





Open Archive Toulouse Archive Ouverte

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible

This is an author's version published in: <http://oatao.univ-toulouse.fr/20457>

Official URL : <http://doi.org/10.1016/j.tetasy.2017.01.014>

To cite this version: Gerard, Doriane  and Guérout, Marc and Casas-Godoy, Leticia and Condoret, Jean-Stéphane  and André, Isabelle and Marty, Alain and Duquesne, Sophie *Efficient resolution of profen ethyl ester racemates by engineered Yarrowia lipolytica Lip2p lipase.* (2017) Tetrahedron: Asymmetry, 28 (3). 433-441. ISSN 0957-4166

Any correspondence concerning this service should be sent to the repository administrator: tech-oatao@listes-diff.inp-toulouse.fr

Efficient resolution of profen ethyl ester racemates by engineered *Yarrowia lipolytica* Lip2p lipase

Doriane Gérard^{a,b,c}, Marc Guéroult^a, Leticia Casas-Godoy^a, Jean-Stéphane Condoret^{b,c}, Isabelle André^a, Alain Marty^{a,*}, Sophie Duquesne^{a,*}

^a LISBP, Université de Toulouse, CNRS, INRA, INSA, Toulouse, France

^b University of Toulouse, INPT, UPS Laboratoire de Génie Chimique, Toulouse, France

^c CNRS, UMR 5503, F-31062 Toulouse, France

ARTICLE INFO

Article history:

Received 22 December 2016

Revised 9 January 2017

Accepted 17 January 2017

Available online 23 February 2017

ABSTRACT

Enzyme-catalyzed enantiomer discrimination is still a great challenge for the development of industrial pharmaceutical processes. For the resolution of ibuprofen, naproxen and ketoprofen racemates, three major anti-inflammatory drugs, only lipases from *Candida rugosa* present a high selectivity if solvent and surfactant use is discarded. However, their catalytic activities are too low. In the present work, we demonstrate that the lipase Lip2p from the yeast *Yarrowia lipolytica* has a higher catalytic activity than *C. rugosa* lipases to hydrolyze the ethyl esters of ibuprofen, naproxen and ketoprofen, but its selectivity is not sufficient [$E = 52$ (*S*); 11 (*S*) and 1.5 (*R*) respectively]. The enantioselectivity was further improved by site-directed mutagenesis, targeted at the substrate binding site and guided by molecular modelling studies. By investigating the binding modes of the (*R*)- and (*S*)-enantiomers in the active site, two amino acid residues located in the hydrophobic substrate binding site of the lipase, namely residues 232 and 235, were identified as crucial for enantiomer discrimination and enzyme activity. The (*S*) enantioselectivity of Lip2p towards ethyl ibuprofen esters was rendered infinite ($E \gg 300$) by replacing V232 by an A or C residue. Substitution of V235 by C, M, S, or T amino acids led to a great increase in the (*S*)-enantioselectivity ($E \gg 300$) towards naproxen ethyl ester. Finally, the variant V232F enabled the efficient kinetic resolution of ethyl ketoprofen ester enantiomers [(*R*)-enantiopreference; $E \gg 300$]. In addition to the increase in selectivity, a remarkable increase in velocity by 2.6, 2.7 and 2.5 times, respectively, was found for ibuprofen, naproxen and ketoprofen ethyl esters.

1. Introduction

α -Substituted aryl and alkyl carboxylic acids are important intermediates encountered in the synthetic pathways of numerous drugs, such as prostaglandin, prostacyclin, semi-synthetic penicillin and thiazolium salts. Among them, derivatives of 2-arylpropionic acids, 2-(4-isobutylphenyl)propionic acid, commonly known as ibuprofen, 2-(6-methoxy-2-naphthyl)propionic acid, also called naproxen, and 2-(3-benzoylphenyl)propionic acid, commonly named ketoprofen are regularly used as non-steroid anti-inflammatory drugs (NAIDs) in the treatment of headaches, rheumatoid arthritis, cephalalgia or muscular cells.¹

All of these molecules have a stereogenic centre at the α -position of the carboxylic function, leading to the co-existence of two enantiomers. In most cases, only one enantiomer has the required biological activity. For instance, the (*S*)-enantiomer of ibuprofen is 160 times more active than its (*R*)-counterpart.^{2,3} Similarly, the (*S*)-enantiomer of naproxen is 28 times more active than the (*R*)-form.⁴ The anti-inflammatory properties of ketoprofen are principally due to the (*S*)-enantiomer, whereas the (*R*)-enantiomer has side effects.⁵ The use of only the active enantiomer is thus often privileged whenever it can be obtained in a pure form. Therefore, a major challenge consists of separating these enantiomers to obtain a pure biologically active substance. Classical methods used to obtain pure enantiomers, such as chemical asymmetric synthesis, stereoselective crystallization or chiral chromatography, are usually expensive. The use of highly enantioselective enzymes (enantioselectivity value, E , ratio of reaction rates for both enantiomers higher than 200) is thus an appealing alternative to separate such enantiomers.

* Corresponding authors at: Laboratoire d'Ingénierie des Systèmes Biologiques et Procédés, Institut National des Sciences Appliquées, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France. Tel.: +33 (0)5 61 55 94 39; fax: +33 (0)5 61 55 94 00.

E-mail addresses: alain.marty@insa-toulouse.fr (A. Marty), sophie.duquesne@insa-toulouse.fr (S. Duquesne).

Several enzymatic routes have been explored for obtaining enantiopure profens and profenols,⁶ such as use of alcohol dehydrogenases,^{7–10} arylmalonate decarboxylases, ene reductases¹¹ and nitrilases, either based on the kinetic resolution of racemates or asymmetric resolution of prochiral precursors.

In the case of 2-arylpropionic acids, lipases have been shown to be good candidates to catalyze the kinetic resolution of (*RS*)-racemates, either by hydrolysis of an ester or by esterification of the acid form. Several lipases from various origins (plant and microbial) have already been reported for the resolution of ibuprofen, naproxen and ketoprofen esters or the corresponding acids.

Lipases from *Rhizomucor miehei*,¹² *Carica papaya*¹³ or evolved *Candida antarctica* lipase,^{14,15} were previously tested for the resolution of (*RS*)-ibuprofen racemate by either hydrolysis or esterification reactions. However, all three of them led to low enantioselectivity ($E < 200$). The lipase from *C. rugosa* (formerly *C. cylindracea*) was shown to be the best enzyme to discriminate (*RS*) ibuprofen racemate.^{16–28} The best result, considering the enantioselective hydrolysis of ibuprofen racemate using free enzyme, presents an enantioselectivity of 247.¹⁶ Nevertheless, this high E value was obtained by the addition of *N,N*-dimethylformamide, which is highly toxic.

C. rugosa, *C. papaya* and *R. miehei* free lipases were also studied for the hydrolysis of naproxen ester racemate.^{13,17,29–35} Among them, lipases from *C. rugosa* were found to be the most enantioselective enzymes for this reaction ($E = 397$).³² Lysophospholipase and carboxylesterase were also studied for the resolution of the naproxen ester.^{36,37} The carboxylesterase NP produced by *Bacillus subtilis* gave a high enantioselectivity ($E = 500$), nevertheless, this high enantioselectivity was obtained by the addition of formaldehyde, which is highly toxic.³⁷

Lipases from *Thermogota maritima*,³⁸ *Aspergillus niger*, *Aspergillus terreus*, *Fusarium oxysporum*,³⁹ *Mucor javanicus*,^{40,41} *R. miehei*,^{40,41} *Trichosporon laibacchii*,⁴² *Pseudomonas cepacia*,⁴ *C. papaya*,¹³ *B. subtilis*⁴³ and from *C. rugosa*¹⁹ were previously tested for the resolution of (*RS*)-ketoprofen racemate. However, all of them led to poor enantioselectivity ($E < 200$). In order to improve upon the enantioselectivity of the lipase from *C. rugosa* ($E = 27$),¹⁹ various strategies were employed, such as enzyme immobilization^{44–46} or two step acetone treatment¹⁸ but only an enantioselectivity of 153 was reached.⁴⁴

The enantioselectivity of *Serratia marcescens* lipase was improved (from 63 to 1084) by the addition of a surfactant Brij 92V.⁴⁷ Similarly, the enantioselectivity of *Pseudomonas* sp. KCTC10122BP lipase⁴⁸ and *Acinetobacter* lipase⁴⁹ were found to be superior to 200 (absolute and 752, respectively) but with use of triton X-100. However, addition of a surfactant generally leads to a complexification of the purification process, and to high production costs.

Variants of *C. Antarctica* lipase CalB,¹⁴ and of the recently metagenome-isolated esterase Est25⁵⁰ enabled an enantioselectivity higher than 200, but very low concentrations of the substrate were used. It appears that the lipases from *C. rugosa* are good candidates for the resolution of the three substrates considered. Despite their significant enantioselectivity, the catalytic efficiencies of the lipases from *C. rugosa* are relatively low, almost one order of magnitude lower than that of Lip2p lipase from the oleaginous yeast *Y. lipolytica*^{51,52} during the resolution of 2-bromophenyl acetic acid ester.

Herein we tried to identify new enzymes able to highly discriminate between the (*R*)- and (*S*)-enantiomers of ibuprofen, naproxen and ketoprofen ethyl esters, and with high catalytic efficiency. The *Y. lipolytica* Lip2p lipase was reported to catalyze the resolution of ibuprofen racemate with a low enantioselectivity ($E = 56$).⁵³ Moreover, variants of this lipase have been shown to have a high catalytic efficiency and enantioselectivity for the resolution of

(*RS*)-2-bromophenyl acetic acid ester racemate.^{52,54} Enzyme variants with increased or totally inverted enantioselectivity, concomitant with a remarkable increase in velocity, were also obtained.⁵⁵ The performances of the *Y. lipolytica* wild-type lipase is compared to wild-type lipases (CRL 1 and 4) from *C. rugosa*, which has been described in the literature as being one of the most efficient enzymes for the resolution of the considered racemates.^{16,19,34,44} In view of the results, the library of Lip2p variants from *Y. lipolytica*, previously built for the resolution of the (*RS*)-2-bromophenyl acetic acid ester racemate, was tested on the three molecules and molecular modelling was used to identify new targets for site-directed mutagenesis and to understand the role of amino acid changes on the selective recognition of the (*RS*)-enantiomers of each racemate.

2. Results and discussion

2.1. Resolution of (*RS*)-ibuprofen naproxen and ketoprofen ethyl ester racemates using wild-type lipases

The reaction scheme of the enantioselective lipase-catalyzed hydrolysis of the racemic mixture of three different esters is presented in Figure 1. Wild-type Lip2p from *Y. lipolytica* was compared to *C. rugosa* lipases (CRL 1 and 4), the most efficient enzymes known to date for the resolution of ibuprofen, naproxen and ketoprofen racemates. CRL lipases were produced in recombinant form by a strain of *Y. lipolytica* as pure isoform of CRL1 and CRL4.⁵¹

All three lipases were produced using the *Y. lipolytica* strain JMY1212,⁵⁶ in which the lipase encoding gene is introduced in the genome at the zeta docking platform, leading to good reproducibility of the enzyme expression and enabling a comparison of enzyme variant activities directly from the supernatant.⁵⁵ In addition, this strain is deleted for the main extracellular protease (XPR2) and the main extracellular lipases (Lip2p, 7 and 8), which in turn enables for the supernatant to be obtained with high protein purity.^{57–59} It was checked that no activity with this strain was obtained whatever the used racemate (data not shown).

The protein contents of *Y. lipolytica* supernatant containing Lip2p, CRL1 or CRL4 were compared by SDS page (data not shown). Enzyme concentration was estimated as 5 times lower for CRL1 and CRL4 expressed in *Y. lipolytica* compared with Lip2p. The two enzymes of *C. rugosa* were then 10 times concentrated for comparison. Enzymes were then tested during the hydrolysis of ibuprofen, naproxen and ketoprofen ethyl esters (Table 1).

Our results confirmed that *C. rugosa* lipase CRL1 was an efficient enzyme with regards to the enantioselectivity to discriminate between the enantiomers of ibuprofen, naproxen and ketoprofen ethyl esters, with a total preference for the (*S*)-enantiomer. Nevertheless, whatever the racemate and even with a 10 times concentration of the supernatant, the rate of reaction was low. Despite sharing 81 % of identity with CRL1, lipase CRL4 was only active on ketoprofen ethyl ester with a total preference for the (*R*)-enantiomer. This reverse enantioselectivity of CRL1 and CRL4 was already observed during the resolution of bromophenyl acetic acid ester racemates.⁵¹

Wild-type Lip2p lipase from *Y. lipolytica* showed a clear preference for the (*S*)-enantiomer in the hydrolysis of ibuprofen ethyl ester racemate ($E = 52$, Table 1). This result is in agreement with the E value of 56 obtained in previous studies⁵³ of esterification of ibuprofen using immobilized Lip2p lipase. The positive influence of the *para* substitution of the phenyl group on the E value of Lip2p lipase had already been observed for the transesterification of 2-bromo-phenyl acetic ethyl and 2-bromo-*p*-tolylacetic ethyl ester.⁶⁰ The presence of a methyl group at the *para*-position of the phenyl group led to an improvement in the selectivity of Lip2p lipase

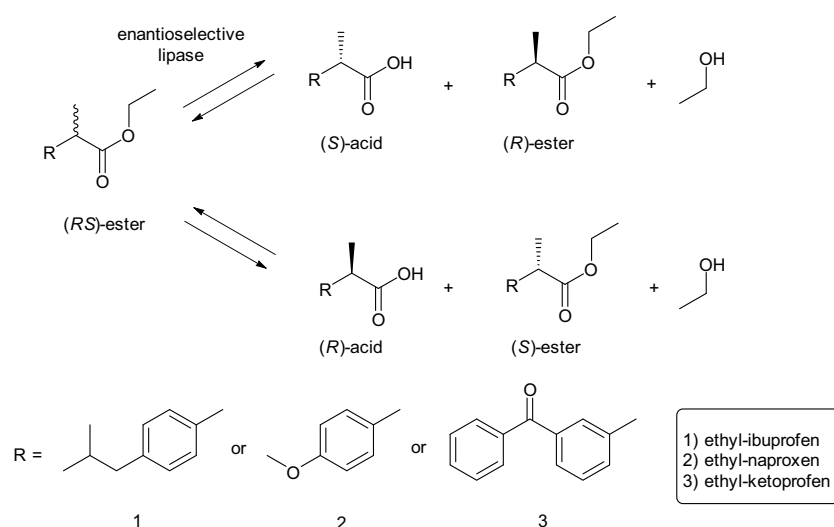


Figure 1. Reaction scheme of the lipase-catalyzed resolution of (*RS*)-ethyl ester racemate of ibuprofen, naproxen and ketoprofen by a (*S*)- or (*R*)-enantioselective lipase (up and down reaction, respectively).

Table 1

Comparison between activities and selectivities of Lip2p from *Y. lipolytica* and *C. rugosa* lipases for the hydrolysis of ethyl-ibuprofen, ethyl-naproxen and ethyl-ketoprofen. The hydrolysis was performed in a biphasic medium: 750 μL of decane containing 50 mM of ester racemate, 750 μL of enzyme supernatant (or the concentrate supernatant for CRL lipases), stirred at room temperature for 100 h

	Ethyl ibuprofen			Ethyl naproxen			Ethyl ketoprofen		
	viS ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	viR ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	<i>E</i> (viS/viR)	viS ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	viR ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	<i>E</i> (viS/viR)	viS ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	viR ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	<i>E</i> (viS/viR) or (viS/viR)
CRL1 10X	0.28	0	>200 (<i>S</i>)	0.15	0	>200 (<i>S</i>)	0.06	0	>200 (<i>S</i>)
CRL4 10X	0	0	Nd	0	0	Nd	0	0.99	>200 (<i>R</i>)
Lip2p 1X	1.56	0.03	52 (<i>S</i>)	0.42	0.04	11 (<i>S</i>)	0.11	0.16	1.5 (<i>R</i>)

Nd = not determined.

towards the (*S*)-enantiomer with an *E* value of 28 against an *E* value of 3 in the absence of the methyl group.⁶⁰ The presence of an isobutyl group at the *para* position of the phenyl group in ibuprofen ester seemed to have the same effect.

The steric hindrance of the naproxen ethyl ester in the Lip2p lipase active site is clearly more important than that of 2-bromophenyl-acetic and ibuprofen ethyl esters, due to the presence of the naphthalene group. As a consequence, the rate of hydrolysis of the preferred (*S*)-enantiomer by wild-type Lip2p is 4 times lower than the one observed for (*S*)-ibuprofen ethyl ester (Table 1). Conversely, the poorly-hydrolyzed (*R*)-enantiomer was slightly better recognized, leading to an enantioselectivity 5 times lower (*E* = 11).

The rate of hydrolysis of the (*S*)-enantiomer of ethyl ketoprofen by wild-type Lip2p is 4 times lower than the one observed for the (*S*)-naproxen ethyl ester, and 16 times lower compared to the (*S*)-ibuprofen ethyl ester. The (*R*)-enantiomer of ethyl ketoprofen was the best recognized enantiomer by Lip2p (*E* = 1.5). Moreover, the catalytic efficiency of Lip2p for the best recognized enantiomer of ibuprofen, naproxen and ketoprofen ethyl ester is one order of magnitude higher than the one obtained with CRL1. Only CRL4, during the ethyl ketoprofen ester resolution, possessed a catalytic efficiency in the same range to that observed with Lip2p.

2.2. Resolution of (*RS*)-ibuprofen ethyl ester racemate by Lip2p enzyme variants

With the aim of improving the enantioselectivity of the Lip2p lipase from *Y. lipolytica* for the resolution of the (*RS*)-ibuprofen ethyl ester racemate, a previously built library of enzyme variants^{54,55} was screened. This library of single enzyme variants

results from a rational engineering strategy that consisted in applying site-directed mutagenesis to 5 amino acids (T88, V94, V285, V232 and D97) located in the substrate binding site. Among these Lip2p variants, position 232 was found to be crucial in terms of the activity and the enantioselectivity during the resolution of 2-bromophenyl acetic acid esters.⁵⁴ In total, 25 single enzyme variants of Lip2p were tested for their ability to resolve the ibuprofen ethyl ester racemate (19 single variants at position 232 and enzyme variants D97A, T88S, V94A, V94L, V285A, and V285L). A double substituted enzyme variant (D97A-V232F), with highly inverted enantioselectivity towards 2-bromo phenyl acetic acid esters⁵⁵ was also tested. It was checked by SDS page gel protein that all of the variants tested are expressed at the same level than the wild-type enzyme (data not shown).

Enzyme variant T88S and enzyme variants at position 94 present lower activity than the wild-type Lip2p and no change in selectivity. Enzyme variant V285A presents a similar behavior as the wild-type enzyme, whereas V285L and D97A variants are no more active. The double substituted enzyme variant D97A-V232F was not active on ethyl ibuprofen, whereas it displayed a total preference for the (*R*)-enantiomer of 2-bromophenyl-acetyl octyl ester and 4.5 times enhancement of its activity.⁵⁵

As already observed during the kinetic resolution of 2-bromo-arylacetic acid esters, position 232 appears to be crucial for the discrimination between ibuprofen enantiomers. Indeed, three Lip2p variants with an amino acid change at this position 232 (V232A, V232C and V232S) present a higher enantioselectivity than the wild-type enzyme. All of them showed an improvement in enantioselectivity, from an *E* value of 52 for the wild-type enzyme to an *E* value higher than 200 for the enzyme variants; this was due

to a better hydrolysis of the preferred (*S*)-enantiomer (2.2–2.6 times higher) concomitant to a lower hydrolysis of the poorly recognized (*R*)-enantiomer (Table 2). Enzyme variants V232A and V232C were no more able to recognize the (*R*)-enantiomer, whereas enzyme variant V232S exhibits only a lower recognition of the poorly recognized (*R*)-enantiomer (2.3 times lower than wild-type Lip2p). In addition to the gain in enantioselectivity, a remarkable increase in velocity was observed for the three variants (2.2–2.6 times increase). The best enzyme was the enzyme variant V232A ($E \gg 300$ with an initial rate of hydrolysis of the (*S*)-enantiomer of $4.1 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$).

During the resolution of 2-bromo-phenyl-acetic octyl esters by Lip2p lipases, the best enzyme variant was found to be the V232S enzyme variant,⁵⁴ with an E value $\gg 200$.

From molecular docking studies, it was assumed that the bulky bromine atom, facing V232 for the (*S*)-enantiomer, was involved in the discrimination of the enantiomers. The size of this amino acid thus appeared to be crucial for the enantioselectivity. It is noteworthy that ibuprofen and 2-bromophenyl-acetic esters are rather similar in structure. The bromine atom present at the α -position of the carboxylic acid in 2-bromophenyl-acetic ester is replaced in ibuprofen by a methyl group, which presents similar steric hindrance. From our results, it appears that the size of the amino acid at position 232 is clearly more important than its polarity. Substitution of the valine present in the wild-type Lip2p lipase by smaller amino acid residues such as A, C or S enables a better recognition of the (*S*)-enantiomer and a poorer recognition of the (*R*)-enantiomer. However, enzyme variant V232G is no more active on ethyl ibuprofen whereas it was found to be more efficient and selective during the hydrolysis of 2-bromophenyl-acetic octyl esters.⁵⁴ As already observed with 2-bromophenyl-acetic octyl ester, the performance of the enzyme variant V232T is similar to that of the wild-type enzyme. Despite the low catalytic efficiency, enzyme variants V232L, V232I, V232F exhibited enantioselectivity inversion during 2-bromophenyl-acetic octyl ester resolution.⁵⁴ Replacement of the valine by a bulkier amino acid such as L, I or F led to enzyme inactivation in the kinetic resolution of ibuprofen.

Using molecular modelling techniques, we constructed three-dimensional models of the covalent intermediates of Lip2p with the (*RS*)-enantiomers of ibuprofen ethyl ester (Fig. 2).

Docking studies revealed distinct binding conformations for the (*RS*)-enantiomers, which differed in the orientation of the aromatic ring, thus impacting the formation of hydrogen bonding interactions between ibuprofen ethyl ester and the oxyanion hole defined by L163 and T88. Only one hydrogen bond was found between the (*R*)-enantiomer of ibuprofen ethyl ester and oxyanion residues of wild-type Lip2p, whereas two hydrogen bonds were observed for the (*S*)-enantiomer (Fig. 3).

This could provide some explanation regarding the (*S*)-enantioselectivity of wild-type Lip2p toward kinetic resolution of the ibuprofen ethyl ester. Mutation of V232 by smaller amino acid residues such as A, C or S, provides more space to better accommodate the (*S*) enantiomer of ibuprofen ethyl ester in the catalytic site

Table 2
Comparison of activity and selectivity of wild-type Lip2p lipase from *Y. lipolytica* and its variants during hydrolysis of (*RS*)-ibuprofen ethyl ester racemate. The hydrolysis was performed in a biphasic medium: 750 μL of decane containing 50 mM of ester racemate, 750 μL of enzyme supernatant (or the concentrate supernatant for CRL lipases), stirred at room temperature for 100 h

	viS ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	viR ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	E (viS/viR)
Wild-type V232	1.56	0.030	52
V232A	4.07	0	>300
V232C	3.55	0	>300
V232S	3.44	0.013	263

(Fig. 2B), with a slight reorientation of the aromatic ring that favors the formation of; (i) hydrogen bonding interactions between residues from the oxyanion hole and the carbonyl group from the ethyl ester (Fig. 3); and (ii) additional van der Waals interactions between I231 and the isobutyl group. Conversely, mutation of V232 did not alter binding mode of the (*R*)-enantiomer in comparison with that observed in wild-type enzyme.

2.3. Resolution of (*RS*)-naproxen ethyl ester racemate by Lip2p enzyme variants

The same strategy used for the resolution of ibuprofen ethyl ester racemate was applied for the resolution of naproxen ethyl ester racemate. The same library of 25 enzyme variants of Lip2p lipase from *Y. lipolytica* was tested. As previously observed for ibuprofen, an amino acid change at positions 88, 94, 97 and 285 had either a neutral or detrimental effect on the enzyme activity and selectivity. Changing V232 by smaller amino acid residues such as A, C or S did not improve either the activity or the selectivity of the enzyme. The hydrolysis rate of the preferred (*S*)-enantiomer was even lower than that of the wild-type Lip2p lipase (data not shown). If a bulkier amino acid residue, such as a L, I or F, was introduced at this position, the activity was completely lost. In conclusion, no improvement in either the activity or in the enantioselectivity, was obtained using this library of enzyme variants.

In order to provide structural insight to improve upon selectivity and activity of Lip2p lipase toward the resolution of naproxen ethyl ester racemate, a molecular modelling study was carried out. The energy-minimized covalent intermediates of Lip2p with the (*RS*)-enantiomers of naproxen ethyl ester were built. Molecular docking was carried out to explore accessible conformational space of both enantiomers in the active site pocket. The top scoring fits identified for each enantiomer were then compared (Fig. 4A). The binding mode of the (*R*)-enantiomer of naproxen ethyl ester was found to be similar to that observed for the (*R*)-ibuprofen ethyl ester. Conversely, the binding mode of the (*S*)-enantiomer was found to be significantly different from that observed for ibuprofen ethyl ester. In this case, the bulkier naphthalene moiety binds in a small pocket near V235. It thus appears that introduction of an amino acid mutation at this position could help further improve the selective recognition of the (*S*)-enantiomer.

On the basis of this computational study, position 235 was mutated by all 19 possible amino acids. It was checked by SDS PAGE that all the variants tested are expressed at the same level than the wild-type enzyme (data not shown).

This position 235 was found to be crucial for the activity and enantioselectivity of the enzyme during the naproxen ethyl esters hydrolysis (Table 3). Indeed, eight enzyme variants presented both an increase in activity and enantioselectivity.

The enantioselectivity was shown to increase due to a concomitant increase in the hydrolysis rate of the preferred (*S*)-enantiomer and decreased in the hydrolysis rate of the poorly-recognized (*R*)-enantiomer. One enzyme variant, namely V235L, exhibited a spectacular increase for the hydrolysis rate of the (*S*)-enantiomer (6.5 times higher than the activity of the wild-type enzyme). Nevertheless, since its initial rate of hydrolysis of the (*R*)-enantiomer was also higher, the resulting E value is only twice the E value of wild-type Lip2p lipase. Enzyme variants V235C, V235M, V235S and V235T were no more able to recognize the (*R*)-enantiomer, leading to an $E \gg 300$. The two best enzyme variants isolated were V235C and V235S, which exhibited a 2.7 times increase in activity for the best recognized (*S*)-enantiomer and an E value higher than 200.

Moreover, to confirm that the (*R*)-enantiomer of the naproxen was absolutely not hydrolyzed by the Lip2p V235S variant, a first hydrolysis of the racemic mixture was conducted with this variant

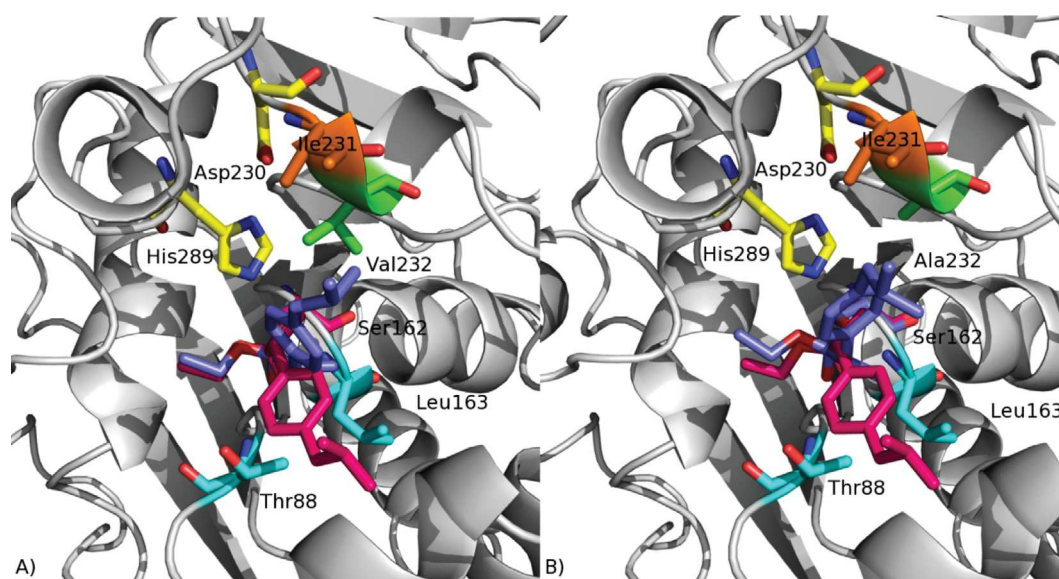


Figure 2. Representation of (*R*)- and (*S*)-ibuprofen ethyl ester enantiomers covalently bound to catalytic S162 of wild-type Lip2p (A) and V232A enzyme variant (B). The (*S*) (blue) and (*R*) (magenta) enantiomers are shown. The catalytic triad (D230, S162, H289) is shown as yellow sticks, residues forming the oxanion hole (L163 and T88) are colored in cyan, amino acid residue at position 232 is colored in green, and I231 in orange. For clarity, hydrogens are not represented.

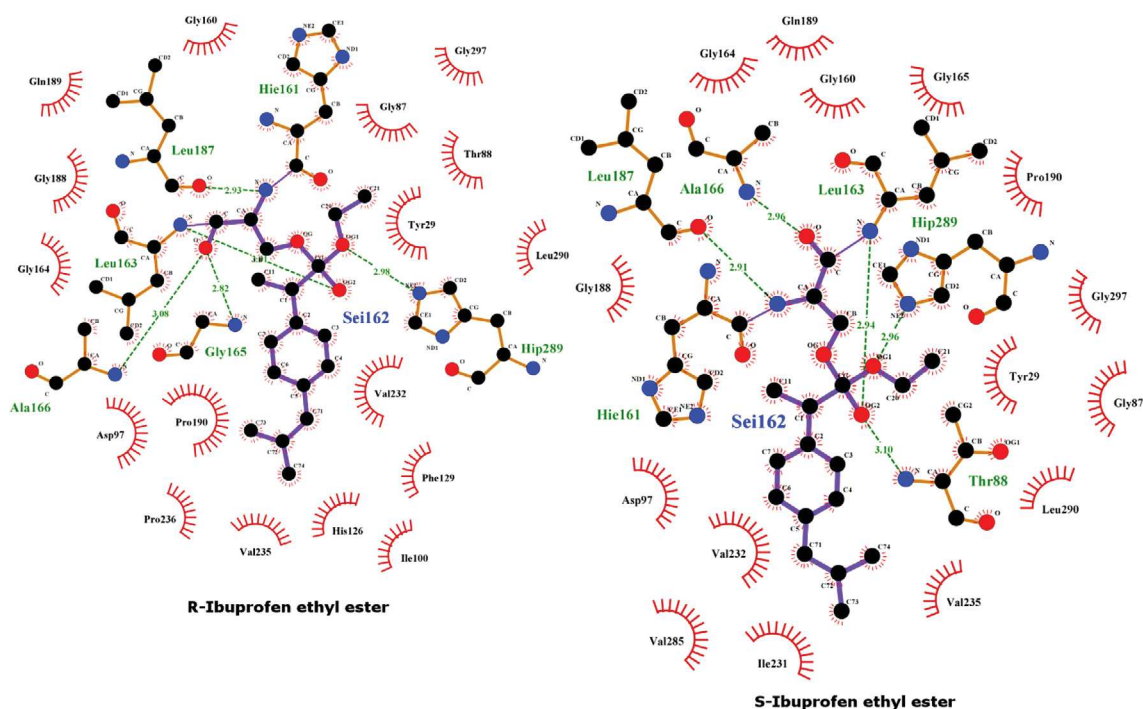


Figure 3. Representation of interactions between (*R*)- or (*S*)-ibuprofen ethyl ester with wild-type Lip2p enzyme. The drawing has been performed using Ligplot.

and the organic phase was recovered after 60 h reaction. This organic phase, which was almost only composed of the (*R*)-enantiomer of the naproxen, was put into contact with fresh enzyme (Lip2p V235S). No hydrolysis of the (*R*)-enantiomer was observed over a period of 50 h.

Detailed analysis of molecular modelling results indicated that substitution of V235 by a small and polar amino acid residue such as S, T or C, favors the formation of an additional hydrogen bonding interaction between the amino acid side chain at position 235 and the oxygen of the methoxy group from the (*S*)-enantiomer

(Fig. 4B), leading to an increase of the affinity for the (*S*)-enantiomer over the (*R*)-enantiomer and thus an enhanced enantioselectivity, as confirmed by experimental data.

The enzyme variants at the position V235 were tested for the resolution of ethyl ibuprofen; these enzyme variants have a low activity compared to the wild type Lip2p. For example, the enzyme variant V235S and V235C are 7 and 15 times less active than the wild type enzyme, respectively. The same negative result was obtained during the resolution of the (*RS*)-2-bromophenyl acetic acid ester racemate.

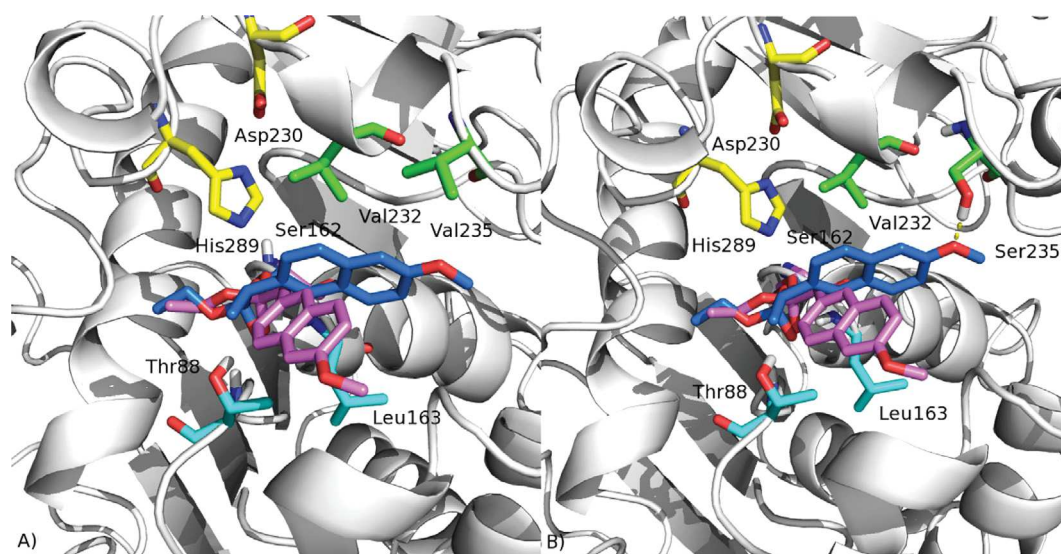


Figure 4. Representation of (*R*)- and (*S*)-naproxen ethyl ester enantiomers covalently bound to catalytic S162 of (A) wild-type Lip2p and (B) Lip2p V235S variant. The (*S*) (blue) and (*R*) (magenta) enantiomers are shown; the catalytic triad is shown in yellow, residues forming the oxyanion hole are colored in cyan and residues 232 and 235 are colored in green. For clarity, hydrogens are not represented.

Table 3

Comparison of activity and selectivity of wild-type Lip2p and the best enzyme variants on position V235 during hydrolysis of (*RS*)-naproxen ethyl ester racemate. The hydrolysis was performed in a biphasic medium: 750 μL of decane containing 50 mM of ester racemate, 750 μL of enzyme supernatant (or the concentrate supernatant for CRL lipases), stirred at room temperature for 100 h

	V_{iS} ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	V_{iR} ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	E (v_{iS}/v_{iR})
Wild-type V235	0.42	0.04	11
V235A	1.10	0.004	274
V235C	1.09	0	>300
V235E	1.31	0.02	60
V235L	2.72	0.14	19
V235M	0.75	0	>300
V235N	1.01	0.02	67
V235S	1.12	0	>300
V235T	0.71	0	>300

2.4. Resolution of (*RS*)-ketoprofen ethyl ester racemate by Lip2p enzyme variants

The two combined libraries used previously were tested. Enzyme variant T88S, V285A and enzyme variants at position V94A had lower activity than the wild-type Lip2p and no change in selectivity. Enzyme variant V285L, D97A are not more active. The two positions V232 and V235, were found to be crucial for both the activity and enantioselectivity of the enzyme towards ethyl ketoprofen (Table 4). Substitution of the valine in position 232 present in the wild-type Lip2p by a smaller amino acid residue such as S (and to a lesser extent A) enables a better recognition of the (*S*)-enantiomer (6 times) and a poorer recognition of the (*R*)-enantiomer (3 times), resulting in an inversion of preferred enantiomer. The single enzyme variant V235A and mainly V235G present the same behavior with an inversion of the preferred enantiomer and a medium enantioselectivity. Nevertheless, the activity of the V235G variant is twice as high than the one obtained with variant V232S. The double enzyme variant V232S-V235G was constructed but the kinetics and enantioselectivity became lower than the wild-type lipase.

Regarding the enantioselective hydrolysis of ketoprofen ethyl ester, the best enzyme was the enzyme variant V232F ($E \gg 300$ with an initial rate of hydrolysis of the (*R*)-enantiomer of $0.4 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$). As observed during the 2-bromo-arylacetic

acid ester resolution, a substitution of valine 232 by a bulky F annihilates the conversion of the (*S*)-enantiomer whereas the recognition for the (*R*)-enantiomer increases (Table 4 and Fig. 5). The double mutation D97A-V232F was interesting in the case of the resolution of the 2-bromo-arylacetic acid ester,⁶¹ but in this case the double mutation is useless, since the monoenzyme variant V232F presents the same enantioselectivity while the rate is slightly higher for the monoenzyme variant (Table 4).

3. Conclusion

Herein we have identified new enzymes for the kinetic resolution of ibuprofen, naproxen and ketoprofen racemates, three molecules of interest for the pharmaceutical industry. Until now, only lipases from *C. rugosa* have been reported to be efficient for this purpose if we exclude solvent and surfactant use. However, if their enantioselectivity is high, their catalytic efficiency remains very low. Herein, the lipase Lip2p, efficiently extracellularly produced by the yeast *Y. lipolytica*, was shown to have reasonable selectivity and one order of magnitude higher activity than the lipase CRL1 from *C. rugosa* during hydrolysis of ibuprofen, naproxen and ketoprofen ethyl esters. The enantioselectivity of this Lip2p was improved by site-directed mutagenesis experiments targeted to the substrate binding site, guided by molecular modelling based on the molecular docking of the (*R*)- and (*S*)-enantiomers of the substrates in the active site. The nature of the amino acids at positions 232 and 235, localized in the hydrophobic substrate binding site, were identified as being crucial for the enantioselectivity of the resolution of these three racemates. The enantioselectivity of Lip2p towards ethyl ibuprofen was increased from 52 for the wild-type enzyme to an enantioselectivity higher than 300 for the variants V232A and V232C. Mutations of V235 to C, M, S, or T lead to a tremendous increase in enantioselectivity, from 11 to an $E \gg 300$ for the resolution of naproxen ethyl ester racemate. One variant of a lipase from *Y. lipolytica* with an infinite enantioselectivity was found (V232F) with an (*R*)-enantioselectivity for the resolution of ethyl ketoprofen ester racemate, while the wild-type lipase had a poor enantioselectivity of 1.5. In addition to the gain in selectivity, a remarkable increase in velocity was demonstrated (at least 2.5 times increase) for all substrates. These results demonstrate the high potential of rational engineering to create

Table 4

Comparison of activity and selectivity of wild-type Lip2p lipase from *Y. lipolytica* and its variants during hydrolysis of (*RS*)-ketoprofen ethyl ester racemate. The hydrolysis was performed in a biphasic medium: 750 μ L of decane containing 50 mM of ester racemate, 750 μ L of enzyme supernatant (or the concentrate supernatant for CRL lipases), stirred at room temperature for 100 h

	viS ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	viR ($\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$)	E (viS/viR) or (viR/viS)	
Wild-type	0.11	0.16	1.5	(R)
V232A	0.11	0.04	2.91	(S)
V232F	0	0.40	>300	(R)
V232S	0.7	0.05	14	(S)
V235A	1.6	0.13	12	(S)
V235G	1.17	0.07	16	(S)
D97A-V232F	0	0.36	>300	(R)

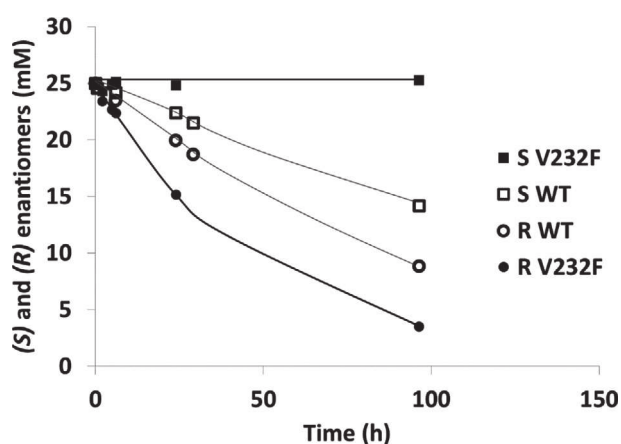


Figure 5. Hydrolysis kinetics of (*RS*)-ketoprofen ethyl ester in a biphasic medium (water:decane, 50:50, v/v) at ambient temperature. Wild-type lipase activity towards the (*S*)- and (*R*)-enantiomers and variant V232F lipase activity towards the (*S*)- and (*R*)-enantiomers. The hydrolysis was performed in a biphasic medium: 750 μ L of decane containing 50 mM of ester racemate, 750 μ L of enzyme supernatant (or the concentrate supernatant for CRL lipases), stirred at room temperature for 100 h.

new biocatalysts with enhanced activity and selectivity, suitable for industrial applications.

4. Experimental

4.1. General

Peptone, tryptone, and yeast extract were purchased from Difco (Paris, France), oleic acid from Prolabo (Fontenay sous Bois, France). Racemic ibuprofen, racemic ketoprofen, (*S*)-naproxen and other chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

4.2. Racemization of (*S*)-naproxen

The protocol used for the racemization of the (*S*)-naproxen is described in earlier studies.^{31,32}

4.3. Synthesis of ibuprofen, naproxen and ketoprofen ethyl ester

The reactant (2.5 g of racemic ibuprofen, naproxen or ketoprofen) was mixed with EtOH (100 mL) ethanol and sulfuric acid (1 mL). The mixture was stirred at 65 °C overnight. Then calcium carbonate (2.5 g) was then added and the mixture was stirred again for 30 min. The precipitate was removed by filtration and the filtrate was dried under reduced pressure. The reaction was followed by Thin Layer Chromatography analysis (TLC) using hexane/

isopropanol (99/1 v/v) as the eluent. ¹H NMR spectra were recorded on a Bruker AC-200.1 (200.1 MHz) spectrometer and confirmed the purity of both esters.

4.4. Construction of Lip2p variants by site-directed mutagenesis

Variants at position 88, 94, 97, 232 and 285 were previously constructed⁵⁴ and only the construction of variants targeting position 235 was performed in the current work. JMP8 plasmid carrying the wild-type LIP2 gene was previously described:⁵⁸ LIP2 encoding the extracellular lipase Lip2p is under the transcriptional control of the strong promoter POX2 inducible by oleic acid also previously described.⁵⁴ Mutagenesis in the LIP2 gene was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). The following primers and their complementary reverse counterparts were used for systematic directed mutagenesis at position 235: 5'-CGTCCCTCA~~XXX~~CCCTTCTGGG-3' with ~~XXX~~ being GCC for V235A, TGC for V235C, GAC for V235D, GAG for V235E, TTC for V235F, GGC for V235G, CAC for V235H, ATC for V235I, AAG for V235K, CTC for V235L, ATG for V235M, AAC for V235N, CCC for V235P, CAG for V235Q, CGA for V235R, TCC for V235S, ACC for V235T, TGG for V235W, TAC for V235Y. *Escherichia coli* DH5 α was used as the host to produce the different plasmids and sequences were controlled by sequencing (GATC, Konstanz, Germany). Plasmids were digested by *NotI* and used for the transformation of strain *Y. lipolytica* JMY1212⁵⁶ by the lithium acetate method as described previously.⁶²

4.5. Production and activity of lipases

The production of lipases from *Y. lipolytica* and *C. rugosa* is described elsewhere.^{51,61} In order to increase the activity, the obtained lipases can be concentrated using centrifugal filter units (Amicon® Ultra Centrifugal Filters, Merck Millipore).

4.6. Enzymatic hydrolysis of ibuprofen, naproxen and ketoprofen ethyl esters

In a 2 mL reactor (Eppendorf), 750 μ L of culture supernatant (or the concentrate supernatant) containing the enzyme and 750 μ L of racemic ethyl ibuprofen, naproxen or ketoprofen (50 mM in decane) were added. The reactors were stirred in a vortex Genie 2 (D. Dutscher, Brumat, France) at room temperature for 100 h. At regular time intervals, the progress of the reaction was monitored by analyzing the organic phase composition after phase separation by centrifugation (dilution 1, 10 and 30 in hexane for the ibuprofen, naproxen and ketoprofen ester, respectively).

4.7. Chromatography analysis

The HPLC device was equipped with a chiral column: Chiralcel OJ-H (25 cm \times 4.6 mm) (Chiral Technologies Europe, Daicel group)

connected to a UV detector (at 254 nm for the analysis of ibuprofen ethyl ester and 270 nm for the naproxen ethyl ester). A flow rate of 1.0 mL/min was used. The mobile phase was composed of a mixture *n*-hexane/isopropanol [98:2 v/v] for ibuprofen and naproxen ethyl ester analysis. Retention time: 4/4.5 min for the (*S*)- and (*R*)-enantiomers of ibuprofen ethyl ester and 20/22 min for the (*S*)- and (*R*)-enantiomers of naproxen ethyl ester. Ibuprofen formed during the reaction and remaining in the decane phase can be analyzed in the same conditions as ibuprofen ethyl ester, the retention time being 7/8 min for the (*S*)- and (*R*)-enantiomers of ibuprofen, respectively.

For the analysis of ketoprofen ethyl ester the mobile phase was composed only of hexane, with retention times of 12 and 13 min for the (*S*)- and (*R*)-enantiomer respectively.

4.8. Determination of enantioselectivity (*E*)

$$E = \frac{v_i S}{v_i R}$$

or

$$E = \frac{v_i R}{v_i S}$$

where *v*_{*i*}*S* and *R* are the initial rates of hydrolysis of the (*S*)- and (*R*)-enantiomers respectively.

4.9. Molecular modelling studies

Since the only available crystallographic structure of *Y. lipolytica* Lip2p corresponds to a closed inactive conformation of the enzyme,⁶³ we used an homology model of the open conformation⁵⁴ to perform docking of the ligands in the catalytic site. Three-dimensional models of the covalent tetrahedral intermediates for each enantiomer (*S*)- and (*R*)-enantiomers of ibuprofen and naproxen ethyl esters, respectively, were built. Each enantiomer was placed in the active site so that it is covalently bonded to the catalytic serine (S162) and fulfills the hydrogen bonding interactions required for productive catalysis. Models of enzyme variants mutated at positions 232 or 235 were constructed from the model of wild type Lip2p by introducing site mutations using the mutator module in VMD.⁶⁴ Each enzyme variant was then minimized using parm99sb-ildn force field⁶⁵ in Gromacs software.⁶⁶ The covalent docking of the enantiomers was carried out using Autodock program.⁶⁷ The conformational space explored during docking was defined by a grid centered on Ser162 sidechain and spacing by 0.375 Å. Autodock parameters were set to the standard values for genetic algorithm and 200 docking poses were extracted. These poses were clustered based on their RMSD. One ligand conformation representative of the best cluster was placed in wild-type Lip2p or its enzyme variants and the whole system was minimized until the maximum force was less than 100 kJ·mol⁻¹. Visualization and graphics were done using VMD software.⁶⁴

Acknowledgments

We thank the Pôles de Recherche et d'Enseignement Supérieur de l'Université de Toulouse and the Région Midi-Pyrénées, France for PhD grant of Doriane Gerard. We also thank the ICEO facility, which is part of the Integrated Screening Platform of Toulouse (PICT, IBISA), for access to the analytical chromatography platform.

References

- Sheldon, R. A. *J. Chem. Technol. Biotechnol.* **1996**, *67*, 1–14.

- Adams, S. S.; Bresloff, P.; Mason, C. G. *J. Pharm. Pharmacol.* **1976**, *28*, 256–257.
- Hutt, A. J.; Caldwell, J. *Clin. Pharmacokinet.* **1984**, *9*, 371–373.
- Battistel, E.; Bianchi, D.; Cesti, P.; Pina, C. *Biotechnol. Bioeng.* **1991**, *38*, 659–664.
- Midha, K. K.; Mckay, G.; Rawson, M. J.; Hubbard, J. W. *Pharm. Sci.* **1998**, *87*, 797–802.
- Kourist, R.; Domínguez de María, P.; Miyamoto, K. *Green Chem.* **2011**, *13*, 2607–2618.
- Könst, P.; Merckens, H.; Kara, S.; Kochius, S.; Vogel, A.; Zuhse, R.; Holtmann, D.; Arends, I.; Hollmann, F. *Angew. Chem., Int. Ed.* **2012**, *51*, 9914–9917.
- Friest, J. A.; Maezato, Y.; Broussy, S.; Blum, P.; Berkowitz, D. B. *J. Am. Chem. Soc.* **2010**, *132*, 5930–5931.
- Galletti, P.; Emer, E.; Gucciardo, G.; Quintavalla, A.; Pori, M.; Giacomini, D. *Org. Biomol. Chem.* **2010**, *8*, 4117–4123.
- Giacomini, D.; Galletti, P.; Quintavalla, A.; Gucciardo, G.; Paradisi, F. *Chem. Commun.* **2007**, 4038–4040.
- Pietruszka, J.; Schölzel, M. *Adv. Synth. Catal.* **2012**, *354*, 751–756.
- Habibi, Z.; Mohammadi, M.; Yousefi, M. *Process Biochem.* **2013**, *48*, 669–676.
- Ng, I. S.; Tsai, S. W. *Process Biochem.* **2006**, *41*, 540–546.
- Qin, B.; Liang, P.; Jia, X.; Zhang, X.; Mu, M.; Wang, X. Y.; Ma, G. Z.; Jin, D. N.; You, S. *Catal. Commun.* **2013**, *38*, 1–5.
- Sandström, A. G.; Wikmark, Y.; Engström, K.; Nyhlén, J.; Bäckvall, J.-E. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 78–83.
- Lee, W. H.; Kim, K.-J.; Kim, M. G.; Lee, S. B. *J. Ferment. Bioeng.* **1995**, *80*, 613–615.
- Chang, C. S.; Tsai, S. W.; Lin, C. N. *Tetrahedron: Asymmetry* **1998**, *9*, 2799–2807.
- Kim, M. G.; Lee, E. G.; Chung, B. H. *Process Biochem.* **2000**, *35*, 977–982.
- Manetti, F.; Mileto, D.; Corelli, F.; Soro, S.; Palocci, C.; Cernia, E.; D'Acquarica, I.; Lotti, M.; Alberghina, L.; Botta, M. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2000**, *1543*, 146–158.
- Bhatia, S.; Long, W. S.; Kamaruddin, A. H. *Chem. Eng. Sci.* **2004**, *59*, 5061–5068.
- Long, W. S.; Kow, P. C.; Kamaruddin, A. H.; Bhatia, S. *Process Biochem.* **2005**, *40*, 2417–2425.
- Fazlena, H.; Kamaruddin, A. H.; Zulkali, M. M. D. *Bioprocess Biosyst. Eng.* **2006**, *28*, 227–233.
- Won, K.; Hong, J. K.; Kim, K. J.; Moon, S. J. *Process Biochem.* **2006**, *41*, 264–269.
- Marszał, M. P.; Siódmiak, T. *Catal. Commun.* **2012**, *24*, 80–84.
- Siódmiak, T.; Ziegler-Borowska, M.; Marszał, M. P. *J. Mol. Catal. B: Enzym.* **2013**, *94*, 7–14.
- Yousefi, M.; Mohammadi, M.; Habibi, Z. *J. Mol. Catal. B: Enzym.* **2014**, *104*, 87–94.
- Tsai, S.-W.; Wei, H.-J. *Biocatal. Biotransform.* **1994**, *11*, 33–45.
- López, N.; Pernas, M. A.; Pastrana, L. M.; Sánchez, A.; Valero, F.; Rúa, M. L. *Biotechnol. Prog.* **2004**, *20*, 65–73.
- Botta, M.; Cernia, E.; Corelli, F.; Manetti, F.; Soro, S. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1997**, *1337*, 302–310.
- Xin, J. Y.; Li, S. B.; Chen, X. H.; Wang, L. L.; Xu, Y. *Enzyme Microb. Technol.* **2000**, *26*, 137–141.
- Wu, J. Y.; Liu, S. W. *Enzyme Microb. Technol.* **2000**, *26*, 124–130.
- Steenkamp, L.; Brady, D. *Enzyme Microb. Technol.* **2003**, *32*, 472–477.
- Chen, C. C.; Tsai, S. W.; Villeneuve, P. J. *Mol. Catal. B: Enzym.* **2005**, *34*, 51–57.
- Xin, J. Y.; Zhao, Y. J.; Shi, Y. G.; Xia, C. G.; Li, S. Ben *World J. Microbiol. Biotechnol.* **2005**, *21*, 193–199.
- Chang, C. S.; Tsai, S.-W. *Enzyme Microb. Technol.* **1997**, *20*, 635–639.
- Sehgal, A. C.; Kelly, R. M. *Biotechnol. Prog.* **2003**, *19*, 1410–1416.
- Steenkamp, L.; Brady, D. *Process Biochem.* **2008**, *43*, 1419–1426.
- Tao, W.; Shengxue, F.; Duobin, M.; Xuan, Y.; Congcong, D.; Xihua, W. X. *J. Mol. Catal. B: Enzym.* **2013**, *85–86*, 23–30.
- Carvalho, P. D. O.; Contesini, F. J.; Ikegaki, M. *J. Microbiol.* **2006**, *37*, 329–337.
- Zhang, W.-W.; Jia, J.-Q.; Wang, N.; Hu, C.-L.; Yang, S.-Y.; Yu, X.-Q. *Biotechnol. Rep.* **2015**, *7*, 1–8.
- Park, H. J.; Choi, W. J.; Huh, E. C.; Lee, E. Y.; Choi, C. Y. *J. Biosci. Bioeng.* **1999**, *87*, 545–547.
- Zhang, Y. Y.; Liu, J. H. *Biochem. Eng. J.* **2011**, *54*, 40–46.
- Ni, Z.; Zhou, P.; Jin, X.; Lin, X. F. *Chem. Biol. Drug Des.* **2011**, *78*, 301–308.
- Xi, W. W.; Xu, J. H. *Process Biochem.* **2005**, *40*, 2161–2166.
- Zhu, S.; Wu, Y.; Yu, Z. J. *Biotechnol.* **2005**, *117*, 397–401.
- Liu, Y. Y.; Xu, J. H.; Wu, H. Y.; Shen, D. J. *Biotechnol.* **2004**, *110*, 209–217.
- Long, Z.; Xu, J.; Zhao, L.; Pan, J.; Yang, S.; Hua, L. J. *Mol. Catal. B: Enzym.* **2007**, *47*, 105–110.
- Kim, G. J.; Lee, E. G.; Gokul, B.; Hahm, M. S.; Prerna, D.; Choi, G. S.; Ryu, Y. W.; Ro, H. S.; Chung, B. H. *J. Mol. Catal. B: Enzym.* **2003**, *22*, 29–35.
- Lee, K. W.; Bae, H. A.; Shin, G. S.; Lee, Y. H. *Enzyme Microb. Technol.* **2006**, *38*, 443–448.
- Yoon, S.; Kim, S.; Park, S.; Hong, E.; Kim, J.; Kim, S.; Yoo, T. H.; Ryu, Y. J. *Mol. Catal. B: Enzym.* **2014**, *100*, 25–31.
- Piamtongkam, R.; Duquesne, S.; Bordes, F.; Barbe, S.; André, I.; Marty, A.; Chulalaksananukul, W. *Biotechnol. Bioeng.* **2011**, *108*, 1749–1756.
- Cancino, M.; Bauchart, P.; Sandoval, G.; Nicaud, J.; André, I.; Dossat, V.; Marty, A. *Tetrahedron: Asymmetry* **2008**, *19*, 1608–1612.
- Liu, Y.; Wang, F.; Tan, T. *Chirality* **2009**, *22*, 929–935.
- Bordes, F.; Cambon, E.; Dossat-Létisse, V.; André, I.; Croux, C.; Nicaud, J. M.; Marty, A. *ChemBioChem* **2009**, *10*, 1705–1713.
- Cambon, E.; Piamtongkam, R.; Bordes, F.; Duquesne, S.; Laguerre, S.; Nicaud, J. M.; Marty, A. *Enzyme Microb. Technol.* **2010**, *47*, 91–96.
- Bordes, F.; Fudalej, F.; Dossat, V.; Nicaud, J.-M.; Marty, A. *J. Microbiol. Methods* **2007**, *70*, 493–502.

57. Fickers, P.; Nicaud, J. M.; Destain, J.; Thonart, P. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 136–142.
58. Pignède, G.; Wang, H.; Fudalej, F.; Seman, M.; Nicaud, J.; Gaillardin, C.; Seman, M.; Nicaud, J.-M. *J. Bacteriol.* **2000**, *182*, 2802–2810.
59. Nicaud, J. M.; Madzak, C.; Van Den Broek, P.; Gysler, C.; Duboc, P.; Niederberger, P.; Gaillardin, C. *FEMS Yeast Res.* **2002**, *2*, 371–379.
60. Guieysse, D.; Sandoval, G.; Faure, L.; Nicaud, J. M.; Monsan, P.; Marty, A. *Tetrahedron: Asymmetry* **2004**, *15*, 3539–3543.
61. Cambon, E.; Piamtongkam, R.; Bordes, F.; Duquesne, S.; André, I.; Marty, A. *Biotechnol. Bioeng.* **2010**, *106*, 852–859.
62. Le Dall, M. T.; Nicaud, J. M.; Gaillardin, C. *Curr. Genet.* **1994**, *26*, 38–44.
63. Bordes, F.; Barbe, S.; Escalier, P.; Mourey, L.; André, I.; Marty, A.; Tranier, S. *Biophys. J.* **2010**, *99*, 2225–2234.
64. Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graph.* **1996**, *14*, 33–38.
65. Lindorff-Larsen, K.; Piana, S.; Palmo, K.; Maragakis, P.; Klepeis, J. L.; Dror, R. O.; Shaw, D. E. *Proteins Struct. Funct. Bioinf.* **2010**, *78*, 1950–1958.
66. Pronk, S.; Páll, S.; Schulz, R.; Larsson, P.; Bjelkmar, P.; Apostolov, R.; Shirts, M. R.; Smith, J. C.; Kasson, P. M.; Van Der Spoel, D.; Hess, B.; Lindahl, E. *Bioinformatics* **2013**, *29*, 845–854.
67. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009**, *30*, 2785–2791.