg mL^{-1}), cell suspension in borate buffer (pH 8.0, 20 mmol L^{-1}, 100 μL) and of $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.1.3. Negative control. Racemic epoxides (10 μL, 2 mmol L^{-1}), BSA (80 μL, 75 pmol L^{-1} or 5.0 mg mL^{-1}), borate buffer (pH 8.0, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.2. Estimated E

2.3.2.1. Enzymatic assays. Enantiomerically pure epoxides **1** and **2** (20 μL, 1 mmol L^{-1}), BSA (70 μL, 85 pmol L^{-1} or 5.7 mg mL^{-1}), cell suspension in phosphate buffer (pH 7.2, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.2.2. Positive control. Diol probes (20 μL, 1 mmol L^{-1}), BSA (70 μL, 85 pmol L^{-1} or 5.7 mg mL^{-1}), cell suspension in phosphate buffer (pH 7.2, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.2.3. Negative control. Enantiomerically pure epoxides (20 μL, 1 mmol L^{-1}); BSA (70 μL, 85 pmol L^{-1} or 5.7 mg mL^{-1}), phosphate buffer (pH 7.2, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.3. Whole cell quick E (introducing competition)

2.3.3.1. Enzymatic assays. Enantiomerically pure epoxides **1** and **2** (10 μL, 2 mmol L^{-1}), non-fluorogenic competition compound (10 μL, 0.2 mmol L^{-1}), BSA (70 μL, 85 pmol L^{-1} or 5.7 mg mL^{-1}), cell suspension in phosphate buffer (pH 7.2, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.3.2. Positive control. Diol probe (10 μL, 2 mmol L^{-1}), competition compound (10 μL, 0.2 mmol L^{-1}), BSA (70 μL, 85 pmol L^{-1} or 5.7 mg mL^{-1}), cell suspension in phosphate buffer (pH 7.2, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.3.3.3. Negative control. Enantiomerically pure epoxides (10 μL, 2 mmol L^{-1}), competition compound (10 μL, 0.2 mmol L^{-1}), BSA (70 μL, 85 pmol L^{-1} or 5.7 mg mL^{-1}), phosphate buffer (pH 7.2, 20 mmol L^{-1}, 100 μL) and $NaIO_4$ (10 μL, 20 mmol L^{-1}).

2.4. Microorganisms and culture conditions for biotransformation reactions

The microorganisms were grown in Erlenmeyer flasks (500 mL) containing 300 mL of liquid culture medium (20 g L^{-1} malt extract for filamentous fungi; 26 g L^{-1} nutrient broth for bacteria and, 1% yeast extract, 2% peptone, and 10% of a solution 200 g L^{-1} of D-glucose for yeasts) with continuous stirring for 2 days at 30 °C in an orbital shaker (150 rpm). The cells were harvested by centrifugation (bacteria, 5000 rpm, 20 min and 18 °C) or by filtration (filamentous fungi) and suspended in phosphate buffer (pH 7.0, 20 mmol L^{-1}).

2.5. Biotransformation reactions

2.0 g of wet cells were added to a 125 mL Erlenmeyer flask containing 40 mL of pH 7.0 phosphate buffer and stirred at 150 rpm at 28 °C. The racemic fluorogenic probe (20 mg/100 μL in acetone) was added to the cell suspension to reach a final concentration of 1 μL mL^{-1}. Conversion was calculated based on the remaining epoxide present in a 2.0 mL sample extracted with ethyl acetate (2 × 1.0 mL) containing benzophenone (0.01 mg mL^{-1}) as internal standard and determined by GC–MS. The diol enantiomeric excess was determined by chiral stationary phase HPLC using a Chiralsel OJ-H (Daicel: 25 cm × 0.46 cm × 10 μm) column with 4:1 isopropanol/hexane as eluent.

2.6. Calculations

The fluorescence signal was converted into umbelliferone concentration according to a calibration curve built with solutions of increasing umbelliferone concentrations (5, 10, 15, and 25 mmol L^{-1}) using phosphate buffer (pH 7.2, 20 mmol L^{-1}) containing sodium periodate and BSA. Thus, the E values were obtained using the equation described below:

$$\text{Quick } E = \frac{(\text{initial rate})_{\text{fast enantiomer}}}{(\text{initial rate})_{\text{slow enantiomer}}}$$

Table 1
Detection of epoxide hydrolases in Brazilian microorganisms whole cells using fluorogenic probes **1** and **2**

Microorganisms	Fluorescent probe	Fluorescence intensity[a]
Candida albicans CCT 0776[b]	1	1919
Aspergillus niger CCT 1435[c]	2	236
Rhyzopus oryzae CCT 4964[c]	2	189
Ama 31[d]	2	154
Ama 32[d]	2	168

[a] Fluorescence intensity = (average of duplicate of the samples) − (average of the duplicate of the negative control) after 10 h of reaction.
[b] Yeasts.
[c] Fungi.
[d] Microorganisms isolated in Amazonia soil not yet identified.

3. Results and discussion

The screening of 103 Brazilian microorganisms applying Reymond's [7,12] methodology adapted to whole cells [11] allowed us to select five microorganisms producing high fluorescent signals with the racemic probes **1** and **2** (Table 1) indicating the presence of epoxide hydrolases for monosubstituted epoxides (probe **1**) and disubstituted epoxides (probe **2**). However, the enantioselectivity (E) of these enzymes remained undetermined, which was the slow step in our screening methodology. Therefore, to increase our efficiency in selecting epoxide hydrolases we choose the quick E assay that was originally designed for chromogenic probes and isolated enzymes [8], requiring adaptations for use with whole cells and fluorescent probes and determination of an optimum pH range.

Experiments using whole cells of the previously selected microorganisms (Table 1) with fluorescent probes **1** or **2** in 20 mmol L^{-1} borate buffer (pH 8.0) lead to non-enzymatic hydrolyses and imprecise E values. Therefore, pH optimization required a compromise between spontaneous hydrolyses and the maximum umbelliferone fluorescent signal that occurs at pH greater than 7. At pH 7.2 umbelliferone has somewhat reduced fluorescent signal but a minimum spontaneous hydrolysis of probes **1** and **2** was observed. Consequently, the experiments were run at pH 7.2 (Fig. 1).

Our first experiments, ignoring the competitive reactions of the enantiomers at the enzymatic binding site, revealed that the

Table 2
Estimated, modified quick E and true enantioselectivity using fluorogenic probes **1** and **2**

Microorganisms	Substrate[a]	Estimated E[b]	Quick E[c]	True E[d]
Candida albicans CCT 0776	1	4.5	1.5	1.2
Aspergillus niger CCT 1435	2	2.7	1.8	1.6
Rhyzopus oryzae CCT 4964	2	1.3	1.1	–
Ama 31	2	1.4	1.1	1.0
Ama 32	2	2.4	1.4	–
A. niger EH	2	1.6	≫100	253

[a] All microorganisms reacted preferentially with the *S*-enantiomer.
[b] Enantioselectivity measured using only fluorogenic probes **1** and **2**.
[c] Enantioselectivity measured using the fluorogenic probes and a competitive reaction.
[d] Enantioselectivity measured using the C.J. Sih's method, the enantiomeric excess of the diols were determined by analysis in chiral phase HPLC.

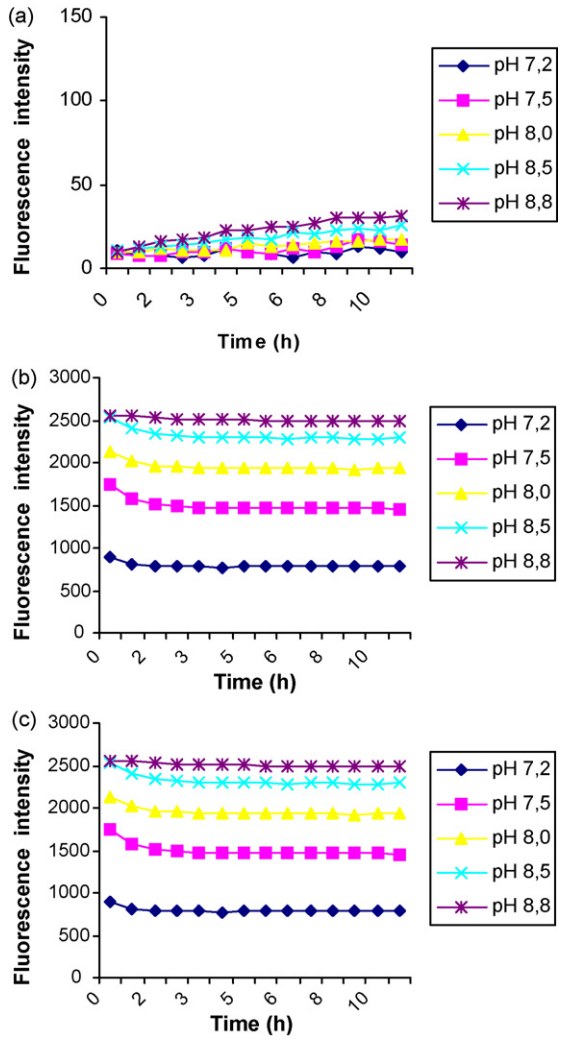


Fig. 1. Kinetics of (a) **1** and (b) **2** spontaneous hydrolyses at various pH, monitoring the released umbelliferone fluorescent signal. (c) Umbelliferone fluorescence at different pH.

estimated *E* was high by as much as 67% (Table 2; Fig. 2). Based on the Kazlauskaz and Janes protocol with chromogenic probes [8], we introduced competition into our experiments using fluorogenic substrates. Searching for a readily available methodology we selected a non-fluorogenic racemic compound to compete with the fluorogenic substrate, bearing in mind that, though "invisible", it was competing for the active site in the separate enantiomeric reactions with comparable reaction rates for low and average *E* values. Thus we introduced two major differences into the currently published methodologies, the competitive compound is racemic and its biotransformation is not monitored.

For better accuracy the substitution pattern and reactivity of the selected competitive compounds were equivalent to those of the fluorescent probes. Therefore, for the quick *E* assays using the fluorogenic probes (*R*)-**1** and (*S*)-**1** the inexpensive monosubstituted epoxide, (±)-1,2-epoxyoctane (**3**), was selected for the competitive reaction. In the experiments using probes (*R*)-**2** and (*S*)-**2** the competitive compound was (±)-3,4-epoxy-1-hexanol (**4**) (Fig. 3).

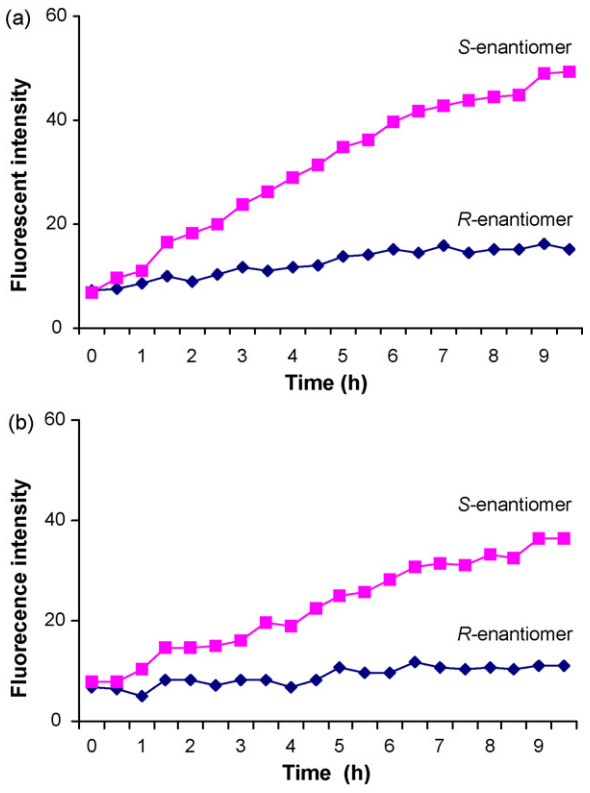


Fig. 2. Time course of the C. albicans CCT0776 epoxide hydrolase reaction with (*S*)-**1** (□) and (*R*)-1 (◊) epoxides. a) non competitive reaction; b) competitive reaction with (±)-1,2-epoxyoctane.

We additionally investigated whether enantiomericaly pure (*R*)-1,2-epoxyoctane or racemic (±)-1,2-epoxyoctane competing with (*S*)-**1** or (*R*)-**1** produced the better results. Measuring reactions with low *E* values, no detectable differences were observed between these conditions. Thus, the use of a racemic competitive compound was selected based on accuracy and availability (Fig. 4).

The next step was to verify whether the competitive reaction was quenching the umbelliferone signal by comparing the fluorescent signal intensity in the presence and absence of the competitive reaction. Both reactions displayed equivalent fluorescent signals, indicating that the competitive reaction was not interfering with the probe signal (Fig. 5).

With these optimizations we have obtained modified quick *E* values of epoxide hydrolases for the 5 selected microorganisms (*Candida albicans* CCT 0776, *Aspergillus niger* CCT 1435, *Rhy-*

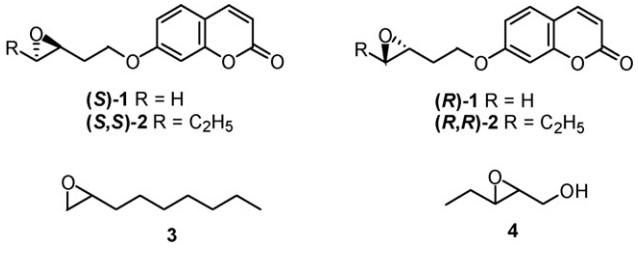


Fig. 3. Fluorogenic probes and competitive compounds used in the quick *E* assay.

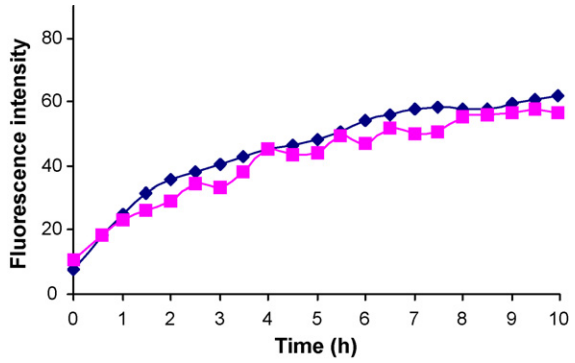


Fig. 4. Umbelliferone fluorescent signal intensities using chiral probe **1**-(*S*), *C. albicans* epoxide hydrolases and racemic (□) or enantiomerically pure (*R*)-1,2-epoxy octane (◊) in the competition reaction.

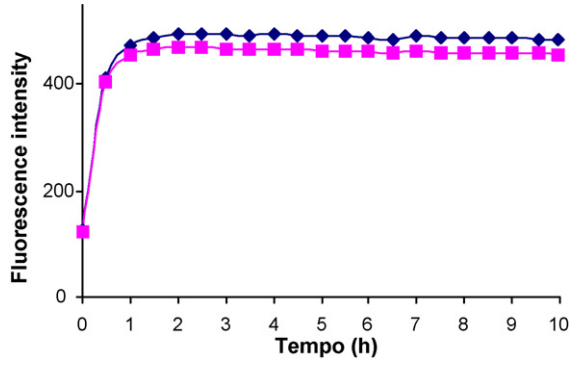


Fig. 5. Comparison of the umbelliferone fluorescent signal intensity in the presence (□) or absence (◊) of competition.

zopus oryzae CCT 4964, Ama 31, Ama 32) and for a epoxide hydrolase recombinant from *A. niger* (Sigma–Aldrich). These are presented in Table 2. The true *E* values were obtained by conventional methodology using the racemic probes **1** and **2**, for which the conversion values and the enantiomeric ratio were determinated by GC–MS and chiral stationary phase HPLC, respectively (Table 2) and they are in excellent agreement with the new modified quick *E*.

These data indicated that monitoring the competitive reaction rate is not absolutely essential and reliable enantioselectivities can be obtained in a high throughput screening for epoxide hydrolases in whole cells and in commercial enzymes using fluorogenic probes.

4. Conclusions

The modified quick *E* assay to determine the enantioselectivity of epoxide hydrolases with fluorogenic probes and whole cells from natural microorganisms or clone collections is a good alternative to traditional methodologies. The obtained *E* values are in good agreement with the true *E* values, allowing a rapid screening of large culture collections requiring minute amounts of substrates and enzymes.

Acknowledgements

We would like to thank FAEP-UNICAMP, FAPESP, Petrobrás, and CNPq (Brazil) for financial support. We are also grateful to Prof. J.-L. Reymond and Prof. Manfred T. Reetz for the donation of the chiral fluorogenic probes and *Aspergillus niger* EH, respectively. We also thank Professor Carol H. Collins for helpful suggestions about English grammar and style.

References

[1] Y. Lu, X. Zhao, Z.-N. Chen, Tetrahedron: Asymmetry 6 (1995) 1093–1096.
[2] A. Fersht, Structure and Mechanism in Protein Science, W. H. Freeman and Company, New York, 1999.
[3] A. Cornish-Bowden, Fundamentals of Enzyme Kinetics, 3rd ed., Portland Press Ltd., London, 2004.
[4] L. Michaelis, M.L. Menten, Biochemische Zeitschrift 49 (1913) 333–369.
[5] C.S. Chen, Y. Fujimoto, G. Girdaukas, C.J. Sih, J. Am. Chem. Soc. 104 (1982) 7294–7299.
[6] J.L. Reymond, Enzyme Assays: High-throughput Screening, in: Genetic Selection and Fingerprints, 1st ed., Wiley-VCH Verlag GmbH & Co, Germany, 2004.
[7] F. Badalassi, D. Wahler, G. Klein, P. Crotti, J.-L. Reymond, Angew. Chem. Int. Ed. 39 (2000) 4067–4070.
[8] L.E. Janes, R.J. Kazlauskaz, J. Org. Chem. 62 (1997) 4560–4561.
[9] Among others HTS assays and epoxide hydrolases references: C. Mateo, A. Archelas, R. Furstoss, Anal. Biochem. 314 (2003) 135–141;
M.T. Reetz, C. Torre, A. Eipper, R. Lohmer, M. Hermes, B. Brunner, A. Maichele, M. Bocola, M. Arand, A. Cronin, Y. Genzel, A. Archelas, R. Furstoss, Org. Lett. 6 (2004) 177–180;
M.T. Reetz, L.-W. Wang, M. Bocola, Angew. Chem. Int. Ed. 45 (2006) 1236–1241.
[10] R. Sicard, L.S. Chen, A.J. Marsaioli, J.-L. Reymond, Adv. Synth. Catal. 347 (2005) 1041–1050.
[11] B. Bicalho, L.S. Chen, J. Grognux, J.-L. Reymond, A.J. Marsaioli, J. Braz. Chem. Soc. 15 (2004) 911–916.
[12] 44 microorganisms were purchased from CCT - Coleção de Culturas Tropicais da Fundação André Tosello and CBMAI - Coleção Brasileira de Microrganismos de Ambiente e Indústrias collections: *Agrobacterium tumefaciens*, *Antrobacter oxydans*, *Antrobacter* sp., *Arquea*, *Bacillus cereus*, *Bacillus*, *Citrobacter amalonaticus*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas oleovorans*, *Pseudomonas putida*, *Salmonella thyphimurium*, *Serratia liquefaciens*, *Serratia marcescens*, *Serratia plymuthica*, *Serratia rubidae*, *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochracius*, *Aspergillus oryzae*, *Aspergillus terreus*, *Cunninghamella ecchinulata*, *Curvularia eragrostides*, *Curvularia lunata*, *Curvularia pallescens*, *Emericela nidulans*, *Geotrichum candidum*, *Mortirela isabelina*, *Nodulisporum* sp., *Rhyzopus oryzae*, *Trametes versicolor*, *Candida albicans*, *Candida utilis*, *Kluyveromyces marxianus*, *Pachysolen tannophilus*, *Pichia canadensis*, *Pichia kluyveri*, *Pichia stipitis*, *Rhodotorula glutinis*, *Sacharomyces boulardii*, *Sacharomyces cerevisae*, *Sacharomyces* sp., *Trichosporon cutaneum*. Another 37 were isolated from Amazonian soil, but have not been identified: Ama 1 to Ama 37 (unpublished work).