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On the Use of Orthoformates as an Efficient Approach to Enhance the Enzymatic Enantioselective Synthesis of (*S*)-Ibuprofen

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Abstract: In this paper, we describe the effectiveness of the combination between an organic solvent system mixture with orthoformates with different chain sizes from one to four carbon atoms. These orthoesters have been used as a "water trapper/alcohol releaser molecule" to reach a notable improvement in enantioselectivity and enantiomeric excess of our target compound, (*S*)-2-(4-isobutylphenyl)propanoic acid (ibuprofen eutomer), during the enzymatic kinetic resolution of *rac*-ibuprofen using immobilized lipase B of *Candida antarctica* as a biocatalyst. At the same time, one of the great problems of biocatalysis in organic media has been solved by eliminating excess water in the medium that allows the reversibility of the reaction. Following the optimization of the reaction conditions, an increase in enantiomeric excess and enantioselectivity was reached by using these acyl donors in the presence of a cosolvent.

Keywords: kinetic resolution; ibuprofen; eutomer; lipase; irreversible esterification; orthoformates; water trapping; cosolvents

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1. Introduction

Nowadays, more than 90% of analgesics (the most widely used pharmaceuticals) belong to the non-steroidal anti-inflammatory NSAIDs group [1,2], and 2-arylpropionic acids (profens) are one of their archetypical compounds. The development of safer profens continues to be a very active research topic, especially after the COVID-19 pandemic [3,4]. It is well established that the pharmacological effectiveness of profens is based on the ability to reduce the synthesis of prostaglandins by inhibiting cyclooxygenase (COX), that is exclusively exerted by the eutomer, in this case the *(S)*-(+)-enantiomer [5,6]. For instance, (*S*)-naproxen is about 28-fold more potent than its (*R*)-antipode [7], as its value is around 100 for (*S*)-ibuprofen [8]. This last compound is commercialized as racemic, as its (*R*)-distomer undergoes a partial (between 35–70%) in vivo metabolic chiral inversion into the eutomer [9,10]. Notably, ibuprofen (Code DB01050 inside DrugBank [11]) has been one of the drugs involved in what is known as the *chiral switch*, the replacement (either partial or complete) of a chiral drug used in the form of a racemate with its eutomer [12]; for this drug, it is possible to find in the pharmaceutical market, both its racemic and enantiopure version, this later one (named dexibuprofen; DrugBank Code DB09213) is commercialized as Seractil®, DexOprifen®, Deltaran®, Ibusoft® or Monactil®. Thus, the relevancy of the pharmacological uses of the *(S)*-(+)-enantiomer [13] and the higher cost associated with its preparation, compared to racemate, are the driving forces for the considerable efforts that have been made for obtaining enantiopure (*S*)-profens, being the employ of biocatalysed protocols as one of the preferred options [14–16](see also the recent article by Zdun et al. [17] and references cited therein illustrating different methodologies).

Among the different options, the most common one is the kinetic resolution (KR) of the racemic profens via lipase-catalysed esterification (Figure 1A) or, conversely, the lipase-catalysed hydrolysis of the corresponding racemic profen esters (Figure 1B).

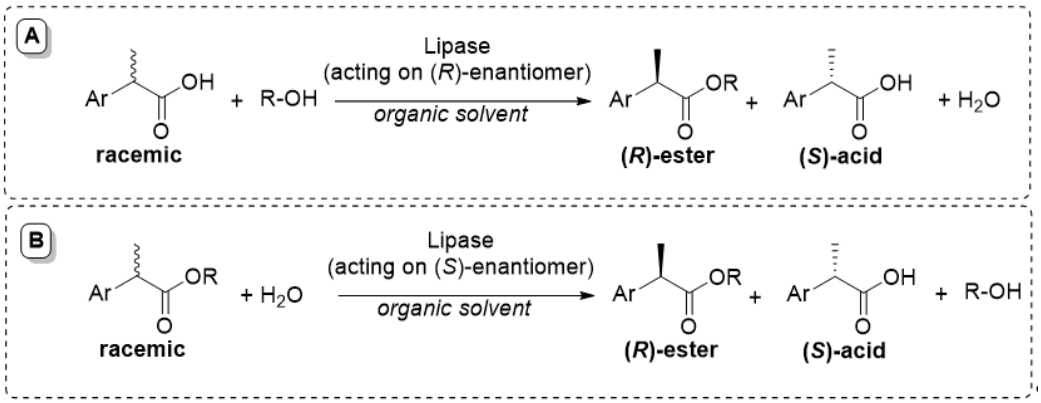

Figure 1. KR of the racemic profens leading to enantiopure (*S*)-(+)-acid (eutomer): (**A**) lipase-catalysed esterification; (**B**) lipase-catalysed hydrolysis of the racemic profen esters.

In this context, a variety of studies addressing the KR of ibuprofen and other racemic profens through esterification (Figure 1A), using the lipase B from *Candida antarctica* (CALB), acting on the (*R*)-enantiomer and therefore "cleaning" the racemate from the non-desired distomer [16,18–23]; conversely, the acylation of the (*S*)-(+)-enantiomer is reported when using *Candida rugosa* lipase (CRL) [24] or *Rhizomucor miehei* lipase (RML) for racemic ibuprofen [25–27], this later one shows an inversion in the stereopreference for *rac*-ketoprofen [26]. Logically, the (*S*)-stereobias of these two lipases is consequently better exploited using the hydrolytic approach shown in Figure 1B [28–31], and some other lipases, such as those of *Yarrowia lypolytica* [32] or *Aspergillus niger* [33], show a similar behaviour.

Regarding the acylation approach depicted in Figure 1A, the inherent reversibility of the esterification procedure demands the extension of the conversion time (at the expense of the chemical yield) to reach a high optical purity of the desired non-converted (*S*)-enantiomer. Moreover, water accumulation on the media, as well as promoting the hydrolysis of the formed ester, tends to form layers of water on the biocatalysts, promoting enzyme inactivation and worsening its performance. This deleterious effect has been reduced by using very hydrophobic supports for enzyme immobilization [34–40], ultrasounds [41–46] or physical methods [47–51]. Additionally, molecular sieves have been used to capture the water formed during the reaction [52–54], but this may increase the risk of reducing too much the available water and leaves conditions unfavourable for the enzyme performance, therefore demanding a careful control of the water activity through the corresponding water adsorption [55–57]. Finally, a chemical technique using dimethyl carbonate, that is able to react with water, has been reported in the KR of racemic naproxen [58].

In view of all of the issues associated with the KR of racemic profens, it seems very reasonable to implement irreversible methodologies to avoid all of the previously described undesirable effects. In this sense, a smart strategy is the use of orthoesters [59] which, upon trapping the water molecules produced as the esterification is progressing, suffer a hydrolysis and continuously release the alcohol required for the esterification. This strategy, schematized in Figure 2, was initially reported by Nicolosi and colleagues, using orthoformates (R^3 = H in Figure 2) for the enantioselective of flurbiprofen [60–62] and later expanded to fenoprofen [63].

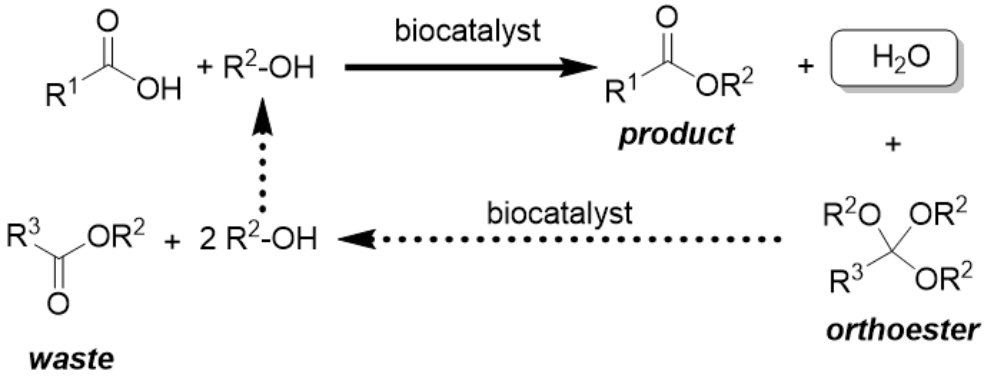

Figure 2. Schematic representation of the irreversible biocatalysed esterification of acids with orthoesters. R^1, R^2 and R^3 may be different alkyl moieties.

Very recently, Zdun et al. [17] have reported the use of methyl and ethyl orthoesters (orthoacetates and orthobenzoates, R^3 = Me or Ph) for the stereoselective esterification of different profens (ibuprofen, ketoprofen, naproxen, flurbiprofen), using Novozym®435, a commercial CALB immobilized on polymethylmethacrylate [64]. In all cases, the enantioselectivity reported was modest, as lipases are more effective in the chiral discrimination of substrates possessing the stereocentre in the alcohol rather than in the acid moiety, as occurs with profens [65]. For esterification of this acids (Figure 1A), it is established that the addition of a relatively polar cosolvent along the main solvent constitutes another approach to optimize the enantioselectivity of lipases in both hydrolysis and esterification reactions with profens [16]. In fact, DMSO was used as a cosolvent to increase the enantioselectivity of CRL in the hydrolysis (Figure 1B) of *rac*-ibuprofen [66]. For esterification catalysed by lipases in organic media (Figure 1A), it is generally recognized that the higher activity and selectivity are obtained using hydrophobic solvents [16], possessing logP values ≥ 2 (logP is the logarithm of the partition coefficient of the solvent in the two phase *n*-octanol–water system). The addition of cosolvents in these hydrophobic solvents shows a positive effect; for instance, the effect of chlorinated solvents was investigated in the kinetic resolution of *rac*-ketoprofen through an enantioselective esterification, by using free lipase B from *Candida antarctica* in a mixture of organic solvents, reporting the best results using 20% of 1,2-dichloropropane in *n*-hexane [67]. In another case [68], the influence of different organic cosolvents (such as isooctane, *n*-hexane, carbon tetrachloride, ethyl acetate, acetonitrile and tetrahydrofuran) was studied with the aim of diminishing the negative effect of ethanol, when used as both solvent and nucleophile, on Novozym®435 [69] catalysed esterification of *rac*-ibuprofen; remarkably, it was reported that the best performance was obtained using only ethanol without any cosolvent added.

In this article, we report the combined use of orthoformates (for trapping the water released and generating the nucleophile in situ) and solvent systems composed of an apolar solvent (isooctane) mixed with cosolvents, in order to improve both the enantioselectivity and the yield in the KR of *rac*-ibuprofen.

2. Results and Discussion

2.1. Cosolvent Screening

The use of cosolvents, combined with the irreversible KR using orthoformates, was carried out to assess a possible positive influence on the enantiodiscrimination of ibuprofen enantiomers with this immobilized CALB. The general scheme of the experimental procedure is depicted in Figure 3.

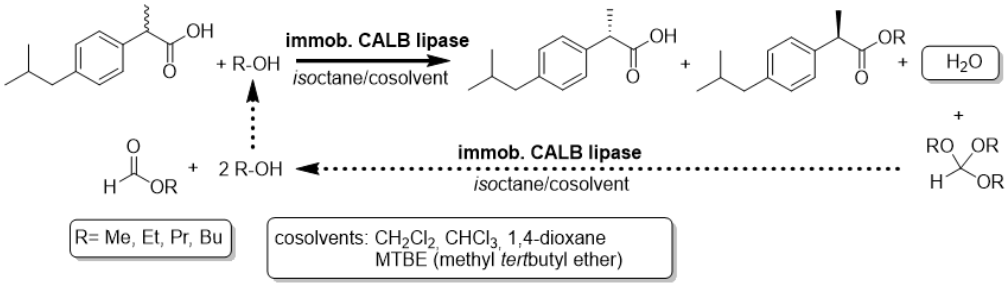

Figure 3. Schematic representation of the irreversible biocatalysed esterification of racemic ibuprofen with orthoformates.

Zdun et al. recently reported on how toluene (logP = 1.67) and *n*-hexane (logP = 3.0), among the different solvents tested, led to the best results in the KR of *rac*-ibuprofen with orthoacetates [17], although the enzymatic performance using *iso*octane (logP = 3.75) was similar to that obtained with *n*-hexane. Due to the fact that this apolar solvent has proven to be highly compatible with lipases in the acyl-transfer processes [29,64,68,70–73], we decided to use it as our standard reaction solvent. Thus, the initial experiments in the KR of *rac*-ibuprofen with triethyl orthoformate (TEOF) were carried out in order to assess the effect of adding different cosolvents to the standard isooctane, together with a small amount (0.5 eq., 7.2 µL, 0.125 mmol) of ethanol (see Section 3.3.1). The addition of this initial amount of EtOH would accelerate the initial enzymatic esterification, with the concomitant production of water molecules, which would be trapped by the enzyme to hydrolyse TEOF and generate more EtOH; interestingly, Zdun et al. [17] did not report any initial addition of the corresponding alcohol. Results are shown in Table 1.

Table 1. Effect of organic polar cosolvents on the kinetic resolution of *rac*-ibuprofen.

Entry	Solvent	Cosolvent (20% *v*/*v*)	Conversion (C), (%)	ee_s [1] (%)	E [2]	EF [3]
1	isooctane	—-	97	75	1.7	0.02
2	isooctane	CH_2Cl_2	44	69	31.8	0.87
3	isooctane	$CHCl_3$	47	70	17.6	0.79
4	isooctane	MTBE	82	47	1.8	0.10
5	isooctane	1,4-dioxane	7.3	1.4	3.7	0.18

Reaction conditions: 5 mL of solvent, *rac*-ibuprofen (0.25 mmol), three equivalents of triethyl orthoformate (TEOF), 0.5 eq. of ethanol, 0.0515 g of immobilized lipase, T = 40 °C, reaction time = 24 h, orbital shaking at 250 rpm (see Section 3.3.1). [1] Enantiomeric excess of the remaining (*S*)-ibuprofen. [2] $E = \ln[(1 - ee_s) \times (1 - C)]/\ln[(1 + ee_s) \times (1 - C)]$, ee_s and C as decimals ($\leq$1) [74,75]. [3] $EF = ee_s/\{[C/(100 - C)] \times 100\}$ [26].

To measure the enantioselectivity, two parameters were used (see Section 3.2); the most popular one is the enantiomeric ratio, the ratio between the specificity constants (k_{cat}/K_M, also called kinetic efficiency) for both enantiomers [74,75]. This parameter, although commonly used and accepted, is not easy to be straightforwardly visualized, as it involves logarithmic scales (see Table 1 footnotes; as a rule of thumb, E values below 15 are inacceptable for practical purposes, being moderate to good in the range of 15–30, and excellent above this value [76]). However, the enantiomeric factor (EF), as reported by López-Belmonte et al. [26], represents a more intuitive metric. This parameter is defined as the correlation between the observed enantiomeric excess and the theoretical enantiomeric excess obtained at the experimental conversion, if only the fast reacting enantiomer would have been converted (formula shown in Table 1 footnote). Thus, the EF is comprised between 0 and 1; the closer to 1, the higher the enantioselectivity.

As can be seen, taking 24 h as the standard reaction time, the use of *iso*octane without any cosolvent (Entry 1) led to the almost total esterification of *rac*-ibuprofen, with a very

poor enantioselectivity. Four cosolvents were tested to check the effect on the reaction; as shown in Table 1, the use of an apolar solvent/chlorinated polar cosolvent (80/20: v/v mixture systems) allowed for an important increase in the selectivity (dichloromethane (DCM), Entry 2; chloroform, Entry 3). Moreover, a very slow reaction with a very little increase, in terms of enantioselectivity was observed in case of 1,4-dioxane (Entry 5). The reaction was faster in presence of *tert*-butyl methyl ether (MTBE, Entry 4), compared to those obtained with the other cosolvents, but exhibited a decrease in enantioselectivity. Zdun et al. [17] have reported on the use of different organic solvents possessing different polarities in the esterification of *rac*-ibuprofen (among other profens) with immobilized CALB and methyl or ethyl orthoacetate. Among them, the solvents described in Table 1 were included, but always as pure solvents, they were not applied as mixtures. These authors reported no conversion using either polar aprotic solvents or chloroform, and only the use of DCM was compatible, although leading to no enantioselectivity. As can be seen in Table 1, the E-value obtained with 20% DCM (31.8, Entry 2) is higher than any case reported by Zdun et al. [17].

A reverse correlation between the cosolvent polarity (P') and overall conversion of *rac*-ibuprofen was noticed in the case of all cosolvents used with the isooctane as the main solvent (Figure 4). Snyder's solvent polarity (P') is based on a combination of parameters, such as the dipole moment, proton acceptor or donor properties, and dispersion forces [77]. In fact, Snyder's polarity index ranks solvents according to a complex theoretical summation of these properties. As a rule, the higher the polarity index, the more polar the solvent.

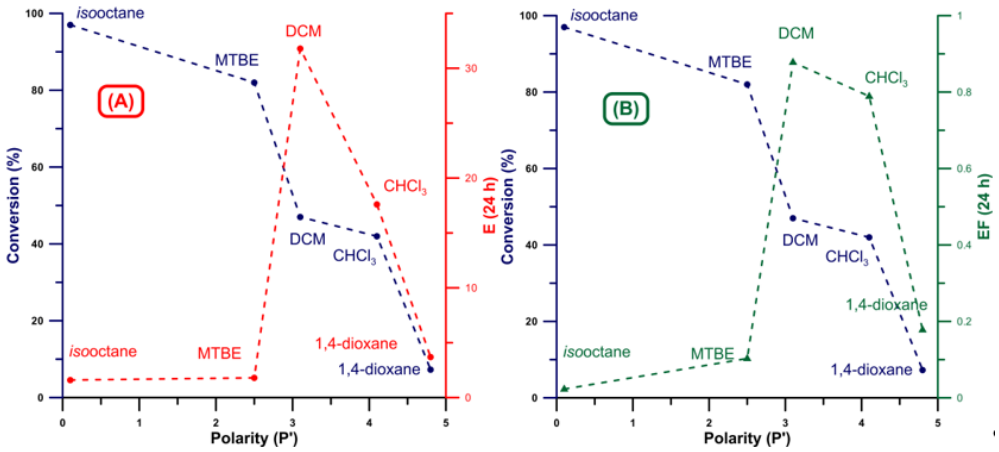

Figure 4. Correlation between the polarity of the cosolvent with conversion (% blue) and enantioselectivity quantified using either E ((**A**), red) or EF ((**B**), green) in the esterification of *rac*-ibuprofen with TEOF. Experimental conditions in Table 1.

These results are in accordance with the data from Zdun et al. [17], which reported how very low yields were obtained using pure polar aprotic solvents, explaining this fact by the distortion of the water layer surrounding the enzyme caused by the penetration of polar solvent molecules in the active site. Moreover, the correlation of P' with E and EF (Figure 4) shows a maximum when using 20% of the chlorinated solvents (chloroform and mostly DCM). It has been reported how the presence of halogen atoms in the solvents' structure may alter the enzymatic conformation and modulate its behaviour [78–81]; this fact could explain the enhanced selectivity using DCM and $CHCl_3$. Interestingly, Jose et al. [68] described the deleterious effect (both in conversion and enantioselectivity) of polar solvents (including DCM) in the direct esterification of *rac*-ibuprofen with EtOH catalysed by this same enzyme; thus, as this effect is not observed in our system using TEOF for the release of EtOH, our esterification method seems to proceed through different pathways. Figure 5 shows the progress curves (% conversion in black; % ee_s in red) obtained for the

esterification using TEOF in 100% isooctane and in the mixture using 20% DCM; this last reaction was selected for further studies.

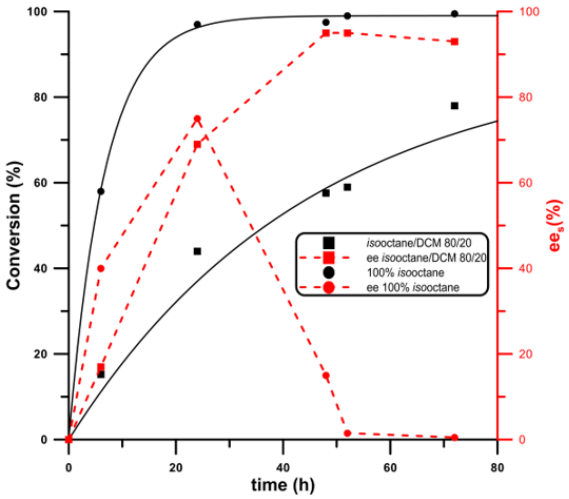

Figure 5. Kinetics of the esterification of *rac-ibu*profen with TEOF using pure isooctane and isooctane/DCM 80/20 (*v*/*v*).

2.2. Effect of the DCM Percentage on the Conversion of Ibuprofen

Aiming to select the best amount of DCM for the next steps of this study, the solvent to cosolvent ratio was studied, increasing the DCM amount from 20% up to 60%. Results are shown in Table 2.

Table 2. Effect of DCM percentage on the kinetic resolution of *rac*-ibuprofen.

Entry	Solvent	DCM % (*v*/*v*)	Conversion (%)	ee$_s$ (%)	E	EF
1	isooctane	0 [1]	97	75	1.7	0.02
2	isooctane	20 [1]	44	69	31.8	0.88
3	isooctane	20 [2]	57	95	21.8	0.72
4	isooctane	40 [2]	22	25	21.2	0.88
5	isooctane	60 [2]	11	11	25	0.89

[1.] Reaction time = 24 h. [2.] Reaction time = 48 h. Reaction conditions: 5 mL of solvent, *rac*-ibuprofen (0.25 mmol), three equivalents triethyl orthoformate (TEOF), 0.5 eq. of ethanol, 0.0515 g of immobilized lipase, T = 40 °C, orbital shaking at 250 rpm (see Section 3.3.2).

The reaction time was either 24 h or 48 h, as the esterification became much slower as the DCM percentage was increased. Considering the results obtained using 20% DCM at 24 h (Entry 2), prolonging the reaction time up to 48 h (Entry 3) led to a slight increase in the conversion, but with a concomitant diminution of enantioselectivity. When moving up to 40% DCM (Entry 4) or 60% DCM (Entry 5), conversions at 48 h were diminishing, as expected according to the progressive increase of the polarity of the reaction medium, previously observed in Figure 4, although enantioselectivity was similar. Accordingly, a 20% of DCM in isooctane was selected as the reference solvent for the next steps.

2.3. Effect of the Nature of the Alkyl Orthoformates in the KR of rac-Ibuprofen

The influence of the alcohol nature in the lipase-catalysed esterification is a well-studied area. In this sense, a detrimental effect of short-polar alcohols on lipases has been fully documented [82–87], mainly related to the penetration of these molecules (MeOH, EtOH) into the active site, resulting in a non-desired interaction with the catalytic histidine (His224 for CALB) [16]. Additionally, MeOH and EtOH has proven to alter the composition

of the carrier (Lewatit VP OC 1600, a macroporous resin composed of methacrylic acid cross-linked with divinylbenzene) used in commercial Novozyme®435 [69,88], promoting aggregation (both for the native and immobilized CALB) and enzyme leaking from the support at high concentrations (higher than 15% *v*/*v*) [82]. Moreover, it is also known that the enzymatic activity generally decreases when using branched (secondary or tertiary) alcohols for the lipase-catalysed esterification of profens [14,16,23,26,89]

Thus, to test the effect of the linear alcohol in our strategy, a low initial concentration (0.5 equivalent) of different alcohols (MeOH, EtOH, *n*-propanol and *n*-butanol) was tested, combined with the corresponding trimethyl orthoformate (TMOF), triethyl orthoformate (TEOF), tripropyl orthoformate (TPOF), or tributyl orthoformate (TBOF), using either pure isooctane or isooctane/DCM (80/20) as the reaction media. The profiles of the progress curves (% conversion vs. time) are shown in Figure 6, while the numeric data including conversion and enantioselectivity are shown in Table 3.

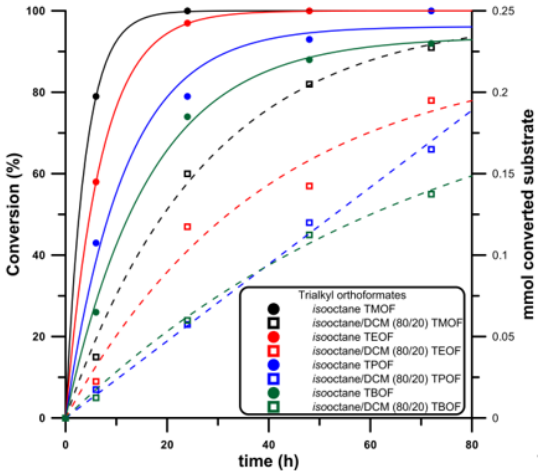

Figure 6. Kinetics of the esterification of *rac*-ibuprofen with different alkyl orthoformates using pure isooctane and isooctane/DCM 80/20 (*v*/*v*). Experimental data in Table 3.

Table 3. Effect of the chain length of alkyl orthoformates on the kinetic resolution of *rac*-ibuprofen in isooctane/DCM.

Entry	Orthoformate	DCM % (*v*/*v*)	Initial Rate (mmol/h)	Conv(%)/ee$_s$ (%) at 24 h	E	EF
1	TMOF	0	6.49×10^{-2}	100/0	1	—-
2	TEOF	0	3.61×10^{-2}	97/75	1.7	0.02
3	TPOF	0	2.06×10^{-2}	79/70	3	0.19
4	TBOF	0	1.42×10^{-2}	74/64	2.7	0.22
5	TMOF	20	9.02×10^{-3}	60/48	3	0.32
6	TEOF	20	5.73×10^{-3}	44/69	31.8	0.88
7	TPOF	20	2.36×10^{-3}	23/28	40	0.94
8	TBOF	20	3.03×10^{-3}	24/25	11	0.76

Reaction conditions: 7 mL of solvent, *rac*-ibuprofen (0.35 mmol), three equivalents trialkyl orthoformate (TMOF, TEOF, TPOF or TBOF), 0.5 eq. of alcohol (methanol, ethanol, *n*-propanol or *n*-butanol), 0.0721 g of immobilized lipase, T = 40 °C, orbital shaking at 250 rpm (see Section 3.3.3).

Experimental data showing progress curves in Figure 6 were adjusted to the single exponential growing model using the program INRATE implemented in the SIMFIT fitting package (version 7.6, release 9), a free-of-charge open source software for simulation, curve fitting, statistics and plotting [90] (accessible at https://simfit.org.uk/simfit.html accessed

on 17 January 2023). From these mathematical fittings, the initial rates were calculated (shown in Table 3). As can be seen, an inverse correlation between the alcohol chain length and esterification rate is observed either using pure isooctane or an 80/20 mixture with DCM. Conversely, an increase in enantioselectivity is observed by increasing the chain length of the nucleophile in case of a reaction without cosolvent. Remarkably, when using the cosolvent, this effect is even higher, reaching a maximum value when using TPOF (E = 40, EF = 0.94). Nevertheless, although the enantioselectivity is higher when using TPOF, compared to TEOF (standard reagent) with TPOF, the esterification rate was slower, reaching only 22% conversion at 24 h. For TPOF, by increasing the reaction time, both E and EF diminished (data not shown in Table 3, so that TEOF can be considered as the best option between the alkyl orthoformates tested.

Figure 7 shows another comparative aspect regarding the use of the four orthoformates. As can be seen, the formation of a turbid heterogeneous suspension was observed at the long reaction times when using TBOF and a solvent system containing *iso*octane and 20% DCM (Figure 7, case (8)). The explanation of this phenomenon will be the subject of further studies to evaluate the physical and/or the chemical damage that the immobilized enzyme could be suffering. In any case, this fact dissuaded us to check the utility of other orthoformates possessing longer alkyl chains and reinforced the fact that TEOF is the best option as shorter reaction times are needed, avoiding any biocatalyst degradation.

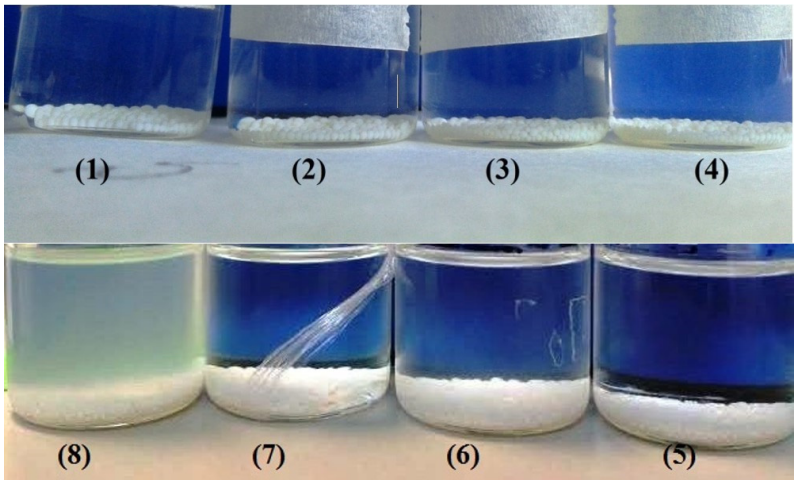

Figure 7. Physical aspect of the reaction mixture (photos were taken after 72 hours). (**1**): TMOF in 100% isooctane, (**2**): TMOF in the presence of 20% DCM, (**3**): TEOF in 100% isooctane, (**4**): TEOF in the presence of 20% DCM, (**5**): TPOF in 100% isooctane, (**6**): TPOF in the presence of 20% DCM, (**7**): TBOF in 100% isooctane, (**8**): TBOF in the presence of 20% DCM, showing a turbidity.

2.4. Effect of the Ibuprofen Concentration

The effect of varying the initial substrate concentration was tested. Results are shown in Table 4, and Figure 8 show the progress curves (% conversion in black; % ee in red; standard condition in blue).

As can be seen from the data in Table 4 and Figure 8, reducing the initial concentration of *rac*-ibuprofen from 10.31 mg mL^{-1} (standard conditions, shown in blue in Figure 8) to a half value led to a slightly lower initial rate and a higher conversion at 24 h (standard reaction time). Anyhow, as the initial substrate concentration had been reduced, this conversion increase (not associated to an increase in the enantioselectivity) did not correspond to a better overall performance. Moreover, by increasing the substrate concentration to 15 mg mL^{-1}, a slightly higher initial rate was obtained, while the enantioselectivity showed a small decrease. Extending the reaction time to 72 h, very good results were obtained, corresponding to 55% conversion and ee_s = 91.6% (E = 22.1, EF = 0.75). As the KR (Figure 3) is intended for "cleaning" the starting racemate front, the non-desired enantiomer conversion values must always be a bit higher than 50% to ensure that fact. Therefore, even at

the expense of an increase in the reaction time from 24 to 72 h, these conditions can be considered optimal, as the enantioselectivity obtained is better than any value reported by Zdun et al. [17]. In further studies, the substrate concentration would be increased to higher values to check the enzymatic performance.

Table 4. Effect of the substrate concentration on the kinetic resolution of *rac*-ibuprofen in isooctane/DCM.

Entry	Substrate Concentration (mg/mL)	Conversion at 24 h (%)	ee_s at 24 h	Initial Rate (mmol/h)	E	EF
1	5	58.3	66.7	4.92×10^{-3}	5.5	0.48
2	10.31 [1]	44	69	5.73×10^{-3}	31.8	0.88
3	15	32	38	6.89×10^{-3}	13.6	0.81

Reaction conditions: 5 mL of solvent (isooctane/DCM 80/20), three equivalents triethyl orthoformate (TEOF), 0.5 eq. of EtOH, 0.0515 g of immobilized lipase, T = 40 °C, orbital shaking at 250 rpm (see Section 3.3.4). [1] *rac*-ibuprofen (0.25 mmol), standard conditions.

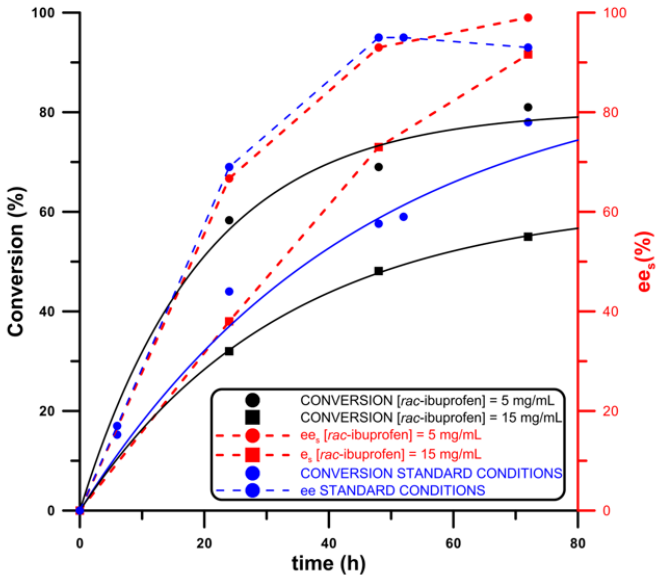

Figure 8. Kinetics of the esterification of *rac-ibu*profen at different concentrations. Experimental data in Table 4.

2.5. Effect of the Enzymatic Loading

The last parameter checked was the biocatalyst/substrate ratio loading. In this respect, many reports on biotransformation recognize that the reaction rate increases by increasing the biocatalyst amount in the reaction, but few reports deal directly with the impact of this parameter on the reaction performance [91,92]. Nevertheless, some recent studies showed that the selectivity was enhanced when diminishing the amount of enzymes; elucidation of the mechanism is not an easy task since many parameters could be considered, such as the change of the particle size of the support and possible aggregation phenomena that might interfere and hinder the specificity, especially in the case of a large quantity [93]. This can increase the diffusion limitation problems and favour the esterification of the slower substrate. Moreover, it is not unlikely that the requirements of water by the system may be different, as the support and enzyme may capture some water molecules and reduce the amount of available water. The same trend was observed by increasing the biocatalyst quantity in our system (Table 5), which led to an overall enantioselectivity decrease, although accompanied by an increase in reaction rate. In fact, comparing Entry 3 in Table 5 with the standard conditions (Table 2, Entry 2), at 24 h, the overall conversion

increased from 44% up to 61% upon doubling up the enzyme amount, but both E and EF values indicated how the enantioselectivity did not improve, but rather decreased (E from 31.8 down to 8, EF from 0.88 down to 0.63).

Table 5. Effect of the enzyme/substrate ratio on the kinetic resolution of *rac*-ibuprofen in isooctane/DCM.

Entry	Enzyme/Substrate	Reaction Time (h)	Conversion (%)	ee_s (%)	E	EF
1	1/1	52	59	95	17.3	0.66
2	1.5/1	48	65	98	13.5	0.53
3	2/1	24	61	83	8	0.53

Reaction conditions: 5 mL of solvent (*iso*octane/DCM 80/20),), *rac*-ibuprofen (0.25 mmol, 0.0515 g), 3 equivalents triethyl orthoformate (TEOF), 0.5 eq. of EtOH, T = 40 °C, orbital shaking at 250 rpm (see Section 3.3.5).

3. Materials and Methods

3.1. Materials

Lipase from *Candida antarctica* (recombinant, expressed in *Aspergillus niger*) immobilized on an acrylic resin (L4777, ≥5000 U/g) was purchased from Merck Life Science S.L.U. (Madrid, Spain). This enzymatic preparation is similar to Novozym®435, initially commercialized by Novozymes A/S (Copenhagen, Denmark). [64,94]. Orthoesters used were commercially available: trimethyl orthoformate (TMOF), triethyl orthoformate (TEOF) and tripropyl orthoformate (TPOF) were obtained from Merck Life Science S.L.U. (Madrid, Spain); tributyl orthoformate (TBOF) was obtained from TCI Europe (Paris, France). Organic solvents used for the HPLC analysis were all HPLC grade: *n*-hexane 96%, HPLC grade (from Scharlab (Barcelona, Spain), isopropanol and trifluoroacetic acid (from Merck Life Science S.L.U., Madrid, Spain)). Organic solvents for the kinetic resolution and alcohols (methanol, ethanol, *n*-propanol and *n*-butanol) were purchased from Merck Life Science S.L.U. (Madrid, Spain). Racemic-ibuprofen (*rac*-ibuprofen) was obtained from TCI Europe, (Paris, France).

3.2. Analytical Methods

Enantiomeric excess and conversion of ibuprofen was monitored by a HPLC analysis (Prominence-i LC-2030 liquid chromatograph, Shimadzu Europe GmbH, Duisburg, Germany) using chiral column Chiralcel OD-H (Daicel Chiral Technologies Europe SAS, Illkirch Cedex, France). The mobile phase was a mixture of *n*-hexane/ isopropanol/TFA: 1000/10/1: *v*/*v*/*v*, with a flow rate of 1 mL/min at a wavelength of 254 nm. Injection volume was 10 to 50 µL.

Reaction progress and the concentrations of both enantiomers (*R*) and (*S*)-ibuprofen (C_S and C_R, respectively, in mg/mL) were determined from the HPLC peak area of *rac*-ibuprofen by using a calibration curve of *rac*-ibuprofen obtained with $R^2 > 0.99$. The enantiomeric ratio *E*, the ratio between the specificity constants (k_{cat}/K_M, also called kinetic efficiency) for the transformation of both enantiomers of the substrate, was calculated using the enantiomeric excess of residual (*S*)-ibuprofen ee_s and the overall conversion c (%), as reported in [74,75]. Furthermore, using these two parameters, the enantiomeric factor (EF) was calculated, as reported in [26]: this parameter is defined as the ratio between the experimental ee_s and the theoretical enantiomeric excess calculated at the measured conversion, if only the fast-reacting enantiomer would have been transformed.

3.3. General Procedures for Optimization

3.3.1. Screening of the Best Cosolvent

To a well stirred suspension of *rac*-ibuprofen (0.0515 g, 0.25 mmol, C_0 = 10.15 mg/mL) in 5 mL of organic solvent (mixture of 80% isooctane/20% of cosolvent: *v*/*v*), 3 equivalents (0.1111 mg, 0.75 mmol) of triethyl orthoformate and 0.5 equivalent of ethanol (7.2 µL,

0.125 mmol) were added and the mixture was vortexed for 1 min. The reaction was started by adding 0.0515 g of immobilized lipase, and the resulting mixture was stirred at 40 °C with orbital shaking (250 rpm). Aliquots were withdrawn and analysed by chiral HPLC (see HPLC analysis) to follow the reaction progress, in order to select the appropriate times for the comparison between the cosolvents, according to the overall conversion *c* (%) and enantiomeric excess of residual acid ee_s (%).

3.3.2. Screening of the Best Percentage of Dichloromethane

By following the same steps of the previous protocol, in which the 5 mL of the organic solvent contain 0%, 20%, 40% and 60% (*v*/*v*) of dichloromethane, respectively, mixed with isooctane, the four enzymatic reactions were carried out in parallel to select the best amount of dichloromethane, according to the reaction velocity expressed by the overall conversion degree *c* (%) and enantiomeric excess of unreacted (*S*)-ibuprofen.

3.3.3. Comparative Kinetics with Different Types of Orthoesters

To the eight suspensions of *rac*-ibuprofen (0.0721g, 0.35 mmol, C_0 = 10.31 mg/mL), in 7 mL of organic solvent (four reactions in isooctane without cosolvent and four reactions with mixture of isooctane/dichloromethane: 80/20: *v*/*v*), 3 equivalents (1.05 mmol) of orthoester (trimethyl orthoformate 0.1114 g, triethyl orthoformate 0.1556 g, tripropyl orthoformate 0.1997 g and tributyl orthoformate 0.2439 g) and 0.5 equivalent (0.175 mmol) of the corresponding alcohol (methanol 7 µL, ethanol 10.2 µL, *n*-propanol 13.1 µL and *n*-butanol 16.21 µL) were added, in order to perform the reactions with and without the cosolvent by using four types of orthoesters. The reaction was started by adding 0.0721g of immobilized lipase. The resulting mixture was stirred at 40 °C with orbital shaking (250 rpm). Aliquots were withdrawn at regular time intervals and analysed by chiral HPLC to determine the progress of the overall conversion *c* (%) and the enantiomeric excess of residual (*S*)-ibuprofen ee_s (%) in each case.

3.3.4. Influence of the Substrate Concentration

To three suspensions of *rac*-ibuprofen (C_0 = 5 mg/mL, 10.31 mg/mL and 15 mg/mL respectively) in 5 mL of organic solvent (mixture of 80/20: *v*/*v*: isooctane/dichloromethane), 3 equivalents (0.1111 mg, 0.75 mmol) of triethyl orthoformate and 0.5 equivalent of ethanol (7.2 µL, 0.125 mmol) were added and the mixture was vortexed for 1 min. The reaction was started by adding 0.0515 g of immobilized lipase. The resulting mixture was stirred at 40 °C with orbital shaking (250 rpm). Aliquots were withdrawn at regular time intervals and analysed by HPLC to follow the reaction progress, in terms of enantiomeric excess and the overall conversion *c* (%) of residual (*S*)-ibuprofen.

3.3.5. Influence of the Enzyme Loading

To the same previous suspension (see Section 3.3.1) with the initial *rac*-ibuprofen concentration of C_0 = 10.31 mg/mL (0.0515 g), different amounts of enzymes were added at different increasing proportions (1/1, 1.5/1, 2/1: *w*/*w* enzyme/ibuprofen respectively). Aliquots were withdrawn to quantify both the enantiomeric excesses and the overall conversion of residual ibuprofen over a selected time interval, as previously detailed in Section 3.3.2).

4. Conclusions

It is worth mentioning that the results presented in our current study seem to be better than those in the literature for the esterification of racemic ibuprofen. In fact, by combining the use of a reaction medium composed by isooctane/DCM 80/20 (*v*/*v*), triethyl orthoformate (TEOF) and a small amount of EtOH, to promote the enzymatic esterification catalysed by immobilized CALB, it was possible to achieve kinetic resolutions with higher enantioselectivity values (up to E = 31.8), compared to recently reported data.

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