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REVIEW ARTICLE

Relevance and bio-catalytic strategies for the kinetic resolution of ketoprofen towards dexketoprofen

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ABSTRACT

This review presents the most relevant investigations concerning the biocatalytic kinetic resolution of racemic ketoprofen to dexketoprofen for the last 22 years. The advantages related to the administration of the dex-enantiomer in terms of human health, the so called "chiral switch" in the pharmaceutical industry and the sustainability of biotransformations have been the driving forces to develop innovative technology to obtain dexketoprofen. In particular, the kinetic resolution of racemic ketoprofen through enantiomeric esterification and hydrolysis using lipases as biocatalysts are thoroughly revised and commented upon. In this context, the biocatalysts, acyl-acceptors (alcohols), reaction conditions, conversion, enantiomeric excess, and enantiomeric ratio among others are discussed. Moreover, the investigations concerning scaling up processes in order to obtain an optically pure enantiomer of the profen are presented. Finally, some guidelines about perspectives of the technology and research opportunities are given.

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Introduction

It is well known that non-steroidal anti-inflammatory drugs (NSAIDs) cause a wide spectrum of adverse reactions despite their broad acceptance and worldwide use. The gastrointestinal side effects, mainly in the stomach and duodenum, such as pyrosis, dyspepsia, gastritis, or diarrhea have been clinically studied and reported. The appearance of gastric or duodenal mucosal injuries in chronically treatments are of major concern since those erosions and ulcers lead to bleeding and/or perforations [1]. The renal functions that depend on prostaglandin synthesis are also affected by NSAIDs in patients with a kidney disease. In fact, the NSAIDS might diminish renal blood flow and the rate of glomerular filtration which is associated with renal failure. Hepatotoxicity and cardiovascular risk of NSAIDs has also been reported [2,3]. Among the different NSAIDs used topically, ketoprofen has often been associated to photosensitivity including phototoxic and photo-allergic reactions [4]. In addition, the asthmatic population is sensitive to NSAIDs and presents a triad of rhinitis, sinusitis, and asthma upon exposure to these drugs [5]. Last year, the U.S. Food and Drug Administration (FDA) published a warning about the increased chance of heart attack and strokes due to high doses and extended intake of non-aspirin NSAIDs such as ketoprofen and ibuprofen, among others [6].

Ketoprofen contains a stereogenic center at the carbon alpha of the carboxyl function and therefore exists as a racemate of the *R*- and *S*-enantiomers which are equivalent in mass. The analgesic effect of ketoprofen has been attributed exclusively to dexketoprofen that is the *S*-enantiomer [7]. However, it is worth noticing that recent clinical investigations demonstrated that racemic ketoprofen undergoes bio-inversion in humans and that the equivalent efficacy with half the dose of dexketoprofen compared with ketoprofen is not straightforward.

Lorier et al. reported differences in the pharmacokinetics of the racemic ketoprofen enantiomers and provided evidence of their bio-inversion [8]. The authors indicated that the ratio between the enantiomers *S*/*R* in plasma increases if the administration of the profen was followed by the intake of food. This observation was attributed to pancreatic and/or intestinal and/or biliary secretions of the drug, followed by reabsorption and conversion of the *R*- to the *S*-isomer.

Barden et al. compared the efficacy of racemic ketoprofen and dexketoprofen reviewing several

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placebo-controlled trials of single dose orally administered ketoprofen (1510 participants) and dexketoprofen (970 participants) in adults with moderate to severe acute post-operative pain [9]. The authors did not observe the expected 2:1 dose ratio between ketoprofen and dexketoprofen for an effective analgesia. They attributed this result to the small number of trials that directly compares both compounds. Previously, Barden and Moore reported a systematic review of dexketoprofen versus ketoprofen and they arrived at a different conclusion [10]. In this study, the authors found evidence that analgesia with dexketoprofen is equivalent to that obtained with double the dose of ketoprofen. In acute pain, there is a hint of superior analgesic effect than double dose ketoprofen. Additionally, a recent investigation provided evidence that dexketoprofen induces less pain than the racemic counterpart during intravenous injection [11].

The clinical investigations concerning the medical use of dexketoprofen provide evidence that the *S*-enantiomer might be a healthier choice than the racemic counterpart. This observation is highlighted here as the motivation in terms of human health that drove numerous investigations towards the synthesis of pure dexketoprofen. The next section discusses economical motivations.

From *R*-/*S*-ketoprofen to dexketoprofen: the chiral switch strategy

The chiral switch occurs when a racemic drug is replaced with a purified single enantiomer version in the marketplace [12]. The new enantiopure drugs come from well-known racemates that possess government approval to be used as pharmaceuticals. The expiration of the patent regarding the synthesis of the racemate is the perfect opportunity to introduce a new patent of the pure enantiomer.

This strategy provides an extended profit for the pharmaceutical company that is producing the racemate, new opportunities to other manufactures and allows bridging studies that would lead to an easier pathway for approval. Ketoprofen and ibuprofen are examples of racemic NSAIDs that underwent the chiral switch. Dexketoprofen and a salt with tromethamine (dexketoprofen trometamol) were introduced in Europe in 1998 and are currently marketed by Menarini Pharmaceuticals in several European countries [12]. However, a careful review of the reports concerning the novel drugs approval of the US Drug Evaluation and Research (CDER) between 2011 and 2017 and a similar survey by Gellad et al. in the 2001–2011 period, allowed to conclude that dexketoprofen has not been approved by the US Food and Drug Administration [13–15].

A short history of such a chiral switch starts with the synthesis of the racemic ketoprofen described for the first time in a British patent in 1966. This was followed by the US patent No. 4,845,281 in 1989 granted to the Italian company Blaschim S.P.A. [16]. In 1996, the company Menarini Pharmaceuticals patented the preparation of dexketoprofen through the kinetic resolution of ketoprofen ethyl ester using a biocatalyst [17]. Simultaneously, that company patented the synthesis of the dexketoprofen tromethamine salt and its use as analgesic and anti-inflammatory agent [18]. By the expiration date of that patent (about twenty years later), other companies such as Galenicum Health S.L. and Lesvi S.L. Laboratories, filled applications for new patents on formulations based on that pharmaceutical [19,20].

Enzymes in biocatalytic processes: more pros than cons

Enzymes are macromolecular biological catalysts that catalyze biochemical reactions *in vivo*. Almost all metabolic processes in the cell need enzymes in order to occur at rates fast enough to sustain life.

They have been naturally adapted to perform under physiological conditions. However, when used as biocatalyst in kinetic resolution they perform in artificial conditions (*in vitro*). Therefore, a major challenge is to transform these *physiological catalysts* into *process catalysts* able to perform under reaction conditions of an industrial process. Enzymes as any catalyst diminish the energy barrier of the chemical reaction. Their chemo-, regio-, diastereo-, and enantioselectivity allow obtaining highly complex molecules which may require multiple steps of protection and de-protection if the reaction is performed through chemical synthesis. Additionally, enzymes are active under mild conditions (temperature, pH, atmospheric pressure) which minimize possible unwanted side reactions. Furthermore, biocatalytic processes are environmentally acceptable since they are completely degradable.

Even though enzymes are complex molecular structures, labile and costly to produce, nowadays these disadvantages have been or are being solved through research and development in different areas. In fact, several strategies have been developed to improve enzyme stability including chemical modification, immobilization to solid matrices, crystallization, aggregation, and techniques of protein engineering. Lipases (EC 3.1.1.3) are one of the most important enzymes employed in organic synthesis. They are hydrolytic

enzymes and exert their activity on the carboxyl ester bonds of triacylglycerols and other substrates. Their natural substrates are insoluble lipid compounds prone to aggregation in aqueous solution. *In vitro*, they can form ester bonds which enable lipases to catalyze various other types of reactions such as esterification, transesterification, interesterification, alcoholysis, and acidolysis [21,22].

Lipases are highly widespread in nature, and are isolated from microorganisms, plants, and animals. Microbial lipases, the most important in biocatalysis, are produced by submerged or solid-state fermentation. The range of optimum temperature is wide, generally between 30 and 60 °C. In terms of pH, most lipases show high activity in neutral or alkaline media. They accept a broad range of non-natural substrates and are versatile for application in organic synthesis [23].

The excellent enantioselectivity of lipases make them useful for obtaining enantiomerically pure compounds as discussed in the next section.

Kinetic resolution of ketoprofen: green biocatalytic strategy

The previous sections provide evidence of the dexketoprofen relevance from the medical side and the importance of producing the pure enantiomer from the racemic ketoprofen with an economical point of view. Additionally, it was discussed that the former synthesis of dexketoprofen patented in 1996 by Menarini Pharmaceuticals involved the biotransformation of the racemic compound assisted by a microorganism. This is not casual at all since the synthesis of enantiomerically pure active pharmaceutical ingredients and their intermediates through bioprocesses are preferred due to the benefit these systems offer as described in the previous section. In fact, the bioassisted and dynamic kinetic resolution are the most widespread methodologies to produce *S*-ketoprofen [24,25]. A recent review by Godoy Dahia et al. [26] indicated that lipases are the most applied biocatalyst in the resolution of racemic mixtures for producing pure enantiomers in the pharmaceutical industry. An exhaustive patent search conducted by the authors indicated that the technology of applying lipases as biocatalysts for kinetic resolution would have already finished its maturity stage [26]. However, new developments are expected with the so-called "third wave of biocatalysis" since numerous scientific investigations are devoted to tailor new biocatalyst and enzymatic process to reach the expectation of a green, sustainable and cost competitive industry. In this context, the following sections summarize the scientific achievements concerning the enzymatic kinetic resolution of racemic ketoprofen and the perspectives of that technology. In particular, the literature regarding the kinetic resolution through enantioselective esterification of the racemic mixture and the enantiomeric hydrolysis of the racemic esters are addressed.

The enantioselective esterification involves the reaction of racemic ketoprofen acid with an alcohol as acyl acceptor (methanol, ethanol, and among others) in the presence of an enantioselective enzyme to achieve the ester of *R*-ketoprofen or *S*-ketoprofen (depending on the enzyme) and water (Figure 1(A)). Among the most commonly used enzymes, lipases are the most suitable due to its stability, availability, acceptance of a broad range of substrates and have no coenzyme requirement for catalysis. The lipase B from *Candida antarctica* (CALB) has been widely used in the esterification of racemic ketoprofen with enantiospecificity towards the *R*-enantiomer, which has the advantage of leaving the desired *S*-isomer without reacting and that no chemical manipulations are required later. The investigations regarding this lipase are discussed in the following sections.

On the other hand, a racemic ketoprofen ester is the starting material during enantioselective hydrolysis. In this case, one of the enantiomers is hydrolyzed faster than the other in order to yield the carboxylic acid (*R*- or *S*- depending on the type of enzyme) and an alcohol (Figure 1(B)). Regularly, two kinds of enzymes are used in this reaction: lipases and esterases. The lipase from *Mucor javanicus* and *Candida antarctica* show enantioselectivity towards *R*-ketoprofen while the lipase from *Candida rugosa* is enantiospecific to the *S*-enantiomer [24].

Background in the kinetic resolution of *R*-/*S*-ketoprofen: a journey through the various biocatalysts and reaction conditions

Kinetic resolution of *R*-/*S*-ketoprofen has been widely investigated through enantioselective esterification (Table 1) and hydrolysis (Table 2) over the last 22 years. Tables 1 and 2 summarize the reaction conditions (biocatalysts, reagents, temperature, co-solvents, surfactants, among others) and enzymatic activity reported in the literature. In the following sections, the enzymes employed, the investigations of the various factors that influence the kinetic resolution (alcohols used in esterification reactions, solvents, emulsifiers, and water activity) and large scale preparations are discussed.

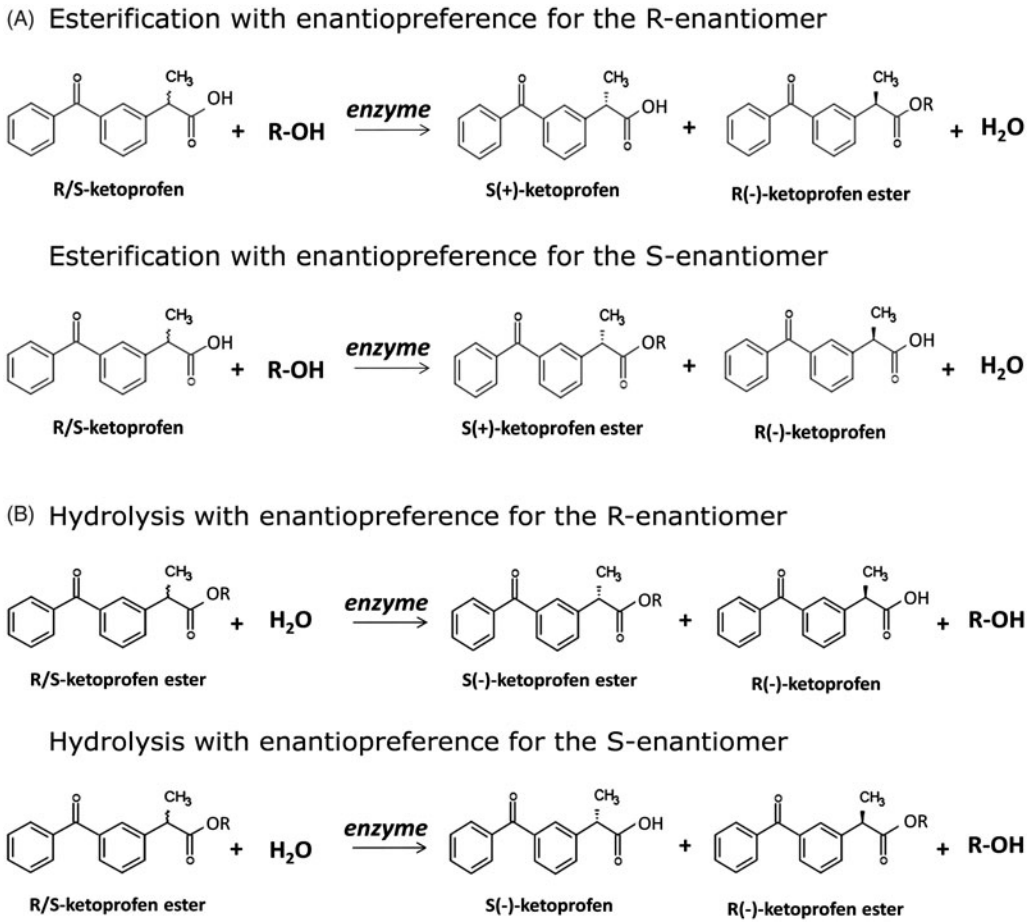

Figure 1. (A) Esterification for the *R*- and *S*-enantiomer. (B) Hydrolysis with enantiopreference for *R*- and *S*-enantiomer.

Assayed biocatalysts in the esterification and hydrolysis towards the kinetic resolution of R-/S-ketoprofen

The information summarized in the Tables 1 and 2 shows that lipases and esterases are the most commonly employed enzymes as biocatalysts which has been stated before. The entries 1, 2, 4–7, 10–12, 14, 15, 17, and 18 in Table 1 showed that CALB is the most frequently studied [27,28,30–33,36–38,40,41,44]. Additionally, the lipases from *Rhizomucor miehei* [29,30,32] (entries 3, 4, and 6 in Table 1, respectively), *Mucor javanicus* [30,38] (entries 4 and 12), *Burkholderia cepacia* [35,42] (entries 9 and 16), *Aspergillus niger* and *terreus* [35,38] (entries 9 and 12) among other microbial lipases have been investigated. The lipase from *Candida rugosa* (formerly known as *Candida cylindracea*) proved activity both in the esterification [30,34,35,38] (entries 4, 8, 9, and 12 in Table 1) and the hydrolysis of the ester of the *S*-ketoprofen [34,36,47–55,62,64,67,68,71,86] (entries 1–9, 16, 18, 19, 22–24, 27, and 42 in Table 2). Interestingly, Japanese firefly luciferases have emerged as novel biocatalysts in the thioesterification of racemic 2-arylpropionic acids with a preference to ketoprofen [39,45,46] (entries 13, 19, and 20). These enzymes obtained from fireflies such as *Luciola lateralis*, *Pylocoeria miyako* and *Hotaria parvura*, among others require the use of cofactors such as ATP, Mg^{2+}, and CoASH as substrates.

The lipase from the latex of papain (*Carica papaya* lipase) also proves activity in the enantioselective hydrolysis of the *R,S*-2,2,2-trifluoroethyl thioester of profens such as ketoprofen, ibuprofen, naproxen, fenoprofen, suprofen, and flurbiprofen (entries 25 and 29 in Table 2) [69,73].

The esterases from thermophile bacteria such as the *Bacillus stearothermophilus* and *Thermotoga maritime*, and from a thermoacidophilic archaeon called *Sulfolobus solfataricus* are able to resist harsh reaction conditions (entries 11, 21, and 37 in Table 2) [57,66,81]. These biocatalysts catalyze the kinetic resolution of ketoprofen esters between 60 °C and 90 °C and a broad pH range from 5 to 9.5.

Enzymes have been applied in their free form, immobilized onto various inorganic and organic materials and as cross-linked crystals. CALB immobilized on beads of polymethylmethacrylate and the lipase from *Mucor miehei* on a macroporous ion-exchange resin are the

Table 1. Biocatalysts, acyl acceptors (alcohols), reaction conditions (molar ratio of substrates, temperature, others), co-solvents, conversion, enantiomeric excess, enantiomeric factor, enantiomeric ratio, specific activity, and reaction rate in the kinetic resolution of racemic ketoprofen through enantiomeric esterification reported in the literature in the last 22 years.

Entry	Biocatalyst	Alcohol (alcohol:profen molar ratio)	Reaction conditions	Co-solvent	Conversion (X%), enantiomeric excess (ee%) and enantiomeric ratio (E)	References
1	SP435A (*Candida antarctica* lipase B, now Novozym® 435)	1-Propanol (1:1)	50 °C, 300 rpm, 3 h. *R*-enantioselectivity	Methyl isobutyl ketone	*X* % = 55.6, *E* = 2.6	[27]
2	Novozym® 435 (*Candida antarctica* lipase B, CALB)	1-Propanol (1:1)	40 °C, 210 rpm. *R*-enantioselectivity	Dipropyl ether	V_{max} (mmol/h g of lipase): R-ketoprofen =2096, *S*-ketoprofen =754	[28]
3	Lipozyme® IM (*Rhizomucor miehei* lipase)	1-Butanol (1:1)	37 °C, stirring, 72 h. *R*-enantioselectivity	Diisopropyl ether	X % = 20, ee % = 9.5, EF =0.38, *E* = 2.4	[29]
4	Lipozyme® IM, Lipozyme® 10000L and Lipase MAP-10 (lipases from *Mucor miehei*), Novozym® 435, CRL lipase (from *Candida rugosa*) and Lipase M (from *Mucor javanicus)*	Ethanol (4:1)	37 °C, 250 rpm, 0.1% v/v water added	Carbon tetrachloride	Lipozyme® IM: X % = 52 (76 h), ee *S*-keto % = 53, *E* = 5 Novozym® 435: X %= 76 (76 h), ee *S*-keto % = 97, *E* = 7 Lipozyme® 10000L: X %= 82 (76 h), ee *S*-keto % = 65, *E* = 2 Lipase MAP-10 X %= 22 (144 h), ee *S*-keto % = 20, *E* = 7 CRL lipase X %= 1 (144 h), ee R-keto % = 0, *E* = 1 Lipase M X %= 23 (144 h), ee *R*-keto % = 20, *E* = 7	[30]
	Novozym® 435	ethanol (4:1)	37 °C, 250 rpm, 75 h (31 hs of reaction with n-hexane as co-solvent); 0.1% v/v water added	Various solvents	Diisopropyl ether: X % = 43, ee % = 17, *E* = 2 Methylene dichloride: X % = 4, ee % = 3, *E* = 7 Isobutyl methyl ketone: % = 19, ee % = 17, *E* = 6 Ethylene dichloride: X % = 31, ee % = 37, *E* = 15 Carbon tetrachloride: X % = 76, ee % = 97, *E* = 7 1,4-dioxane: X % = 3, ee % = 2, *E* = 3 Acetonitrile: X % = 7, ee % = 5, *E* = 4 Ethanol: X % = 6, ee % = 2, *E* = 2 Toluene: X % = 67, ee % = 84, *E* = 6 *n*-Hexane: X % = 89, ee % = 81, *E* = 2	
	Novozym® 435	Methanol, ethanol, 1-propanol, 1-butanol, 1-hexanol, octanol (4:1)	37 °C, 250 rpm	*n*-Hexane with 20% v/v ethylene dichloride	Methanol: X % = 59 (9 h), ee % = 75, *E* = 6 Ethanol: X % = 46 (9 h), ee % = 58, *E* = 10 1-Propanol: X % = 38 (9 h), ee % = 48, *E* = 9 1-butanol: X % = 29 (18 h), ee % = 31, *E* = 10 1-hexanol: X % = 35 (18 h), ee % = 33, *E* = 6 Octanol: X % = 25 (18 h), ee % = 23, *E* = 8	
5	Novozym® 435	1-Propanol (4:1)	30 h	Cyclohexane	X% = 68, ee *S*-keto % = 92	[31]
6	Novozym® 435 and Lipozyme® IM20 (*Mucor miehei* lipase)	Solventless: 1-dodecanol (1:1) and 1-propanol (1:1) Solvents: 1-dodecanol (1:1)	Solventless: 75 °C and stirring, under reduced pressure for continuous water removal. Solvents: 60 °C R-enantioselectivity	Solventless and co-solvents with log P from 1.36 to 3.5	No reaction under solventless conditions with 1-dodecanol. With 1-propanol, IR =48 Co-solvents: higher log P, faster reaction	[32]
7	Novozym® 435	ethanol	45 °C, 300 rpm, 5 days. *R*-enantioselectivity	Dichloropropane	X % = 60, ee *S*-keto % = 90, *E* = 12	[33]

(*continued*)

Table 1. Continued

Entry	Biocatalyst	Alcohol (alcohol:profen molar ratio)	Reaction conditions	Co-solvent	Conversion (X%), enantiomeric excess (ee%) and enantiomeric ratio (E)	References
8	Commercial lipase from *Candida rugosa*	1-propanol (4:1)	30 °C, magnetic stirring, 500 h. *S*-enantioselectivity	Isooctane	X % = 17, ee *R*-keto % = 79	[34]
9	*Candida rugosa* lipase, lipase MY (*Candida rugosa*), lipase PS (*Burkholderia cepacia*), lipase AK (*Pseudomonas fluorescens*) and lipase A (*Aspergillus niger*)	1,4-butanediol (25:1)	37 °C, 24 h *Candida rugosa* lipase, lipase MY and lipase AK posses *S*-enantioselectivity Lipase PS and lipase A possess *R*-enantioselectivity	Isooctane	*Candida rugosa* lipase: X % = 46.15, ee *R*-keto % = 70.3, lipase MY: X % = 49.32, ee *R*-keto % = 73.2 and lipase AK: X % = 3.54, ee *R*-keto % = 16.4 Lipase PS: X % = 1.02, ee *S*-keto % = 66.6 and lipase A: X % = 9.13, ee *S*-keto % = 87.3	[35]
	Lipase MY	1,3-propanediol, 1,4-butanediol, 1,5-pentanediol, 1,6-hexanediol (5:1)	37 °C, 24 h	Isooctane, cyclohexane	Data using isooctane as co-solvent: 1,3-propanediol: X % = 36.15, ee *R*-keto % = 53.0 1,4-butanediol: X % = 65.47, ee *R*-keto % = 33.5 1,5-pentanediol: X % = 39.80, ee *R*-keto % = 57.7 1,6-hexanediol: X % = 28.45, ee *R*-keto % = 72.0 Data using cyclohexane as co-solvent: 1,3-propanediol: X % = 27.83, ee *R*-keto % = 48.8 1,4-butanediol: X % = 30.00, ee *R*-keto % = 36.6 1,5-pentanediol: X % = 12.36, ee *R*-keto % = 25.1 1,6-hexanediol: X % = 8.37, ee *R*-keto % = 65.3	
10	Novozym® 435	Ethanol (3:1)	45 °C, 160 rpm. *R*-enantioselectivity	1,2-dichloropropane	*E* = 10.5	[36]
11	Free CALB	n-butanol (0:1–5:1)	40 °C, 250 rpm, 24 h batch reactor	Hexane: 1,2-dichloropropane (80: 20% v/v)	X % (*R*-ketoprofen) = 81, ee_p = 66.4, ee *S*-keto % = 59.4	[37]
12	Lipase from A*spergillus* niger, *Aspergilus* terreus, *Fusarium* oxysporum, *Mucor javanicus*, *Penicillum* solitum and *Rhizopus* javanicus, Novozym® 435 (lipase B from *Candida antarctica* and crude commercial *Candida rugosa* lipase	1-Propanol (1:1)	35 °C, 180 rpm, 212 h (except *Candida rugosa* lipase and Novozym® 435: 54 h) Lipases from A*spergillus* niger, A*spergillus terreus,* Candida *antarctica* with *R*-enantioselectivity Lipases from *Fusarium oxysporum*, *Mucor javanicus*, *Penicillum solitum*, *Rhizopus javanicus*, *Candida rugosa* posses *S*-enantioselectivity	Isooctane	Lipase from A*spergillus* niger: X % = 18.3, ee *S*-keto % = 8.9, *E* = 2.7 Lipase of A*spergillus terreus*: X % = 14.1, ee *S*-keto % = 3.3, *E* = 1.5 Lipase of *Fusarium oxysporum*: X % = 15.0, ee *R*-keto % = 4.0, *E* = 1.6 Lipase of *Mucor javanicus*: X % = 12.2, ee *R*-keto % = 3.0, *E* = 1.5 Lipase of *Penicillum solitum*: inactive Lipase of *Rhizopus javanicus*. inactive Crude commercial *Candida rugosa* lipase: X % = 42.0, ee *R*-keto % = 41.3, *E* = 5.4 Novozym® 435: X % = 62.0, ee *S*-keto % = 55.0, E = 3.3	[38]
13	Firefly luciferase from *Luciola lateralis*, *Luciola cruciata* and *Photinus pyralis*	No	30 °C, 60 min ATP, Mg^{2+}, CoASH, potassium phosphate buffer pH 7 (active also at pH 9–10) *R*-enantioselectivity	No	*Luciola lateralis*: X % = 61.8, ee *S*-keto % = 99.6, *E* = 24 *Luciola cruciata:* X % = 39.0, ee *S*-keto % = 53.5, *E* = 19 *Photinus pyralis:* X % = 24.7, ee *S*-keto % = 28.8, *E* = 20	[39]

(*continued*)

Table 1. Continued

Entry	Biocatalyst	Alcohol (alcohol:profen molar ratio)	Reaction conditions	Co-solvent	Conversion (X%), enantiomeric excess (ee%) and enantiomeric ratio (E)	References
14	CALB immobilized on an enzymatic membrane reactor (EMR)	1-Butanol (2:1)	40 °C, flow rate 40 mL/min, pH 7.0 *R*-enantioselectivity	Hexane: 1,2-dichloropropane (80:20% v/v)	X % = 73, ee *S*-keto % = 57.0, *E* = 27	[40]
15	Novozym® 435 and enzymatic membrane reactor	1-Butanol (1:20)	Batch: 60 °C, stirring, 24 h. EMR: 40 °C, 200 bar *R*-enantioselectivity	Ionic Liquids (ILs), supercritical CO_2	Batch: *E* = 5, ee *S*-keto % = 40 EMR: ee_p %: from 100 to 60	[41]
16	Immobilized lipase of *Burkholderia cepacia*	Various alcohols from C1 to C12	37 °C, 200 rpm, 24 h. *R*-enantioselectivity	Heptane	Methanol: SA =341.1, *E* = 1.11 Ethanol: SA =279.7, *E* = 1.15 1-propanol: SA =230.3, *E* = 1.07 1-butanol: SA =230.3, *E* = 1.16 1-pentanol: SA =179.7, *E* = 1.31 1-hexanol: SA =169.4, *E* = 1.58 1-heptanol: SA =181.0, *E* = 1.61 1-octanol: SA =165.0, *E* = 1.91 1-nonanol: SA =167.2, *E* = 1.56 1-dodecanol: SA =356.4, *E* = 1.06 Isopropanol: SA =197.4, *E* = 1.12 Isobutanol: SA =213.9, *E* = 1.39 Isoamylol: SA =186.4, *E* = 1.31 Isooctanol: SA =185.3, *E* = 1.25	[42]
		1-Octanol		Solvents of varying log P	Tetradecane: SA =115.2, *E* = 1.38 Dodecane: SA =191.9, *E* = 1.36 Undecane: SA =241.3, *E* = 1.09 Decane: SA =159.0, *E* = 1.31 Nonane: SA =164.5, *E* = 1.11 Isooctane: SA =192.0, *E* = 1.07 Octane: SA =181.1, *E* = 1.21 Heptane: SA =169.4, *E* = 1.42 Hexane: SA =208.4, *E* = 1.30 Cyclohexane: SA =120.6, *E* = 1.34 Benzene: SA =93.2, *E* = 1.05 1-pentanol: SA =70.2, *E* = 1.11 1-butanol: SA =68.0, *E* = 1.22 Acetone: SA =87.7, *E* = 1.12 Acetonitrile: SA =43.9, *E* = 1.17	
17	Novozym® 435	2-Propanol (21:1)	45 °C, 200 rpm, 72 h. *R*-enantioselectivity	Solventless	X % = 18.7, ee *S*-keto % = 3	[43]
18	Novozym® 435	Methanol, ethanol (9:1) 1-propanol (13:1)	45 °C, 200 rpm, 72 h. *R*-enantioselectivity	Solventless	Methanol: X% = 10, ee *S*-keto % = 1, SA $=1.84 \times 10^{-3}$ Ethanol: X% = 57, ee *S*-keto % = 54, SA $=1.05 \times 10^{-2}$ 1-propanol: X% = 59, ee *S*-keto % = 56, SA $=1.09 \times 10^{-2}$	[44]
19	Firefly luciferase from *Luciola lateralis*	No alcohols used	ATP, Mg^{2+}, CoASH, potassium phosphate buffer pH 7, 27 °C, various arylpropionic acids *R*-enantioselectivity	Solventless	Specific activity =20.5 nmol min^{-1} mg^{-1} in the thioesterification of rac-ketoprofen	[45]

(*continued*)

Table 1. Continued

Entry	Biocatalyst	Alcohol (alcohol:profen molar ratio)	Reaction conditions	Co-solvent	Conversion (X%), enantiomeric excess (ee%) and enantiomeric ratio (E)	References
20	Firefly luciferase from *Pylocoeria miyako* (PmL WT) and *Hotaria parvura* (HpL WT) and three mutants of each one (PmL_I350F, PmL_M397S, PmL_I350F-M397S for PmL and HpL_F351I, HpL_S398M, HpL_F351I-S398M for HpL)	No alcohols used	ATP, Mg^{2+}, CoASH, potassium phosphate buffer pH 7, phosphoenolpyruvic acid, NADH, adenylate kinase, pyruvate kinase, lactate dehydrogenase *R*-enantioselectivity	Solventless		[46]

X%: conversion; ee *S*-keto %: enantiomeric excess towards the *S*-ketoprofen; ee *R*-keto %: enantiomeric excess towards the *R*-ketoprofen; $ee_p = ([R]_{ester} - [S]_{ester})/([R]_{ester} + [S]_{ester})$; *E*: enantiomeric ratio; EF: enantiomeric factor; SA: specific activity (U/g protein); IR: Initial Rate (μmol h^{-1} g^{-1}); ATP: adenosine triphosphate; CoASH: coenzyme A.

commercial biocatalysts known as Novozym® 435 and Lipozyme® IM, respectively.

The literature shows that the immobilization of lipases and esterases onto a variety of materials has been investigated although they did not reach a commercial status. The investigation of alumina, silica, and agarose [49] (entry 3 in Table 2); silica gel, celite, and a silanized ceramic called Toyonite [52,68] (entries 6 and 24 in Table 2); membranes [40,41] (entries 14 and 15 in Table 1) and ionic exchange resins such as Sephadex C-50 and Amberlite [64,68] (entries 18 and 24 in Table 2), can be found in the literature.

More recently, a series of innovations in this area have been reported. Hu et al. developed hollow nanospheres (500 nm) composed by alginate-graft-poly(ethyleneglycol)/α-cyclodextrins in order to encapsulate the *Aspergillus terreus* lipase (entry 39 in Table 2) [83]. These spheres are able to entrap the enzyme within their structure during their synthesis. This biocatalyst showed stability after extended storage and high reusability during the hydrolysis of *R*-/*S*-ketoprofen vinyl ester.

Zhang et al. reported the immobilization of *Mucor javanicus* and *Rhyzomucor miehei* by mixing an emulsion of these lipases in sodium bis-(2-ethylhexyl) sulfosuccinate (called AOT) with gelatin obtained from porcine skin in a phosphate buffer (entry 40 in Table 2) [84]. The immobilized enzymes were more tolerant to organic solvents compared with the free enzymes in the hydrolysis of *R*-/*S*-ketoprofen vinyl ester.

Recently, Cao et al. presented magnetic nanocrystals composed of a cellulose-magnetite mixture as suitable carriers to immobilize *Pseudomonas cepacia* lipase through cross-linking with glutaraldehyde (entry 41 in Table 2) [85]. The biocatalyst was removed out of the reaction medium using magnetic forces and was capable of six reuses in the hydrolysis of ketoprofen methyl ester.

It should be recalled that a suitable biotransformation for industrial application should reach an enantiomeric ratio E at least in the range of 15–30 [88]. This condition involves a conversion below 50% and an enantiomeric excess higher than the conversion value. Comparing the E ratios of the esterification presented in Table 1, it is possible to conclude that only in a few cases the lipases comply with that requirement under narrow conditions of reaction. For instance the esterification of ketoprofen with ethanol catalyzed with Novozym® 435 reaches an E 15 in ethylene dichloride at 37 °C [30] (entry 4 in Table 1) and firefly luciferases show an E in the 19–24 range (entry 13 in Table 1) [39]. The lipase from *Candida rugosa* catalyzes the esterification of the *S*-enantiomer with *E* values ranging from 16

Table 2. Biocatalyst, reagents, reaction conditions (temperature, time, others), co-solvents, conversion, enantiomeric excess, enantiomeric ratio, enantiomeric factor, and specific activity in the kinetic resolution of racemic ketoprofen through enantiomeric hydrolysis reported in the literature in the last 22 years.

Entry	Biocatalyst	Reagents	Reaction conditions	Emulsifier or solvent	Conversion (X%), enantiomeric excess (ee%) and enatiomeric ratio (E)	Reference
1	Commercial *Candida rugosa* lipase (CRL) and in the form of a cross-linked enzyme crystal (CRL-CLEC)	*R*-/*S*-ketoprofen chloroethyl ester and buffer sodium acetate 0.1 M at pH 5	40 °C, 200 rpm, 40 h. *S*-enantioselectivity	No	CRL: ee % *R*-keto = 54.7, X % = 36.7, E_{app} = 5.2 CRL-CLECs: ee % *R*-keto = 88.6, X % = 49.3, Eapp = 64:	[47]
2	Commercial an purified lipase from *Candida cylindracea* (now *Candida rugosa)*	*R*-/*S*-ketoprofen ethyl ester and 0.1 M Tris/HCl buffer pH 7.0	30 °C, 700 rpm, 96 h. *S*-enantioselectivity	No	Commercial: X % = 19 Purified: X % = 39	[48]
3	Lipase from *Candida cylindracea* immobilized on alumina, silica and agarose	*R*-/*S*-ketoprofen ethyl ester and 0.1 M Tris/HCl buffer pH 7.0	35 °C, 700 rpm, 168 h. *S*-enantioselectivity	No	Alumina: X % = 14.5, ee *S*-keto % = 95 Silica: X % = 20.5, ee *S*-keto % = 97 Agarose: X % = 21.1, ee *S*-keto % = 95	[49]
4	Lipase OF (from *Candida rugosa*)	*R*-/*S*-ketoprofen 2-chloroethyl ester and buffer 0.1 M citrate/0.2 M Na_2HPO_4	30 °C, 160 rpm *S*-enantioselectivity	No	pH 4.0: *E* = 1.2, A = 172 pH 2.2: *E* = 13.6, A = 202	[50]
5	Lipase OF (from *Candida rugosa*)	Ketoprofen 2-chloroethyl ester and 50 mM of sodium phosphate buffer at pH 7.0	30 °C, 160 rpm, 10 h. *S*-enantioselectivity	With and without emulsifiers	Data without using an emulsifier: X % = 8.7, ee *S*-keto % = 8.3, *E* = 1.2 Data with non-ionic emulsifiers: Tween-80: X % = 46, ee *S*-keto % = 60, *E* = 6.7 Tween-60: X % = 24, ee *S*-keto % = 6.4, *E* = 1.2 Dodecyl phosphate: X % = 8.0, ee *S*-keto % = 3.2, *E* = 1.1 OP-10: X % = 43, ee *S*-keto % = 4.5, *E* = 1.1 Span-60: X % = 9.2, ee *S*-keto % = 3.0, *E* = 1.1 Data with anionic emulsifiers: AOT: X % = 2.2, ee *S*-keto % = 26, *E* = 1.7 Sodium dodecyl sulfate: X % = 5.0, ee *S*-keto % = 2.0, *E* = 1.1 Data with cationic emulsifiers: BODMAC: X % = 4.9, ee *S*-keto % = 6.0, *E* = 1.1 Benzethonium chloride: X % = 3.9, ee *S*-keto % = 68, *E* = 5.4	[51]
6	Lipase AK (from *Pseudomonas fluorescens*), lipase PS (from *Pseudomonas cepacia*), lipase M (from *Mucor javanicus*), lipase AY (from *Candida rugosa*), lipase SP 435 (from *Candida antarctica*), lipases OF and CCL (from *Candida cylindracea*), lipase LIP (from *Pseudomonas aeruginosa*), and α-chymotrypsin (from bovine pancreas)	*R*-/*S*-ketoprofen monochloroethyl ester and 0.1 M phosphate buffer pH 7.0	30 °C, 120 rpm	Acetone	Lipase M: E = 17 Lipases AY, OF and CCL: E ≤ 2 Lipases AK, PS, LIP and α-chymotrypsin: inactive	[52]
	Lipase M (from *Mucor javanicus*) and lipase M immobilized onto Hyflo Super-cel and Toyonite 200-M and 200-A	*R*-/*S*-ketoprofen monochloroethyl and trifluoroethyl ester and 0.1 M phosphate buffer at pH 7.0	30 °C, 120 rpm, 3 h (except lipase immobilized onto Toyonite 200-M, 0.5 h) *R*-enantioselectivity	Various co-solvent with free lipase acetone as co-solvent with immobilized lipase	Lipase M: Monochloroethyl ester of *R*-/*S*-ketoprofen: Non-emulsified: X % = 32, ee *R*-keto % = 40, *E* = 3 Tertbutyl methyl ester: X % = 18, ee *R*-keto % = 62, *E* = 5	

(*continued*)

Table 2. Continued

Entry	Biocatalyst	Reagents	Reaction conditions	Emulsifier or solvent	Conversion (X%), enantiomeric excess (ee%) and enatiomeric ratio (E)	Reference
					Tertbutyl alcohol: X % = 23, ee *R*-keto % = 49, *E* = 3 Methanol: X % = 38, ee *R*-keto % = 82, *E* = 17 Acetonitrile: X % < 5 DMSO: X % = 50, ee *R*-keto % = 54, *E* = 6 DMF: X % = 44, ee *R*-keto % = 86, *E* = 27 Acetone: X % = 31, ee *R*-keto % = 93, *E* = 41 Trifluorethyl ester of *R*-/*S*-ketoprofen: Acetone: X % = 32, ee *R*-keto % = 94, *E* = 50 Immobilized lipase: Trifluorethyl ester of *R*-/*S*-ketoprofen: Toyonite 200-M: X % = 66, ee *R*-keto % = 46, *E* = 8 Toyonite 200-A: X % = 53, ee *R*-keto % = 86, *E* = 55 Hyflo Super-Cel: X % = 43, ee *R*-keto % = 91, *E* = 44	
7	Lipase OF (from *Candida rugosa*) and two-step acetone-treated CRL	*R*-/*S*-ketoprofen ethyl ester and 50 mM MES buffer at pH 6.0	24 h of reaction *S*-enantioselectivity	No	Lipase OF: X% = 11.6, ee *S*-keto % = 36, *E* = 2.2 Two-step acetone-treated CRL: X% = 11.3, ee *S*-keto % = 100, *E* > 100	[53]
8	Lipase from *Candida rugosa* wild-type and mutants (Phe344Val; Phe344,345Val and Phe345Val)	*R*-/*S*-ketoprofen methyl ester and 0.2 M phosphate buffer pH 7.9	40 °C, stirring, 168 h *S*-enantioselectivity	No	Wild-type: X % = 33, ee *S*-keto % = 89.28, *E* = 27.2 Phe344Val: X % = 38, ee *S*-keto % = 86.28, *E* = 23.0 Phe344,345Val: X % = 9, ee *S*-keto % = 61.84, E = 4.5 Phe345Val: no reaction	[54]
9	Lipases from *Candida rugosa*: OF, MY, Type VII	*R*-/*S*-ketoprofen 2-chloroethyl ester and 0.1 M citrate/0.2 M Na_2HPO_4 buffer at pH 7.0, 4.0, and 2.5	30 °C, 160 rpm, 120 h *S*-enantioselectivity	No	pH 7.0: Lipase OF: X % = 13.0, ee *S*-keto % = 4.5, *E* = 1.1 Lipase MY: X % = 20.9, ee *S*-keto % = 23.6, *E* = 1.7 Type VII: X % = 9.5, ee *S*-keto % = 12.0, *E* = 1.3 pH 4.0: Lipase OF: X % = 20.5, ee *S*-keto % = 26.9, *E* = 1.9 Lipase MY: X % = 24.5, ee *S*-keto % = 60.0, *E* = 4.8 Type VII: X % = 8.5, ee *S*-keto % = 27.6, *E* = 1.8 pH 2.5: Lipase OF: X % = 29.2, ee *S*-keto % = 77.6, *E* = 13.2 Lipase MY: X % = 8.7, ee *S*-keto % = 86.9, *E* = 15.5 Type VII: X % = 10.4, ee *S*-keto % = 89.4, *E* = 19.8	[55]
	Lipases from *Candida rugosa*: OF, MY, Type VII	*R*-/*S*-ketoprofen 2-chloroethyl ester and 0.1 M citrate/0.2 M Na_2HPO_4 buffer at pH 70, 4.0, and 2.5	30 °C, 160 rpm, 24 h *S*-enantioselectivity	Tween-80	pH 7.0: Lipase OF: X % = 69.9, ee *S*-keto % = 16.5, *E* = 1.9 Lipase MY: X % = 10.6, ee *S*-keto % =54.2, *E* = 3.6 Type VII: X % = 10.0, ee *S*-keto % = 50.1, *E* = 3.2 pH 4.0: Lipase OF: X % = 29.0, ee *S*-keto % = 41.9, *E* = 2.9 Lipase MY: X % = 8.9, ee *S*-keto % = 86.2, *E* = 14.7 Type VII: X % = 8.5, ee *S*-keto % = 86.3, *E* = 14.7 pH 2.5: Lipase OF: X % = 33.7, ee *S*-keto % = 95.0, *E* = 62.2 Lipase MY: X % = 4.5, ee *S*-keto % = 92.6, *E* = 27.2 Type VII: X % = 4.9, ee *S*-keto % = 93.8, *E* = 32.8	
10	Esterase from Pseudomonas sp. S34	*R*-/*S*-ketoprofen ethyl ester and 50 mM Tris-HCl	30 °C, 28h *S*-enantioselectivity	Triton X-100	X% = 47, ee *S*-keto % > 99	[56]

(*continued*)

Table 2. Continued

Entry	Biocatalyst	Reagents	Reaction conditions	Emulsifier or solvent	Conversion (X%), enantiomeric excess (ee%) and enatiomeric ratio (E)	Reference
11	Esterase from *Bacillus stearothermophilus* JY144	*R*-/*S*-ketoprofen ethyl ester and 50 mM Tris-HCl	37 °C, 25 h *S*-enantioselectivity	Triton X-100	X % = 50, ee *R*-keto % > 98	[57]
12	Esterase from *Trichosporon brassicae*	*R*-/*S*-ketoprofen ethyl ester and 50 mM potassium phosphate buffer	30 °C, 180 rpm, 10 h	Tween 80	Optimum relative activity: 310%	[58]
13	Novozym® 435	Ketoprofen propyl ester in a basic aqueous solution ($NaHCO_3$ 1 N)	50 °C, 10 h, 150 rpm. Solvent-free two-phase system *R*-enantioselectivity	No	X % = 56, *E* = 8	[59]
14	Recombinant esterase from *Pseudomonas fluorescens*	*R*-/*S*-ketoprofen ethyl ester and 50 mM buffer Tris-HCl	35 °C, 180 rpm, 35 h *S*-enantioselectivity	Various alcohols and surfactants	ee *S*-keto % > 99	[60]
15	Recombinant esterase from *Pseudomonas* sp.	*R*-/*S*-ketoprofen ethyl ester and 50 mM Tris-HCl pH 8.5	30 °C *S*-enantioselectivity	Triton X-100	SA =2360	[61]
16	Lipase from *Candida rugosa*	*R*-/*S*-ketoprofen ethyl ester, 50 mM phosphate/NaCl buffer pH 7.0 and 30 mM hydroxypropyl-β-cyclodextrin	37 °C, 200 rpm, 5 days *S*-enantioselectivity	No	X % = 49, ee *S*-keto % = 99	[62]
17	Lipase from *Acinetobacter*	*R*-/*S*-ketoprofen ethyl ester dissolved in ethanol and 40 mM Tris/HCl buffer pH 7	30 °C, 200 rpm, 48 h *S*-enantioselectivity	Triton X-100	X % = 35, ee *S*-keto % = 96	[63]
18	Free lipase OF (from *Candida rugosa*) and immobilized onto SP-Sephadex C-50	*R*-/*S*-ketoprofen 2-chloroethyl ester dissolved in ethanol and 7 mM citrate/15 mM Na_2HPO_4 buffer pH 3.5	30 °C, 160 rpm, 10 h (with Tween-80) and 7 h (non-emulsified) *S*-enantioselectivity	Non-emulsified operation and addition of Tween-80	Data obtained with a non-emulsified system: Free lipase OF: X % = 11.4, ee *S*-keto % = 17.1, *E* = 1.4 Immobilized lipase OF: X % = 8.7, ee *S*-keto % = 70.1, *E* = 6.1 Data obtained with the addition of Tween-80: Free lipase OF: X % = 32.2, ee *S*-keto % = 80.2, *E* = 13 Immobilized lipase OF: X % = 22.3, ee *S*-keto % = 94.9, *E* = 50	[64]
19	Lipase from *Candida rugosa* immobilized onto various supports	*R*-/*S*-ketoprofen ethyl ester and 0.1 M Tris/HCl buffer pH 7	168 h of reaction *S*-enantioselectivity	No	Alumina: X % = 14.5, ee *S*-keto % = 95 Silica: X % = 20.5, ee *S*-keto % = 97 Agarose: X % = 21, ee *S*-keto % = 94	[34]
20	Lipase from a mutant strain of *Trichosporon laibacchii*	*R*-/*S*-ketoprofen ethyl ester	23 °C, 150 rpm, 72 h *S*-enantioselectivity	Tween-80	X% = 47, ee *S*-keto % = 94, *E* = 82.5	[65]
21	Recombinant esterase Est3 from *Sulfolobus solfataricus*	*R*-/*S*-ketoprofen methyl esters and Tris-HCl buffer pH 7.0	60 °C, 32 h *R*-enantioselectivity	Various solvents and detergents	X % = 20; ee *R*-keto % = 88, *E* = 10	[66]
22	Free lipase OF (from *Candida rugosa*) and immobilized onto pH-sensitive supports (polymers)	*R*-/*S*-ketoprofen 2-chloroethyl ester and 0.1 M citrate/0.2 M Na_2HPO_4 buffer pH 7	30 °C, 160 rpm, 10 h *S*-enantioselectivity	No	Free lipase OF: Relative activity % = 1.0, *E* = 1.2 Support 1: Relative activity % = 1.53, *E* = 10.5 Support 2: Relative activity % = 1.55, *E* = 5.6 Support 3: Relative activity % = 1.38, *E* = 3.9 Support 4: Relative activity % = 1.42, *E* = 4.7 Support 5: Relative activity % = 1.46, *E* = 5.5	[67]
23	Lipase from *Candida rugosa*	*R*-/*S*-ketoprofen methyl ester and 0.1 M citrate/0.2 M Na_2HPO_4 buffer pH 7.5	30 °C, 160 rpm *S*-enantioselectivity	Cyclohexane	*E* = 38	[36]
24	Free lipase OF (from *Candida rugosa*) and immobilized onto inorganic porous supports (silica gel and Toyonite 200-M), macroporous adsorption resins (Amberlite XAD-7 and HZ 802) and an epoxy resin (HZ PGET)	*R*-/*S*-ketoprofen 2-chloroethyl ester and 0.1 M citrate/0.2 M Na_2HPO_4 buffer pH 2.5	30 °C, 120 rpm, 5h *S*-enantioselectivity	Tween-80	Free lipase OF: X % = 18.2, ee *S*-keto % = 95, *E* = 48 Lipase OF immobilized onto various supports Silica gel: X % = 33.4, ee *S*-keto % = 97.4, *E* = 125 Toyonite 200-M: X % = 21.1, ee *S*-keto % = 96.8, *E* = 79 HZ 802: X % = 11.2, ee *S*-keto % = 96.9, E = 153 XAD-7: X % = 4.7, ee *S*-keto % = 98.6, E = 46 HZ PGET: X % = 5.3, ee *S*-keto % = 95.5, E = 48	[68]

(*continued*)

Table 2. Continued

Entry	Biocatalyst	Reagents	Reaction conditions	Emulsifier or solvent	Conversion (X%), enantiomeric excess (ee%) and enatiomeric ratio (E)	Reference
25	Partially purified *Carica papaya* lipase (PCPL)	*R*-/*S*-ketoprofen 2,2,2-trifluoroethyl thioester	45 °C *R*-enantioselectivity	Isooctane	$E = 30$	[69]
26	Lipases/esterases from *Bacillus* strain NK13 crude and purified fractions	*R*-/*S*-ketoprofen chloroethyl ester and 1 M PBS pH 7.0	30 °C, 140 rpm, 72 h Enantioselectivity depends on the fraction	Ethyl acetate	Some lipases are *S*-enantioselective while others are *R*-enantioselective. High yield and low optical purity	[70]
27	Native and surfactant-coated lipase Type VII (from *Candida rugosa*)	*R*-/*S*-ketoprofen methyl ester and 0.1 M citrate/0.2 M Na_2HPO_4 buffer pH 7.0 coupled with photo-oxidation of methanol	30 °C, 120 rpm, 5 h *S*-enantioselectivity	Isooctane	Without photo-oxidation: Native: E = 51, X % = 11 Surfactant-coated: $E = 52$, X % = 12 With photo-oxidation: Native: $E = 53$, X % = 47 Surfactant-coated: $E = 50$, X % = 49	[71]
28	Lipase from *Acinetobacter sp*. ES-1	*R*-/*S*-ketoprofen ethyl ester and 40 mM Tris-HCl buffer pH 7.0	37 °C, 200 rpm, 24 h *S*-enantioselectivity	Triton X-100	X % = 42, ee *S*-keto % = 98	[72]
29	Lipases from *Carica papaya* partially purified (pCPL)	*R*-/*S*-ketoprofen 2,2,2-trifluoroethyl thioester	45 and 60 °C, stirring	Isooctane	45 °C: $E = 30$ 60 °C: $E = 20$	[73]
30	Esterase Est25 from metagenomic library	*R*-/*S*-ketoprofen ethyl ester and 50 mM Tris-HCl buffer pH 7.5	25 °C, 1 h *R*-enantioselectivity	Non-emulsified and non-ionic emulsifiers	Non-emulsified: ee *R*-keto % = −4.6 Tween-20: ee *R*-keto % = −0.3 Tween-40: ee *R*-keto % = −0.3 Tween-60: ee *R*-keto % = −0.7 Tween-80: ee *R*-keto % = −2.3 Triton X-15: ee *R*-keto % = −1.0 Triton X-45: ee *R*-keto % = −1.5 Triton X-100: ee *R*-keto % = −3.5 Triton X-165: ee *R*-keto % = −0.37 Triton X-305: ee *R*-keto % = −0.7	[74]
31	Lipase from *Serratia marcescens*	*R*-/*S*-ketoprofen methyl/ethyl/2-chloroethyl/isopropyl/butyl/octyl ester	30 °C, 1000 rpm, 24 h *S*-enantioselectivity	Dimethyl sulfoxide (DMSO)	Methyl ester: X% = 51.3, ee *S*-keto % = 69.1, $E = 12$ Ethyl ester: X% = 48.2, ee *S*-keto % = 91.6, $E = 63$ 2-chloroethyl ester: X% = 41.0, ee *S*-keto % = 50.4, $E = 4$ Isopropyl ester: X% = 31.1, ee *S*-keto % = 59.2, $E = 5$ Butyl ester: X% = 42.4, ee *S*-keto % = 78.8, $E = 15$ Octyl ester: X% = 47.9, ee *S*-keto % = 85.2, $E = 30$	[75]
32	Recombinant esterase EST-Y29 from metagenome library	*R*-/*S*-ketoprofen ethyl ester	*R*-enantioselectivity	Ethanol 5% and DMSO 5 and 10%	Increasing amount of organic solvents led to a gradual decrease of EST-Y29 activity, except for DMSO.	[76]
33	Esterase from *Pseudomonas sp*	*R*-/*S*-ketoprofen ethyl ester and Tris-HCl buffer pH 8.0 in a bicontinuous microemulsion system	80 rpm, 40 °C, 50 h *S*-enantioselectivity	Cetyltrimethylammonium bromide (CTAB) and 1-pentanol	X % = 84.6, ee *S*-keto % = 50	[77]
34	Immobilized *T. laibacchii* lipase	*R*-/*S*-ketoprofen ethyl ester and phosphate buffer pH 7.5	45 °C, 33 h *S*-enantioselectivity	Tween-80	$E = 87.4$, ee *S*-keto % = 94.4	[78]
35	*Bacillus subtilis* lipase A	*R*-/*S*-ketoprofen vinyl ester and PBS buffer pH 7.0	20 °C, 200 rpm, 24 h *R*-enantioselectivity	No	X % = 32.1, ee *R*-keto % = 87.9	[79]
36	*Candida antarctica* lipase B: wild-type and mutants M1, M2, M3, M4, and M5	*R*-/*S*-ketoprofen 4-nitrophenyl ester	Wild-type: 20 h, *R*-enantioselectivity Mutants: 1 h, *S*-enantioselectivity	No	Wild-type: X % = 0.7, ee *R*-keto % = 26.2, $E = 1.7$ M1: X % = 10.3, ee *S*-keto % = 65.2, $E = 5.1$ M2: X % = 12.8, ee *S*-keto % = 94.6, $E = 42.1$ M3: X % = 10.2, ee *S*-keto % = 93.6, $E = 33.6$ M4: X % = 13.0, ee *S*-keto % = 93.9, E = 36.7 M5: X % = 36.0, ee *S*-keto % = 99.1, $E > 200$	[80]

(*continued*)

Table 2. Continued

Entry	Biocatalyst	Reagents	Reaction conditions	Emulsifier or solvent	Conversion (X%), enantiomeric excess (ee%) and enatiomeric ratio (E)	Reference
37	Esterase from *Thermotoga maritima*	*R*-/*S*-ketoprofen ethyl ester and 50 mM sodium acetate buffer pH 5.5	70 °C, 200 rpm, 23 h *S*-enantioselectivity	No	X % = 42.1, *E* = 91.4, ee *S*-keto % = 95.7	[81]
38	Esterase Est25 wild-type and mutants (L255F, L255W, L255A, and L255D)	*R*-/*S*-ketoprofen ethyl ester and 50 mM Tris-HCl buffer pH 8.0	30 °C S-enantioselectivity	Triton X-100	Wild-type: X % = 90, ee *S*-keto % = 0, *E* = 1 L255F: X % = 90, ee *S*-keto % = 0, *E* = 1 L255W: X % = 50, ee *S*-keto % = 80, *E* = 18 L255A: X % = 12, ee *S*-keto % = 70, *E* = 6 L255D: inactive	[82]
39	Lipase from *Aspergillus terreus* free and immobilized onto Alg-g-PEG/α-CD hollow spheres	*R*-/*S*-ketoprofen vinyl ester and 20 mM Tris-HCl buffer pH 7.4	30 °C, 200 rpm, 96 h *R*-enantioselectivity	Acetone	Free: X % = 16.0, ee *R*-keto % = 82, *E* = 11.4 Immobilized: X % = 45.9, ee *R*-keto % = 96, *E* = 128.8	[83]
40	*Rhizomucor miehei* and *Mucor javanicus* immobilized on microemulsion organogels	Ketoprofen vinyl ester	30 °C, 24 h *R*-enantioselectivity	Various organic solvents	*Mucor javanicus*, X % = 35, ee *R*-keto % = 99, *E* > 200 *Rhizomucor miehei*, X % = 51, ee *R*-keto % = 86, *E* = 35 Highly reusable biocatalysts	[84]
41	*Pseudomonas cepacia* immobilized on magnetic cellulose nanocrystals	Ketoprofen ethyl ester, PBS buffer pH 6.5	35 °C, 200 rpm, 2.5 h *R*-enantioselectivity	Triton X-100	Yield % = 43.4, ee *R*-keto % = 83.5 6 cycles of reuse	[85]
42	Mutants lipases from *Candida rugosa* (CRL1 and CRL4) *and* lipase Lip2p from *Yarrowia lipolytica* wild-type and mutants (V232A, V232F, V232S, V235A, V235G, V97A-V232F)	*R*-/*S*-ketoprofen ethyl ester	Room temperature, stirred, 100 h CRL1, V232A, V232S, V235A, V235G: *S*-enantioselectivity CRL4, Lip2p, V232F, D97A-V232F: *R*-enantioselectivity	Decane	CRL1: *E* > 200 CRL4: *E* > 200 Lip2p: *E* = 1.5 V232A: *E* = 2.91 V232F: *E* > 300 V232S: *E* = 14 V235A: *E* = 12 V235G: *E* = 16 V97A-V232F: *E* > 300	[86]
43	Esterase Est25 wild-type and mutants (F72G, L255W, and F72G/L255W)	*R*-/*S*-ketoprofen ethyl ester and 200 mM Tris-HCl buffer (pH 8.0)	30 °C, 30 min *S*-enantioselectivity	Triton X-100	Est25: *E* = 1.1 F72G: *E* = 1.3 L255W: *E* = 16.1 F72G/L255W: E = 55.4	[87]

X%: conversion; ee *S*-keto %: enantiomeric excess towards the *S*-ketoprofen; ee *R*-keto %: enantiomeric excess towards the *R*-ketoprofen; $ee_p = ([R]_{ester} - [S]_{ester})/([R]_{ester} + [S]_{ester})$; *E*: enantiomeric ratio; E_{app}: apparent value dependent on the presence of effectors and product inhibition;EF: Enantiomeric Factor; A: One unit (U) of activity is the amount of enzyme that releases 1 μmol of product per hour; SA: Specific activity (U/g protein).

to 51 with various alcohols in isooctane and cyclohexane at 37 °C (entry 9 in Table 1) [35].

In contrast with the observations discussed above, hydrolysis of racemic esters of ketoprofen is by far the most suitable to obtain pure enantiomers. In this regard, the hydrolysis reaches *E* values above 50 with the lipase from *Candida rugosa* ($E > 100$) and the lipase from *Mucor javanicus* ($E > 200$) among others (entries 1, 6, 7, 9, 18, 23, 24, 27, and 40 in Table 2) [36,47,52,53,55,64,68,71,84]. Moreover, excellent results have been achieved with lipases from mutant strains of *C. rugosa, C. antarctica* and *T. laibachii* reaching *E* values higher than 200 (entries 8, 20, and 36 in Table 2) [54,65,80].

Free and immobilized lipases must be compared under similar reaction conditions in order to obtain reliable conclusions about the catalytic performance. This task is not an easy one since in most cases the authors do not compare the activity of the immobilized biocatalyst with the free counterpart. Nevertheless, the following is a comparison of the optimum catalytic performances of free and immobilized versions of the same enzyme that are found in the literature.

The esterification of racemic ketoprofen with *n*-butanol in hexane:1,2-dichloropropane catalyzed with free CALB showed 81% conversion with 59% enantiomeric excess towards the *S*-ketoprofen [37]. The commercial Novozym® 435 presented 29% conversion and 31% enantiomeric excess under similar reaction conditions [30]. Moreover, CALB immobilized on a membrane reactor showed 73% conversion and 57% of enantiomeric excess [40]. These results show that, in particular, the immobilized biocatalysts (either commercial or not) possess *E* values from 10 to 27 versus $E \sim 2$ for free CALB indicating an improved performance of the supported CALB.

Another straightforward example of the benefits of immobilization is the case of the lipase from *Burkholderia cepacia* [42]. This lipase is not active in its free form but shows activity in the esterification of ketoprofen with various alcohols and co-solvents.

The immobilized lipase from *Candida rugosa* on alumina, silica and agarose also shows an improved enantioselectivity in the hydrolysis of *R*-/*S*-ketoprofen ethyl ester. The free lipase shows 36% enantiomeric excess at 11.6% conversion [53]. However, the immobilized lipase reached an enantiomeric excess of 95–97% towards the *S*-enantiomer [34,49].

Kinetic resolution in non-aqueous environments

Most of the organic compounds such as ketoprofen are not soluble in aqueous media [89]. The overcome of such constraint using lipases in organic media has been the topic of numerous investigations as will be discussed in this section.

Tables 1 and 2 show the variety of solvents with different polarities that have been assayed in the kinetic resolution of ketoprofen. Regularly, the hydrophobicity/hydrophilicity of the organic solvents is measured with the parameter known as "log P". This parameter is defined as the logarithm of the partition coefficient of a given component in an octanol-water two-phase system. In general, the activity of the lipases is higher in hydrophobic solvents with a log *p* values greater than 2. Moreover, the higher the hydrophilicity of the solvent the lower the catalytic performance of the lipases. This observation is attributed to the fact that polar hydrophilic substances are able to remove water out of the enzyme which in turn affects their structure causing its denaturation [30,32,42,52,66,84]. Various investigations demonstrated that mixtures of two miscible solvents improved the enantiomeric ratio, the reaction rate, and the substrate solubility. These systems are mixtures of a non-polar solvent with a halogenated or hydrophilic solvent [30,33,35,37,40,90].

In the particular case of esterification reactions and returning to those systems with acceptable enantiomeric ratios *E* (discussed in the previous section), it is worth noting that the best results are obtained with non-polar hydrophobic solvents. Lipases demonstrate their best performance in ethylene dichloride, cyclohexane and isooctane (entries 4 and 9 in Table 1) [30,35]. Even though a wide variety of solvents has been assessed, results were not close to the expected for a satisfying kinetic resolution of ketoprofen.

In contrast, the addition of an organic solvent or emulsifier during hydrolysis reactions allowed an increasing enantiomeric ratio, even surpassing 200 (entries 5, 6, 9, 18, and 40 in Table 2) [51,52,55,64,84]. However, it should be commented that during *Candida rugosa* lipase catalyzed hydrolysis, very good results were obtained without the addition of a co-solvent or an emulsifier [34,47,53–55,62,64] (entries 1, 7–9, 16, 18, and 19 in Table 2). It appears that it is an economical and environmental sustainability advantage of the hydrolysis over the esterification.

To the knowledge of the authors, the investigation reported by Lozano et al. is the only one devoted to the use of ionic liquids (ILs) and supercritical CO_2 in the esterification of racemic ketoprofen with *n*-butanol in a membrane type of reactor (entry 15 in Table 1) [41]. Additional details of this investigation are provided in the "Scaling-up of the kinetic resolution towards the commercial production of dexketoprofen" section.

Concluding remarks about the use of organic solvents regards their toxicity and environmental concerns

during the disposition of these hazardous substances. In fact, toxicity is the main reason for the control of residual solvents and their elimination is driven by regulations that limit their concentration in pharmaceuticals [91,92]. Moreover, most organic solvents are volatile, flammable, hazardous to humans, and the environment and they are the main component of generated waste in the pharmaceutical industry. There is no doubt that these observations point out the relevance of greener solvent free processes as discussed in the following sections.

Interfacial activation of lipases with the addition of surfactants

The effect of the addition of surfactants has also been investigated in the hydrolysis of ketoprofen esters in order to improve the catalytic performance of the biocatalysts. In general, the active site of the lipases and esterases contains a catalytic triad that is buried completely beneath a helical segment or lid. The enzyme's active site is exposed by the movement of the helical lid that occurs in the presence of aqueous-oily environments. The addition of emulsifiers in the reaction medium somehow mimics that microenvironment, promotes the exposure of the active site and the accessibility of the substrate.

In this context, several authors reported the addition of the non-ionic surfactants Tween-80 [55,58,64–66,68,74,78] (entries 9, 12, 18, 20, 21, 24, 30, and 34 in Table 2) and Triton X-100 [56,57,61,63,66,72,74,82,85,87] (entries 10, 11, 15, 17, 21, 28, 30, 38, 41, and 43 in Table 2) in the hydrolysis of *R*-/*S*-ketoprofen esters catalyzed by lipases and esterases. In this regard, many investigations provide evidences of the high enantiomeric excess and enantiomeric ratio of the enzymes in the presence of the surfactants (Table 2). For example, the lipase OF from *Candida rugosa* achieves an E 62 (entry 9 in Table 2) with the addition of Tween-80 and no added co-solvent [55]. Additionally, the esterase from *Bacillus stearothermophilus* in Triton X-100 shows and enantiomeric excess above 98 and 50% conversion that corresponds to an optimum performance ($E > 400$) as discussed before (entry 11 in Table 2) [57].

Liu et al. reported a dependence of lipase activity with this kind of surfactant [51]. Non-ionic surfactants enhanced the catalytic performance while the cationic (bis-octadecyl dimethyl ammonium chloride called BODMAC, benzethonium chloride, and cetyltrimethylammonium bromide) and anionic (sodium dodecyl sulfate and bis-2-ethylhexylsodium sulfosuccinate typically called AOT) surfactants showed inhibitory effects on the lipase. Actually, the hydrolysis with such emulsifiers shows enantiomeric ratios $E \leq 5$ (entries 5 and 33 in Table 2) [51,77]. These effects are attributed to ionic interactions between the surfactant and the lipase, which induce unfolding and denaturing of the enzyme. On the other hand, non-ionic surfactants present only hydrogen-bonding and hydrophobic interactions with the enzyme. These interactions activate the enzyme and may change their enantioselectivity.

Kim et al. used chiral cyclodextrins to produce a complex with *R*-/*S*-ketoprofen ethyl ester that was able to disperse in an aqueous medium (entry 16 in Table 2) [62]. Hydroxypropyl-β-cyclodextrin was the most efficient chiral selector and disperser within the assayed cyclodextrins. This phenomenon was attributed to the formation of an inclusion complex between the cyclodextrins and 2-arylpropionic acid that enhances the solubility in order to perform the chiral resolution.

Kinetic resolution in solvent-free systems

Tables 1 and 2 show that the kinetic resolution of racemic ketoprofen is performed with the addition of varied organic solvents as discussed in the "Kinetic resolution in non-aqueous environments" section. However, the solvents are not completely removed during the actual manufacturing process and the residual amount of those substances in the pharmaceuticals (both in the active pharmaceutical ingredient and the excipients) is tightly regulated. The United States Pharmacopeia USP 30 indicates that those solvents belonging to *Class I* should be avoided (carbon tetrachloride; 1,2-dichloroethane; 1,1-dichloroethene, among others) [92,93]. Methanol, hexane, trichloroethylene, and acetonitrile (among others) that are within the *Class II* of solvents must be kept at low concentrations. Ethanol, acetone, 1-butanol, heptane, methyl isobutyl ketone, dimethyl sulfoxide among others, occur in *Class III* since they are considered less toxic and a lower risk to human health than the other classes of solvents.

Therefore, the solvent may sometimes be a critical element in the synthetic process and thus, a solvent-free system is of great interest as discussed before.

In this context, a solvent free-system consists of a simple mixture of reactants that allows a high substrate concentration, is cost saving, reduces environmental hazards, and allows the recovery of products without further complex purification steps (since fewer components would be present in the reaction mixture at the end of the manufacture).

There are few studies concerning lipase-catalyzed esterification in solvent-free systems (entries 6, 17, and 18 in Table 1) [32,43,44]. In this regard, Toledo

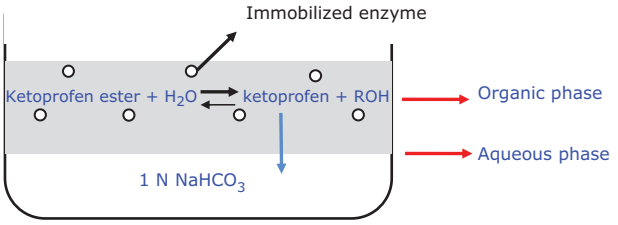

Figure 2. Schematic diagram of a solvent-free two-phase system for the hydrolysis of ketoprofen esters (from reference [59]).

et al. reported an enantiomeric ratio $E \sim 4$ using solely ethanol and 1-propanol as reactants and solvents [44].

The esters of ketoprofen are soluble in aqueous buffered systems therefore the hydrolysis can readily be performed without the addition of an organic solvent with excellent results as discussed before (entries 19, 1–4, 7–9, 13, 16, 22, and 35 in Table 2) [34,47–50,53–55, 59,62,67,79].

Jin et al. developed an interesting solvent-free two-phase system to hydrolyze ketoprofen esters catalyzed with Novozym® 435 [59]. The system involves an upper phase containing the butyl ester and a lower phase containing an aqueous solution of $NaHCO_3$ (Figure 2). The stereoselectivity of the biocatalyst allowed the production of *S*-ketoprofen ester as an unreacted substrate and *R*-ketoprofen as a product of the enzymatic reaction. The last one is transferred to the aqueous layer upon deprotonation in the basic media. The biphasic hydrolysis reached a low enantiomeric ratio (E 8) similarly to the results obtained in the esterification without added co-solvent.

Influence of the nature of the alcohol in the biocatalytic performance

There is no doubt that a key factor in the esterification is the alcohol nature of the reaction. The influence of the alcohol moiety is related to the mechanism of the action of lipases, which is known as a Ping–Pong Bi-Bi with two tetrahedral intermediates and an acyl–enzyme complex. As the formation of the acyl–enzyme intermediate is a crucial step in the esterification, the final reaction yield depends on the accessibility of the alcohol to the acyl–enzyme complex [94]. Microbial lipases catalyze the esterification of *R*-/*S*-ketoprofen preferentially with primary alcohols; the reaction rate diminishes with secondary alcohols and is practically nil with tertiary alcohols.

More recently, this research group performed a thorough investigation of the esterification of *R*-/*S*-ketoprofen with various alcohols catalyzed with Novozym® 435 [43,44]. The alcohols were used as reactants and solvents therefore no co-solvents were added in order to develop a greener process. The interaction of the alcohols with the biocatalyst was studied at a molecular level through *in situ* infrared spectroscopy and molecular modeling. The evidence undoubtedly demonstrates the dissolution of the polymeric support, loss of active protein, strong adsorption of the alcohols, modification of the secondary structure of the protein, and smoothing of the inner structure of the biocatalyst's beads upon extended contact with the alcohols [95]. Nevertheless, the specific activity and enantiomeric excess towards dexketoprofen remained unaltered upon extended contact with ethanol, 1- and 2-propanol as acyl acceptors. Theoretical calculations demonstrated that methanol introduces steric and electronic hindrance within the step of the coordination of the *R*-/*S*-ketoprofen with the catalytic triad.

Park et al. demonstrated that the highest rate of esterification occurred with primary alcohols but it was nil with secondary alcohols. The authors did not observe a correlation between the length of the carbon chain of the primary alcohols (C1–C8) and the activity of CALB [30].

In this context, the investigations of Li et al. suggested that primary and secondary alcohols could match the hydrophobic acyl-binding tunnel of the active site of the lipase from *Burkholderia cepacia* increasing the catalytic activity. However, the structure of the carbon chain of polyols and tertiary alcohols caused steric hindrance in the active site so that the esterification is inhibited [42]. In contrast with this observation, Chang and Hsu observed that the lipase from *Candida rugosa* was active with diols such as 1,4-butanediol and 1,3-propanediol [35]. However, the enantiomeric ratio of the esterification of ketoprofen with those alcohols is strongly influenced by the co-solvent. For instance, 1,3-propanediol with isooctane provides an E 51 but this value drops below zero in cyclohexane. In contrast, *E* equals 2 in the esterification with 1,4 butanediol in isooctane but the *E* value is 18 when cyclohexane is used.

Those conclusions indicate that there is not an univocal trend between the length and structure of the alcohols carbon chain and the catalytic performance. Therefore, the influence of that parameter must be investigated for each individual enzyme.

Influence of the water content in the catalytic activity

It is well known that a certain water activity (a_W) is required in order to retain the catalytically active

three-dimensional structure of the enzymes and it is crucial in enzymatic synthetic reactions in organic media. A molecular dynamics investigation of the structure of CALB in pure water and methanol, tert-butyl alcohol, methyl tert-butyl ether, and hexane with various water activities demonstrated that the hydration of the enzyme was similar regardless of the solvent. However, the solvent and a_w influence the structure and flexibility of the enzyme [96]. Investigations performed by Chamorro et al. demonstrated that a small amount of water leads to an enhancement of the catalytic performance of CRL which the authors relate to a more accessible active site. This enzyme lacks enantioselectivity unless a little amount of water is present in the reaction medium. In fact, conversion and specific activity for *S*-ketoprofen increases from 5% and 6.9 mM $U^{-1}\,h^{-1}$ to 27.5% and 47.7 mM $U^{-1}\,h^{-1}$ in the absence and presence of water, respectively. However, these parameters remain constant for *R*-ketoprofen [97].

Conversely, Foresti et al. proposed the presence of a network of H-bonding water pool near the active site which may interact with the histidine residue of CALB, disrupting the close interaction serine–histidine within the active triad. Theoretical calculations revealed that the addition of water to the double bond of the histidine (His224) is favored by -8.4 kcal mol^{-1} [98]. This observation indicates that high water content in the reaction media can be considered as an inhibiting factor. Banik et al. demonstrated that polar solvents compete with water molecules for interaction with the surface of CALB and are able to displace the molecules of water that form the first hydration shell of the enzyme [99]. The authors also concluded that high density of the solvent molecules near the active site region and at the entrance of the active site pocket indicates a clear affinity of the solvent for the active site region. The fact that the solvent molecules have a strong affinity toward the active site indicates that it can compete with substrate molecules and can act as an inhibitor resulting in a reduced catalytic activity.

Water is produced during esterification reactions. As it is accumulated in the reaction media, it decreases both the reaction rate and the ester yield. Molecular sieves, a saturated solution of certain salts and also the continuous consumption of water through a side chemical reaction have been strategies proposed to maintain constant water activity [100].

For instance, Park et al. obtained the highest enantiomeric ratio (E 15) in the esterification with ethanol in a solvent mixture with 0.15% v/v water or with the addition of $NaSO_4{\cdot}10H_2O$ [30].

Additionally, De Crescenzo et al. performed the enzymatic esterification of racemic ketoprofen under reduced pressure [32]. This process allows the continuous removal of the produced water by vaporizing the solvent rich in water under reduced pressure and recycling the dry solvent in the reaction media after passing it through a water trap.

Scaling-up of the kinetic resolution towards the commercial production of dexketoprofen

The successful industrial application of a biotransformation comprises a series of requirements that are summarized as follows [101]:

- The application of free crude or purified enzymes in aqueous or organic media in a batch or fed-batch reactor. The use of liquid–solid or liquid–liquid biphasic systems is of much interest for simple and straightforward industrial processes.
- Continuous application of immobilized enzymes in fluidized or fixed bed reactors.
- The use of enzyme membrane reactors when the biotransformation requires the recycling of a cofactor or expensive enzymes.
- The highest product concentration in the shortest possible time during a biocatalytic process (value creation).
- All the following steps in the downstream processing must be designed to minimize losses during isolation and purification (value conservation). Process intensification including process integration between value creation and value conservation is the key to cost control.

This section discusses the scientific investigations that, from the point of view of the authors, accomplish with some of those premises and therefore, might be considered as possessing potential for industrial applications.

In this context, Liu et al. tested a stirred tank reactor, a packed column and an air-bubbled column reactors in the biocatalytic hydrolysis of 2-chloroethyl ester of racemic ketoprofen [64]. The air-bubbled column reactor containing the immobilized lipase from *Candida rugosa* allowed a long term operation and maintains more than 50% of its initial activity for 300 h. This setup developed a productivity of 6.7 g ketoprofen per day per gram of immobilized enzyme, compared to 2.3 g for the packed column reactor.

D'Antona et al. reported large scale preparation of *S*-ketoprofen through a two-step kinetic resolution [33]. Initially, the racemic mixture of ketoprofen is esterified with ethanol in 1,2-dichloropropane until 58% conversion and 77% enantiomeric excess towards *S*-

ketoprofen is obtained. The catalyst employed, Novozym® 435, is separated and the solution is evaporated to dryness under vacuum. The oily residue is mixed with dicloromethane and sodium bicarbonate that generates two phases. Concentrated sulfuric acid is added until ketoprofen enantiomerically enriched in the *S*-enantiomer precipitates. The recovered solid is mixed with a similar amount of racemic ketoprofen and the process starts again. This two-step procedure reaches 60% overall yield and 96% enantiomeric excess towards the *S*-enantiomer.

Zhu et al. studied a two-step process involving both hydrolysis and esterification reactions (Figure 3) [36]. The hydrolysis of the methyl ester of ketoprofen catalyzed by the lipase from *Candida rugosa* is performed in a first step reactor. A second hydrolysis is performed under similar conditions although the ketoprofen methyl ester came from the re-esterification of the ketoprofen (enriched in *S*-ketoprofen) produced in the first hydrolysis step. The second hydrolysis ended when the enantiomeric excess of *S*-ketoprofen reached a desired value. Under these experimental conditions, the authors could achieve an *E* value of 38.

Regarding the esterification reaction, the operation was similar. The first esterification step is followed by a second esterification but employing the ketoprofen (enriched in *S*-ketoprofen) from the first one and the biocatalyst Novozym® 435. In this attempt, the authors only reached an *E* value of 10.5. A similar proposal was reported by Wu et al. that performed the esterification of *R*-/*S*-ketoprofen followed by the enzymatic hydrolysis of the ester product [90]. In the first step of the esterification, the ester conversion reached 58.3% and an enantiomeric excess of 83% to *S*-ketoprofen which corresponded to an *E* value of approximately 10. After this reaction, hydrolysis was performed of the ester product (which was enriched in *R*-enantiomer) in order to increase the *R*-ketoprofen concentration. This process achieved 90% of enantiomeric excess of *R*-ketoprofen when 30% of the ester was converted.

An alternative methodology, based on enzymatic membrane reactors (EMR), was developed by Ong et al. [40]. The substrates are fed in an EMR and the products separated through the membrane in a continuous flow type operation. The EMR technology offers high efficiency, ease of scale-up, applicability in continuous and

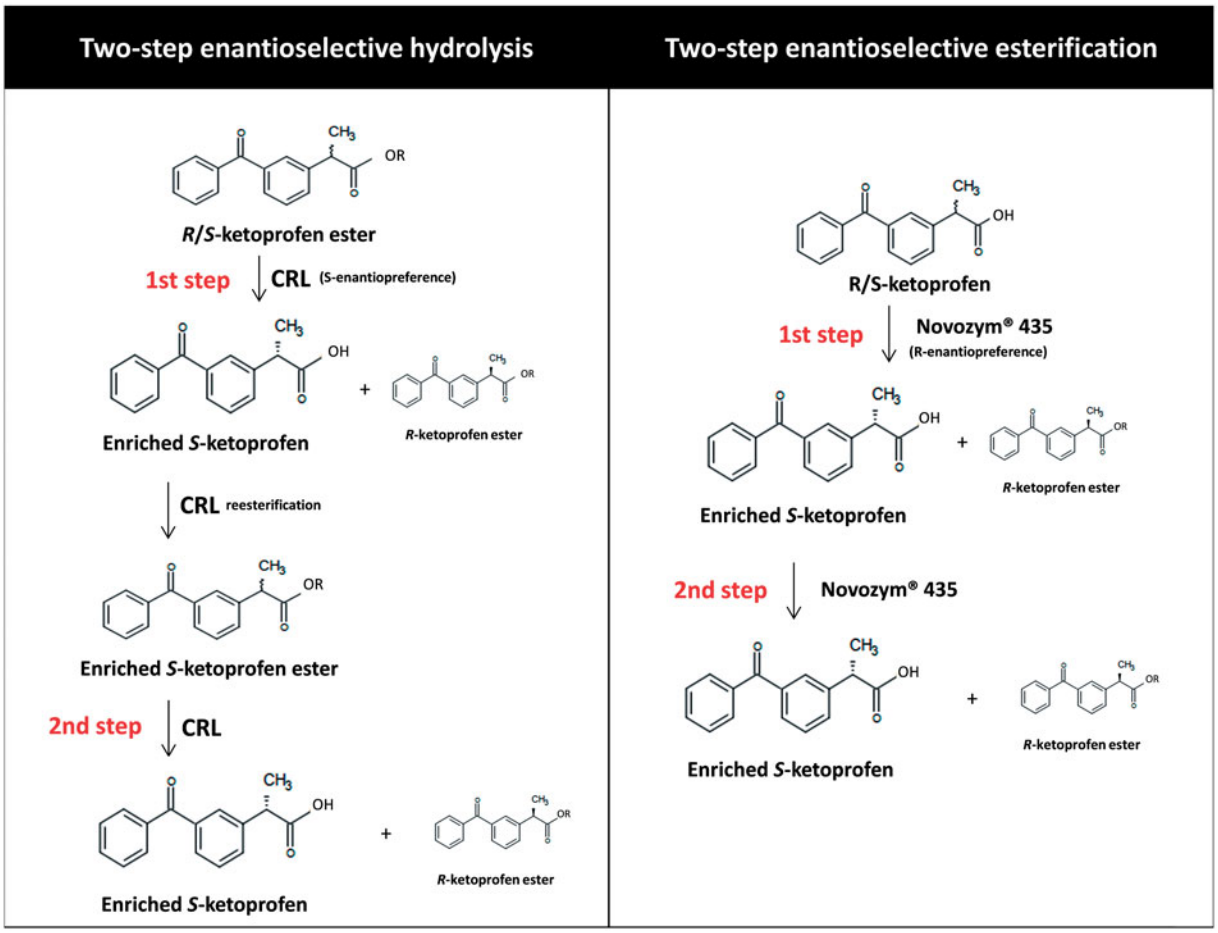

Figure 3. Two-step biochemical kinetic resolution of racemic ketoprofen (from reference [36]).

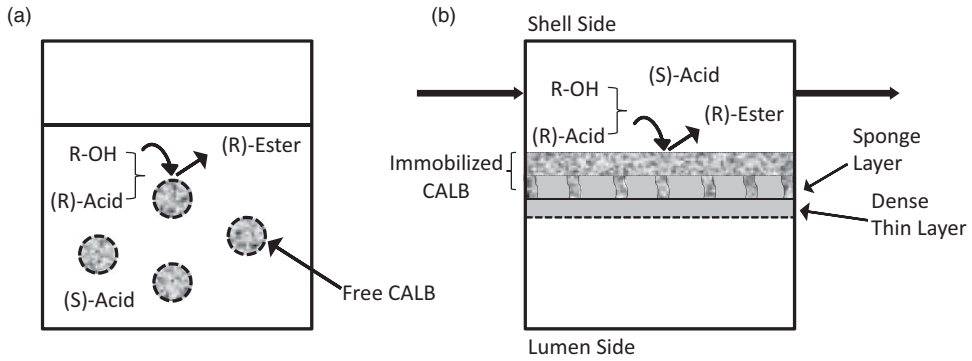

Figure 4. Comparison between the enzymatic esterification of *R*-/*S*-ketoprofen in a batch reactor (a) and enzymatic membrane reactor EMR (b) (from reference [40]).

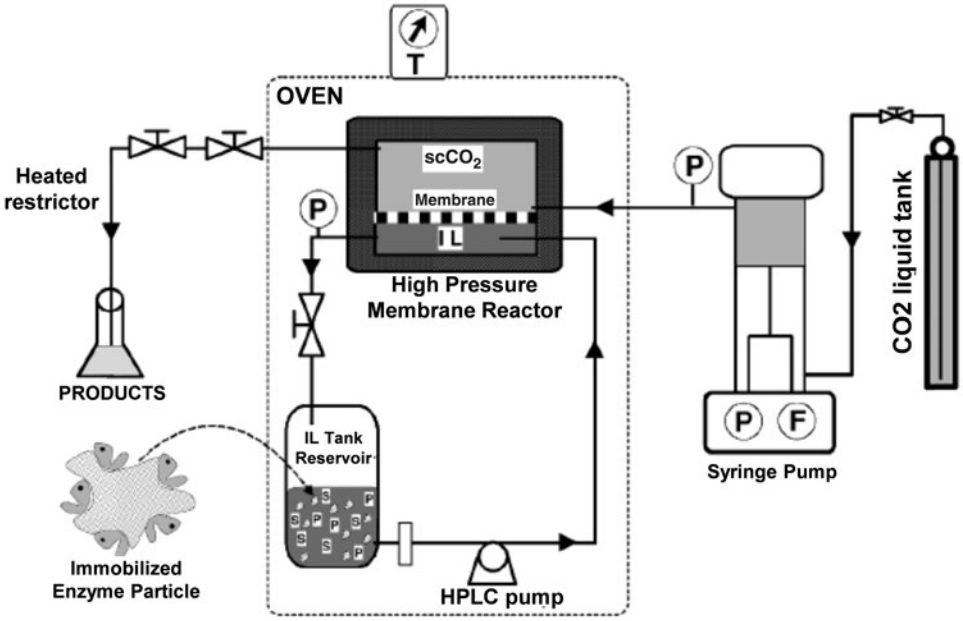

Figure 5. Enzymatic membrane reactor working with ILs and $scCO_2$ (from reference [41]).

steady state mode, easier retention, reuse of the enzyme, reduction of substrate/product inhibition, enzyme-free-end product, flexibility of the system configuration, enhanced stability of enzyme, and resistance to dilution by solution. The Figure 4 compares the enzymatic esterification of *R*-/*S*-ketoprofen in a batch reactor and an EMR where the esterification occurs at the layer of CALB immobilized on the membrane. This setup allows the esterification with an *E* 27 versus 10 of the operation in a conventional batch reactor.

Lozano et al. described an enzymatic membrane reactor based on ionic liquids (ILs) and supercritical CO_2 which was applied to the enantioselective esterification of *R*-/*S*-ketoprofen with 1-butanol (Figure 5) [41]. The $scCO_2$ flow was able to extract continuously the *R*-ketoprofen butyl ester from the IL medium through a polymeric membrane. The enantiomeric excess towards *S*-ketoprofen reaches 23% after a 10 h reaction (calculated from the data provided by the article) and remains constant up to 25 h of operation. This technique is a green chemistry tool to easily carry out the separation of ketoprofen isomers although more research must be conducted in order to improve the enantiomeric excess towards the *S*-enantiomer.

Hydrolysis of the *R*-/*S*-ketoprofen ester catalyzed by an esterase enzyme has been performed in bi-continuous microemulsion conditions by Sathishkumar [77]. This system features continuous oil and water phases intertwined in a dynamic extended network in which oil and water are separated by extended fluid interfacial films comprised of a monolayer of surfactant molecules. Bi-continuous microemulsions are capable of solubilizing the enzyme and hydrophilic substrates, as well as the hydrophobic reactants. Ionic and polar species

diffuse into the water phase and non-polar species diffuse into the oil phase. The esterase from *Pseudomonas sp* is dissolved into the aqueous phase, whereas the substrate and the product of the hydrolysis are dissolved in the organic medium. Although the conversion was higher and faster than in regular emulsified hydrolysis (such as those discussed in the "Interfacial activation of lipases with the addition of surfactants" section), the enantiomeric ratio *E* was 1.7. This observation indicates that this system requires further optimization in order to be useful for industrial applications.

Final remarks

This contribution is an overhaul of the kinetic resolution of racemic ketoprofen for the past 22 years. The most commonly employed biocatalysts are based on lipases in their free and immobilized form. Esterases were also applied in the hydrolysis of the esters of racemic ketoprofen. The exhaustive analysis of results presented in this review, allows the conclusion that the hydrolytic reaction is the most suitable for the kinetic resolution of ketoprofen. The optimal reaction medium conditions have been widely studied regarding the effect of different polarity solvents, the addition of emulsifiers, the nature of the alcohols used in the esterification, and the control of water activity. Scale-up attempts have been performed by applying two-step resolution techniques, enzymatic membrane reactors, and bi-continuous microemulsion processes.

The literature illustrates that a limited amount of supports have been investigated in the immobilization of enzymes for the kinetic resolution of ketoprofen. In this context, the application of biomass wastes from the agro-industry (modified or not) as novel supports might be "cutting edge" research that has not been explored in the kinetic resolution of racemic profens.

A further unexplored field is the application of deep eutectic solvents (known as the third generation of ILs) as replacement for conventional ILs and organic co-solvents. Deep eutectic systems possess straightforward preparation, biodegradability, and low cost. The feasibility of esterification with alcohols as reactants and solvents has recently been explored. Nevertheless, further investigation is necessary to improve the enantiomeric ratio of the reaction and to recover the *S*-enantiomer.

In the opinion of the authors these observations are an opportunity to develop a sustainable technology.

Disclosure statement

No potential conflict of interest was reported by the authors.

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