Article

High Throughput Enzymatic Enantiomeric Excess: Quick-*ee*

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High throughput techniques to evaluate enantiomeric excess (*ee*) and enantiomeric ratio (*E*) of enzymatic reactions are fast and efficient tools that can be applied large quantities of enzymes, microorganisms and clones. The Quick-*ee* technique proposed in this study produces the *ee* and conversion values separately.

Keywords: enantiomeric excess (*ee*), enantiomeric ratio (*E*), high throughput screening (HTS)

Introduction

There is a growing need for enzymatic and multienzymatic cascade processes in the chemical and pharmaceutical industries to replace chemical steps with steps that incorporate green chemistry principles.[1-5] When enantiomerically pure products are required, enzymes are the catalysts of choice for enantio-, diastereo- and site-selective transformations, due to their specificity in discriminating prochiral centres and faces.[6-10]

Currently, tailored enzymatic performance is achieved using genetic engineering to modify catalytic sites using several techniques, including site-directed evolution.[11,12] The transformation process requires modification of the DNA fragment encoding the enzyme, which is inserted into a vector (plasmid, phosmid or virus) and introduced into host cells, producing several clones with different specificities. Sorting these clones leads to highly specific enzymes and products with high enantiomeric excesses (*ee*) and yields.[13] Such transformations can be further improved to broaden their catalytic applications by generating enzymes that accept several substrates (substrate promiscuity).[14,15] These attributes enhance the application of this biotechnology in industry, where molecular engineering and green chemistry work together to add value to products.[16,17] These biocatalyst issues (*ee*, enantiomeric ratio (*E*), etc.) require access to fast and sensitive methodologies,[18-21] such as high throughput screening (HTS).[22,23] Methodologies to rapidly obtain the enantiomeric ratio require chiral substrates

and initial rate monitoring (V_0) for each enantiomer.[24,25] These HTS techniques usually employ chromogenic or fluorogenic probes, allowing for the simultaneous evaluation of 6, 24, 96, or 384 reactions.[26-28] Based on this idea, Kazlauskas and co-workers introduced Quick-*E* for hydrolases through the application of chiral chromogenic probes. A chromogenic competitor was also introduced into the experiment, which behaved as the enantiomer, a statement not exactly true because they are different compounds, thus producing a good *E* evaluation.[26]

Similar methodology was proposed by Reymond *et al.*[27-29] However, this methodology does not use a competitor and is based on fluorogenic probes, which are usually more sensitive (approximately 10^3).[29] The lack of enantiomeric competition for the enzyme active site results in large deviations from true *E* values.

In this study, we applied both concepts, exploiting both the sensitivity of fluorogenic probes and competition, for the HTS evaluation of *ee*, which is referred to here as Quick-*ee*.

Experimental

Cultivation of microorganisms

Bacteria were inoculated into nutrient broth. Yeasts and fungi were inoculated into yeast extract-malt and cultivated for 16 h at 28 °C with stirring at 200 rpm. The cells were then transferred to a Petri dish containing nutrient agar (NA) or yeast extract-malt extract agar (YMA) and incubated at 30 °C for an additional 16 h period.

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Fluorescence assays

Cells were harvested with a Drigalsky's spatula and suspended in borate buffer (pH 7.4, 0.2 mmol L^{-1}). (R)-**1** ((R)-7-(3,4-diacetoxybutyloxy)-2H-1-benzopyran-2-one), (S)-**1** ((S)-7-(3,4-diacetoxybutyloxy)-2H-1-benzopyran-2-one) and **2** (7-(2-acetoxyethyloxy)-2H-1-benzopyran-2-one)), synthesized according to Reymond's methodology,[29] were each dissolved in H$_2$O:MeCN 1:1 to prepare solutions of 1 mmol L^{-1} and 2 mmol L^{-1} for the assays with and without competition, respectively. All assays were performed in triplicate in 96 well microtitre plates, with 200 μL in each well. Fluorescence was measured using an Analytik Jena AG FlashScan 530 spectrophotometer, equipped with λ$_{ex}$ 360 nm and λ$_{em}$ 460 nm filters (24 h, 28 °C).

The assays (enzymatic assay, negative control and positive control) were monitored for 24 h at 28 °C, simultaneously.

Enzyme and microorganism screening

Enzymatic assay. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), bovine serum albumin (BSA) (80 μL, 5.0 g L^{-1} in borate buffer, pH 7.4), racemic probe (**1**) (10 μL, 2 mmol L^{-1}), and enzymatic or cell suspension (100 μL, 0.2 g L^{-1}).

Negative control. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (80 μL, 5.0 g L^{-1} in borate buffer, pH 7.4), racemic probe (**1**) (10 μL, 2 mmol L^{-1}) and borate buffer (100 μL, pH 7.4).

Positive control. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (80 μL, 5.0 g L^{-1} in borate buffer, pH 7.4), racemic alcohol (**3**) (10 μL, 2 mmol L^{-1}), and enzymatic or cell suspension (100 μL, 0.2 g L^{-1}).

Assays without competition

Enzymatic assay. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (80 μL, 5.0 g L^{-1} in borate buffer, pH 7.4), fluorescent probe (R)-**1** or (S)-**1** (10 μL, 2 mmol L^{-1}), and enzymatic or cell suspension (100 μL, 0.2 g L^{-1}).

Negative control. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (80 μL, 5.0 g L^{-1} in borate buffer, pH 7.4), fluorescent probe (R)-**1** or (S)-**1** (10 μL, 2 mmol L^{-1}), and borate buffer (100 μL, pH 7.4).

Positive control. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (80 μL, 5.0 g L^{-1} in borate buffer, pH 7.4), alcohol (R)-**3** or (S)-**3** (10 μL, 2 mmol L^{-1}), and enzymatic or cell suspension (100 μL, 0.2 g L^{-1}).

Quick-*E* and Quick-*ee* (without competition)

Enzymatic assay. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (70 μL, 5.7 g L^{-1} in borate buffer, pH 7.4), fluorescent probe (R)-**1** or (S)-**1** (10 μL, 1 mmol L^{-1}), competitor (**2**) (10 μL, 1 mmol L^{-1}), and enzymatic or cell suspension (100 μL, 0.2 g L^{-1}).

Negative control. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (70 μL, 5.7 g L^{-1} in borate buffer, pH 7.4), fluorescent probe (R)-**1** or (S)-**1** (10 μL, 1 mmol L^{-1}), competitor (**2**) (10 μL, 1 mmol L^{-1}), and borate buffer (100 μL, pH 7.4).

Positive control. To each well were added NaIO$_4$ (10 μL, 20 mmol L^{-1} in water), BSA (70 μL, 5.7 g L^{-1} in borate buffer, pH 7.4), alcohol (R)-**3** or (S)-**3** (10 μL, 1 mmol L^{-1}), alcohol competitor (**4**) (10 μL, 1 mmol L^{-1}), and enzymatic or cell suspension (100 μL, 0.2 g L^{-1}).

Biotransformations

To the implementation of the Quick-*ee* methodology, the racemic fluorogenic probe **1** (10 μL, 2.0 mmol L^{-1} in H$_2$O/MeCN, 1:1) were added to the cell suspension (190 μL, 2.0 g L^{-1}) to reach a final concentration of 100 mmol L^{-1}. The diol enantiomeric excess (*ee*) was determined by high performance liquid chromatography (HPLC) using a CHIRALPAK-IC (Daicel: 25 cm × 0.46 cm) column, which contains a chiral stationary phase, with 6:4 ethanol:hexane as the eluent, an injection volume of 20 μL, a flow rate of 1.0 mL min^{-1} and l$_{abs}$ = 320 nm.

Calculations

$E_{estimated}$ and Quick-*E*

The E value determined in assays without competitor **2** ($E_{estimated}$) and E value determined in assays with competitor **2** (Quick-*E*) were determined from the initial reaction rates (V_0) for each enantiomer (equation 1), which were obtained from the slope of the curve of reactant concentration *versus* time (t) at t = 0 (V_0).

$$E = \frac{(V_0)_{fast\ enantiomer}}{(V_0)_{slow\ enantiomer}} \tag{1}$$

ee with competition (Quick-*ee*) and without competition (*ee*$_{estimated}$)

The enantiomeric excess values were calculated (equation 2) for each point using the fluorescence signal measurement for each assay (relative fluorescence unit, RFU).

$$ee\,\% = \frac{(RFU_{fast\,enantiomer} - RFU_{slow\,enantiomer})}{(RFU_{fast\,enantiomer} + RFU_{slow\,enantiomer})} \times 100 \quad (2)$$

% conversions

The conversions (c) were calculated at each point of the curve by taking into account the fluorescent signals of the assays and the positive control (equation 3).

$$c\,\% = \frac{(RFU_{fast\,enantiomer} + RFU_{slow\,enantiomer})}{(RFU_{positive\,control\,(fast\,enantiomer)} + RFU_{positive\,control(slow\,enantiomer)})} \times 100 \quad (3)$$

Results and Discussion

Screening of enzymes and microorganisms

For the validation of the Quick-*ee* experiments, we have selected eight enzymes [Lipase basic kit, SIGMA-ALDRICH: *Aspergillus* (84205), *Candida rugosa* (62316), *Mucor miehei* (62298), *Pseudomonas cepacia* (62309), *Pseudomonas fluorescens* (95608), *Rhizopus arrhizus* (62305), *Rhizopus niveus* (62310), *Hog pâncreas* (62300)] (Figure 1a); and four microorganisms [*Serratia liquefaciens* (CCT-1479), *Pseudomonas fluorescens* (CCT-7393), *Cunninghamella echinulata* (CCT-4259) and *Tricosporon cutaneum* (CCT-1903)] for the hydrolysis of the fluorogenic probe **1** (Figure 1b).[19,22]

The best conversions were obtained with the microorganisms *Serratia liquefaciens* (CCT-1479), *Pseudomonas fluorescens* (CCT-7393), and the lipase from *Aspergillus* (Sigma-Aldrich 84205) (Figures 1a and 1b, respectively). However, although *Serratia liquefaciens* (CCT-1479) converted the probe **1** in good yields, the enantioselectivity was poor. Thus, for implementing the methodology of Quick-*ee*, we used the microorganism *Pseudomonas fluorescens* (CCT-7393) and the enzyme lipase from *Aspergillus* (Sigma-Aldrich 84205).

Quick-*ee*

Assay conversions (c) and enantiomeric excess (*ee*) values at a specific time are parameters of fundamental importance in the kinetic characterization of an enzyme. Therefore, it is interesting to investigate new methodologies to obtain these parameters using high throughput screening.
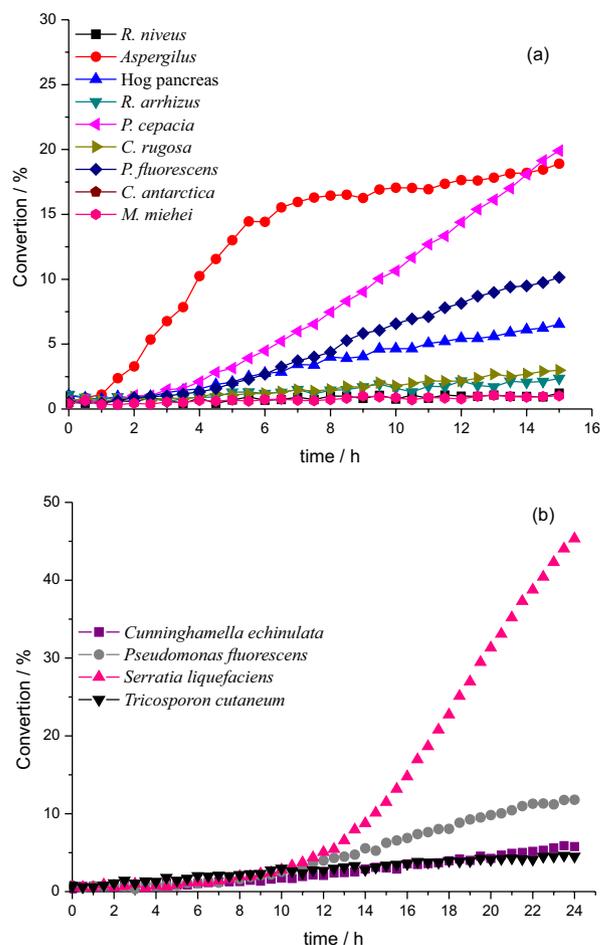
Figure 1. Enzymatic assay conversions in borate buffer (pH 7.4) containing fluorogenic probe **1** (100 mmol L^{-1}), BSA (5.0 g L^{-1}), and NaIO$_4$ (20 mmol L^{-1}) (a) enzymes or (b) cells (0.1 g L^{-1}).

Two methodologies employing fluorogenic probes were developed. The first is based on Reymond's methodology[26] and calculates *E* from the ratio between the initial rates of each enantiomer, which are evaluated separately. However, this methodology does not take into consideration the enantiomeric competition for the active site, which can produce large deviations from the real *ee*. Kazlauskas' methodology[27] of using chromogenic probes introduces competition to minimize effects resulting from the lack of competition. However, both methodologies do not reveal the *ee* and conversions at a particular reaction time. To overcome previous limitations, we fused the Reymond's and Kazlauskas's[26,27] methodologies by implementing the assay with fluorogenic probe **1** and a nonfluorogenic competitor **2** to obtain *ee* and conversion (Figures 2 and 3).

Choosing the nonfluorogenic competitor

The nonfluorogenic competitor **2** was selected by taking into consideration its structural similarity to the probe **1** (Scheme 1).
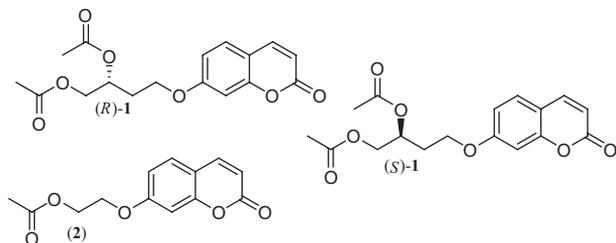
Figure 2. Fluorogenic probes (*R*)-**1** and (*S*)-**1** and non fluorogenic competitor 2 employed in Quick-*ee* assays.
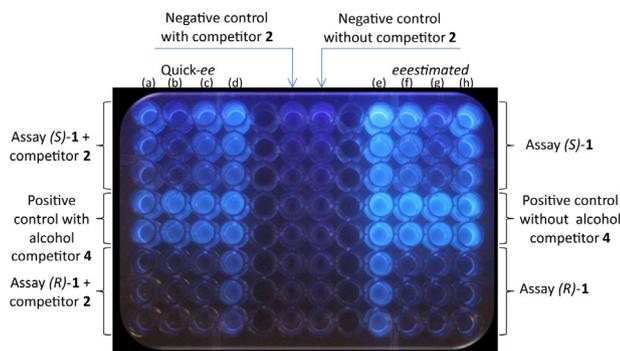
Figure. 3. Representation of microtitre plate assay to obtain *ee* with a competitor **2** (Quick-*ee*) and without a competitor **2**. Representation: (a) and (h) *Pseudomonas fluorescens* (CCT-7393); (b) and (g) *Cunninghamella echinulata* (CCT-4259); (c) and (f) lipase from *Aspergillus* (Sigma-Aldrich 84205); (d) and (e) *Serratia liquefaciens* (CCT-1479).

Thus (*R*)-**1** and **2** (or (*S*)-**1** and **2**) can compete for the hydrolase enzymatic site producing diols (*R*)-**3** and (*S*)-**3** and alcohol **4**, respectively. However diols (*R*)-**3** and (*S*)-**3** are cleaved by the periodate present in the reaction mixture, producing aldehyde **5**, which undergoes β-elimination, catalysed by BSA, producing a fluorescent signal (Scheme 1). The fluorescent signal intensity is concentration dependent and reveals the amount of umbelliferone produced.[27] The same enzymatic cascade does not occur with alcohol **4**, which is not oxidized, does

not undergo β-elimination and, consequently, does not produce a fluorescent signal (Scheme 1).

Quick-*ee* experiments

Biocatalytic hydrolyses of (*R*)-**1** and (*S*)-**1** with *Aspergillus* lipase (SIGMA ALDRICH 84205), (Figure 4a and 4b) revealed that (*S*)-**1** is preferentially hydrolysed, producing a fluorescent signal of higher intensity than (*R*)-**1** at any reaction time observed until 24 h, with and without competitor **2** (Figures 4a and 4b, respectively). The same is true for the reaction with *Pseudomonas fluorescens* (CCT-7393) cells (Figures 4c and 4d).

The real *ee* = 30 value for *Aspergillus* lipase (Sigma-Aldrich 84205) (determined by chiral HPLC, in the Supporting Information (SI) section, Figure S5) was determined at 30% conversion and the Quick-*ee* was 45 (Figure 5b) (Table 1). This value is closer to real *ee* (*ee*$_{real}$) than the estimated *ee* (*ee*$_{estimated}$ = 55), obtained without competitor (Figures 5a, 5b) (Table 1). The same is true for the reaction with *Pseudomonas fluorescens* (CCT-7393) cells (Figures 5c, 5d) (Table 1).

E values were obtained from the initial speed ratios of each enantiomer in each assay. *E* values improved in the presence of competitors (Quick-*E*) (Table 2) This is assigned to the competition of both fluorogenic probes ((*R*)-**1** and (*S*)-**1**) and its competitor (**2**) to the enzyme active site.

Conclusions

The Quick-*ee* was validated for enzymes and microorganisms and the results in the presence of a competitor were closer to real *ee* for low *E* reactions. This methodology provides an easy access evaluation of numerous samples, such as libraries of mutants and clones.

Scheme 1. Enzymatic reaction with fluorogenic probes (*S*)-**1**, (*R*)-**1** and non-fluorogenic competitor **2**.
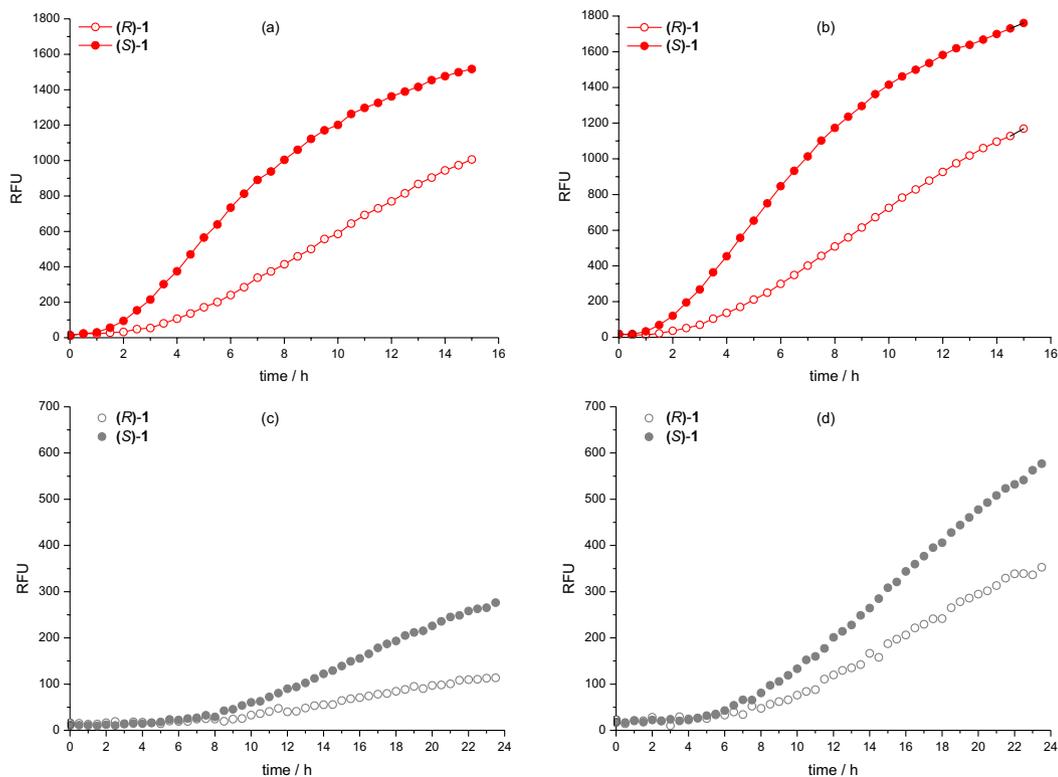
Figure. 4. Fluorescence signals from assays using the *Aspergillus* enzyme (Sigma-Aldrich 84205) (a) with competitor **2** and (b) without competitor **2**; *Pseudomonas fluorescens* (CCT-7393) microorganism (c) with competitor **2** and (d) without competitor **2**, showing the preference for (*S*)-**1**.
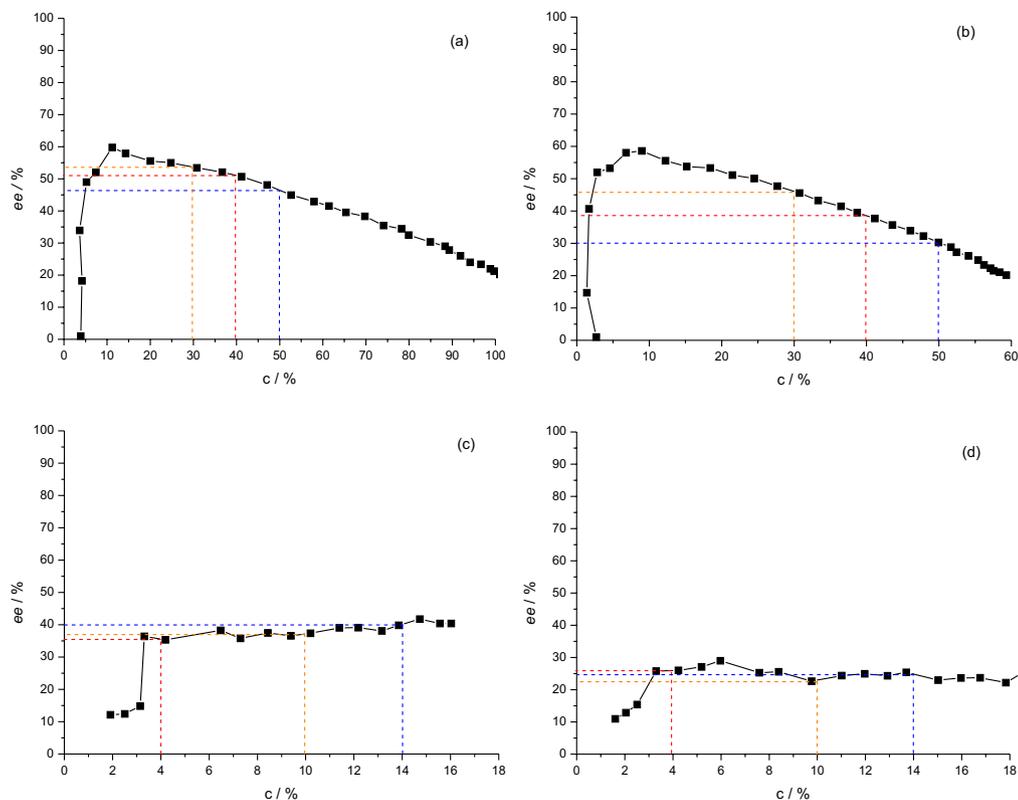
Figure 5. Enantiomeric excess and conversions with *Aspergillus* lipase (Sigma-Aldrich 84205) (a) without competitor **2** and (b) with competitor **2** (Quick-*ee*). Just like in *Pseudomonas fluorescens* (CCT-7393) whole cells (c) without competitor **2** and (d) with competitor **2** (Quick-*ee*). In both cases, minor *ee* values are observed in experiments with the competitor **2** for the same conversion level.

Table 1. *ee* values for assays without competition (*ee*$_{estimated}$), with competition (Quick-*ee*), and the real *ee* (*ee*$_{real}$)

Enzyme / Microorganism	Conversion / %[c]	*ee*$_{estimated}$[d]	Quick-*ee*[d]	*ee*$_{real}$	Deviation / % (*ee*$_{estimate}$)	Deviation / % (Quick-*ee*)	Enantioselective for
Lipase from *Aspergillus*[a]	30	55	45	30	83.3	50.0	(*S*)-**1**
Pseudomonas fluorescens[b]	4	35	27	25	40	8	(*S*)-**1**

[a]Enzyme; [b]microorganism; [c]equation 3; [d]equation 2.

Table 2. *E* values from experiments with and without competitors

Enzyme / Microorganism	*E*$_{estimated}$[c]	Quick-*E*[c]	*E*$_{real}$[d]	Deviation / % (*E*$_{estimated}$)	Deviation / % (Quick-*E*)	Enantioselective for
Lipase from *Aspergillus*[a]	3.15	2.33	2.09	50.7	11.5	(*S*)-**1**
Pseudomonas fluorescens[b]	2.61	1.56	1.67	56.3	6.6	(*S*)-**1**

[a]Enzyme; [b]microorganism; [c] $E = \dfrac{(V_0)_{fast\ enantiomer}}{(V_0)_{slow\ enantiomer}}$; [d] $E = \dfrac{\ln(1-c[1+ee_{product}])}{\ln(1-c[1-ee_{product}])}$.

Supplementary Information

Supplementary data (chromatograms of biotransformation assays) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgements

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