Effect of Co-solvents in the Enantioselective Esterification of (R/S)-ibuprofen with Ethanol

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Abstract: The commercial biocatalyst Novozym® 435 was used for the kinetic resolution of (R/S)-ibuprofen through the esterification with short chain alcohols (ethanol, 1-propanol and 2-propanol) in the absence of organic co-solvent. The best enzymatic performance was obtained by employing ethanol as reagent and solvent. Due to the deleterious effect of this alcohol on the integrity of the commercial biocatalyst previously reported different organic co-solvents (isooctane, n-hexane, carbon tetrachloride, ethyl acetate, acetonitrile and tetrahydrofuran) were screened in order to minimize the volume of ethanol to be used. Thus, the effect of the chemical nature of the co-solvent on enantioselective esterification of (R/S)-ibuprofen with ethanol was evaluated. The results show that the best performance was obtained with the reaction system without co-solvent added. Additionally, this investigation demonstrated the need to address multiple physico-chemical properties of the solvents to analyze their effects on biocatalysis.

Keywords: Ibuprofen, Novozym435, Ethanol, Organic solvents.

INTRODUCTION

Ibuprofen is the nonsteroidal anti-inflamatory drug (NSAIDs) most used worldwide. This pharmaceutical is marketed primarily as a racemate, but it has been reported that the S(+)-ibuprofen is 160 times more active than its enantiomer in prostaglandin synthesis "*in vitro*" [1]. This observation, common to other chiral drugs, has driven academic research and pharmaceutical companies to develop methods of production of single enantiomers. Previously, we reported that the commercial biocatalyst Novozym® 435 is able to conduct enantioselective esterification of ibuprofen using ethanol as substrate and solvent, alternatively to the traditionally used organic medium, resulting in a cheaper eco-friendly process [2, 3].

The reaction system replacing the ethanol with other alcohols of short chain, specifically 1-propanol and 2-propanol was evaluated based on the results previously reported by some of us regarding the degradation of Novozym® 435 in contact with ethanol [3]. Several investigations concerning the use of different alcohols in the enzymatic esterification of profens clearly indicate that higher enzyme activities are obtained when using primary alcohols, among which the linear chain primary alcohols provided the best performance. In contrast, no activity was found when using tertiary alcohols or polyols. However, there are inconsistencies regarding enzymatic activity with secondary alcohols [4-8]. Several research groups reported an increase in enzymatic activity by reducing the number of carbon atoms of the alcohol used [4, 6, 9-11]. In this sense, 1-propanol is the most widely used alcohol and has been chosen as optimum. Few records regarding the use of alcohols with less than three carbon atoms have been found because they attributed a dehydrating effect of the enzymes [4].

The study of the effect of alcohols of three carbon atoms in the kinetic resolution of (R/S)-ibuprofen along with previous reports of some of us regarding the degradation of the commercial biocatalyst in association with these alcohols leads to the conclusion that a strategy to minimize their effect on the biocatalyst must be designed [3, 12]. In this sense, the present contribution evaluates the reaction system to decrease the concentration of ethanol in contact with the commercial biocatalyst. However, the decrease in the amount of ethanol (typically 1 mL of alcohol is used along with 0.500 g of ibuprofen) necessarily requires the use of a co-solvent in order to dissolve the ibuprofen and to reach an intimate contact between the biocatalyst and the substrates. In this context, isooctane, n-hexane, carbon tetrachloride, ethylacetate, acetonitrile and tetrahydrofuran were chosen as organic co-solvents in the present investigation. The choice of these six solvents was based firstly on previous reports in the literature which demonstrated the feasibility of their use in biocatalysis. Furthermore, it is widely known and reported that the nature of the solvent is one of the key parameters in the performance of enzymatic reactions influencing both the activity and the selectivity of the enzyme [4-9, 13-15]. Although there is abundant literature regarding the effect of

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several solvents on enzymatic reaction systems to date clear or general trends have not been reported. The more general observations on the esterification catalyzed by lipases in organic media suggest that higher activities and selectivities are obtained in hydrophobic solvents. The hydrophobicity of a solvent is represented with the value of log P that is the logarithm of the distribution constant of the solvent in a two phase octanol-water system [16]. The log P = 2 is a "hinge" value (turning behavior) in enzymatic esterification, finding good values of conversion and enantioselectivity with solvents whose log P is equal or higher than 2, and very low activity or no reaction for values of log P <2 [4-8, 13]. However, heterogeneous behaviors are reported for both regions of this parameter. In this sense, Lopez-Belmonte et al. and Ceynowa`s group that studied the racemic resolution of 2-arylpropionic acids catalyzed by immobilized lipases observed a linear dependence of the conversion with no trend in the enantioselectivity for log P equal or higher than 2 [5, 6]. Moreover, Liu et al., Kim et al. and D`antona et al. observed no tendency for any of the reaction parameters with the nature of the solvent in the esterification of R/S-ibuprofen with various alcohols using *Candida rugosa* lipase, *Candida sp* and Novozym® 435, respectively [4, 13, 15]. The literature also reports exceptions in the presence of certain solvents with low log P values [7-9, 17]. In this regard, Zhao et al. investigated the esterification of ibuprofen with 1-octanol catalyzed with APE1547 (esterase), achieving the best conversion when using solvents with intermediate values of log P (~ 2.5). In contrast, those solvents with high or low Log P such as, acetonitrile (log P = -0.33) and isooctane (log P = 4.5) provided lower conversions [9].

Persson et al. studied the esterification of 2-phenyl propionic acid with 1-heptanol using catalysts based on lipases of various origins, finding very different behaviors depending on the catalyst [18]. The authors determined that the enantioselectivity of Novozym® 435 was independent of the log P of the solvent. However, the authors observed an increase in the enantioselectivity with log P when using immobilized *Candida rugosa* lipase.

The numerous reports on this matter exhibit experimental results without further explanation of the observed phenomena. At first it was postulated that the more hydrophilic solvents have greater ability to remove water molecules essential to the enzyme molecules thus affecting its activity [19, 20]. However, Liu and colleagues studied the esterification of racemic ibuprofen with 1-propanol in various solvents and evidenced the influence of the chemical nature of the solvent on the performance of the reaction even under the control of water activity (a_w). This observation demonstrates that there is a solvent effect on the enzyme activity due to the interaction with essential water molecules along with other specific effects on the enzyme [4]. Thus, the influence of the reaction environment is highly complex since it involves interactions between the solvent, the enzyme and the substrates which makes impossible to predict the behavior. In this context, the esterification of (R/S)-ibuprofen with ethanol along with various organic solvents, both hydrophobic and hydrophilic, was investigated in the present contribution. Additionally, the esterification of (R/S)-ibuprofen with other alcohols without the use of co-solvents has also been investigated.

EXPERIMENTAL

Esterification Reactions

The effect of the nature of the alcohol was evaluated by reacting ibuprofen (0.5 g) with 1.00 mL of n-propanol (Sigma Aldrich 99.5 %) and isopropanol (J.T. Baker 99.93 %), respectively, without adding co-solvent. The reaction conditions employed were those previously found to be optimal for the reaction with ethanol, i.e. initial water content of 4.6 % v/v, 160 mg Novozym® 435 (Novo Nordisk, Denmark) per mL of alcohol, 45 °C, 200 rpm, and 48 hours [2]. Further tests were performed with 1.30 and 3.20 mL of these alcohols in order to evaluate the effect of the volume of alcohol. Additionally, blank tests were performed with 1.00 mL of alcohol in the absence of catalyst.

The evaluation of different organic co-solvents were performed in closed 100 mL vials kept at constant temperature (45 °C) and stirring continued (200 rpm) in a shaker bath (Julabo SW22, Germany). In all cases 0.5 g (2.42 mmol) of (R/S)-ibuprofen was reacted with a defined volume of absolute ethanol (Carlo Erba 99.8%) in the presence of organic solvent (acetonitrile, isooctane, n-hexane, carbon tetrachloride, ethyl acetate, tetrahydrofuran). In all cases, the minimum volumes of solvents needed to dissolve 0.5 g of ibuprofen were used. Those volumes correspond to: 20.00 mL of isooctane (99.5% Carlo Erba) 17.00 mLof n-hexane (Sintorgan 98.5%), 5.00 mL of carbon tetrachloride (UVE 99.9%), 2.50 mL of ethyl acetate (Carlo Erba Analyticals 99.5 %), 2.00 mL of acetonitrile (Carlo Erba 99.9 %) and 2.00 mL tetrahydrofuran (Merck> 99 %). Distilled water was added in a 4.76 % v/v only in those reaction systems wherein the solvent was miscible in water. Reactions were initiated with the addition of Novozym® 435 which was used in a ratio corresponding to 160 mg of biocatalyst per 1 mL of alcohol. The volumes of absolute ethanol (0.14, 0.20, 0.35, 0.60, 0.80 and 1.00 mL) corresponded to the following alcohol: acid molar ratios: 1:1, 1.42: 1, 2.47:1, 4.25:1, 5.70:1 and 7.08:1, respectively. Additionally, tests to determine the degree of advance and enantioselectivity of the non catalyzed reaction were assayed. These experiments were performed with alcohol: acid molar relation equal to 7.08:1 in the absence of biocatalyst.

Analysis of Samples

The conversion (X %) and enantiomeric excess of the substrate (ee %) were determined after 48 h. of reaction. The analysis of both enantiomers was conducted by chiral HPLC analysis in a Nucleodex beta-PM column (Macherey-Nagel, Germany) with an UV detector operated at 230 nm. The mobile phase (methanol/ 0.1% TEAA pH 4.0 (60/40 v/v)) was operated at a flow rate of 0.700 ml/min. All samples were run a minimum of 4 times with a relative error of 2.4 %. Enantiomeric excess (ee) referred to the form (S) of the remaining ibuprofen was calculated according to the following equation, where [S] and [R] account for the concentrations of the (S) and (R) enantiomers respectively.

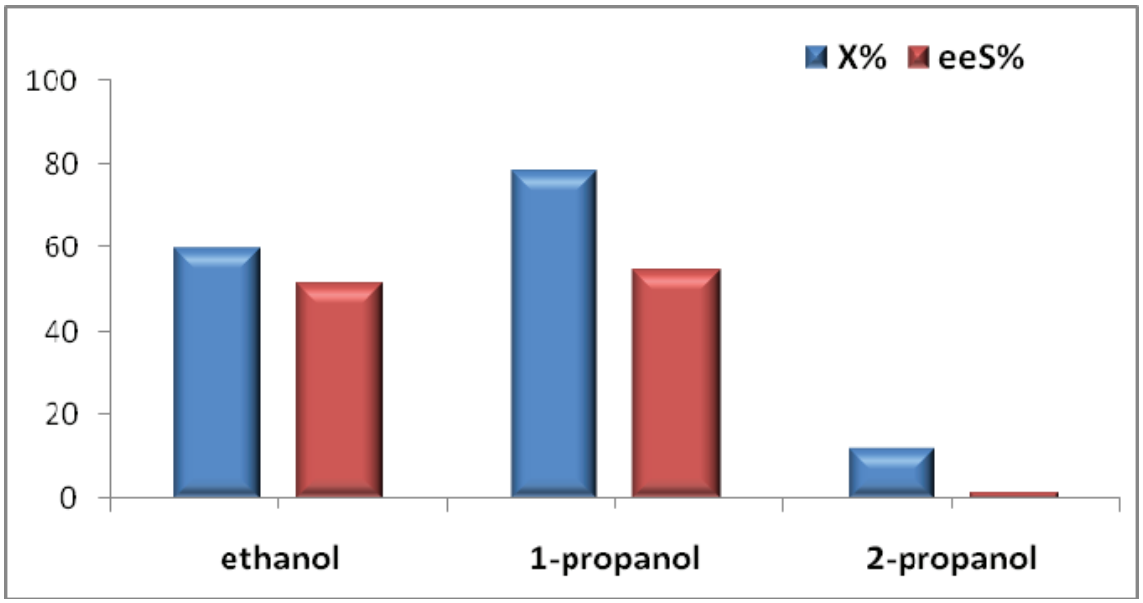

Fig. (1). Effect of the nature of the alcohol on the conversion (X %) and enantioselectivity (eeS %) in the esterification of R/S-ibuprofen. Conditions of reaction: 0.5 g (R/S)-ibuprofen, 1.00 mL alcohol, 160 mg Novozym® 435, 45 °C, 200 rpm, 4.76 % v/v distilled water, 48 h of reaction.

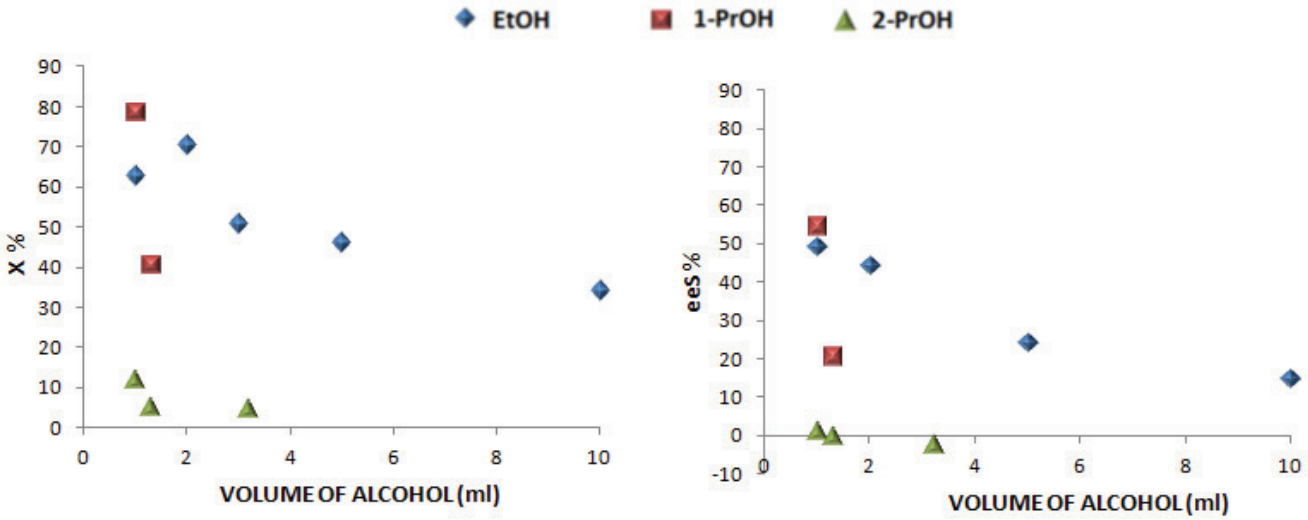

Fig. (2). Effect of volume of 1-propanol (1-PrOH) and 2-propanol (2-PrOH) of the conversion (X %, left) and enantiomeric excess (eeS %, right) in the esterification of (R/S)-Ibuprofen catalysed by Novozym® 435. Comparatively shows the effect of the volume of ethanol (EtOH). Reaction conditions: 0.5 g (R/S)-ibuprofen, variable volume of alcohol, 160 mg Novozym® 435 per mL of alcohol, 45 °C, 200 rpm, 4.76 % v/v distilled water, 48 h reaction.

$$ee\ S\% = [([S] - [R])/([S] + [R])] \times 100$$

The conversion of profen was also verified by titration of the final reaction mixture with a basic solution of KOH in ethanol of known concentration [2].

RESULTS

Effect of the Nature of the Alcohol

Fig. (**1**) shows the values of conversion and enantiomeric excess towards the S(+) enantiomer in the esterification of (R/S)-ibuprofen with different short chain alcohols with no added co-solvent. The results clearly show that the secondary alcohol possesses a negative impact in the biocatalytic performance most probably due to a steric hindrance of the alcohol on the active site as previously demonstrated for ketoprofen esterification [12]. Therefore, the impact of increased volumes of the alcohols over the minimum volume required for the dissolution of the substrate and intimate contact of the catalyst with the reaction system was assessed. The Fig. (**2**) evidenced that a volume higher than 1.00 mL of alcohol has a negative effect on the progress of reaction and selectivity regardless of the nature of the alcohol.

Effect of the Molar Ratio of Substrates

Fig. (**3**) shows the values of conversion (X%) and enantiomeric excess to the species S(+)-ibuprofen (eeS%) obtained

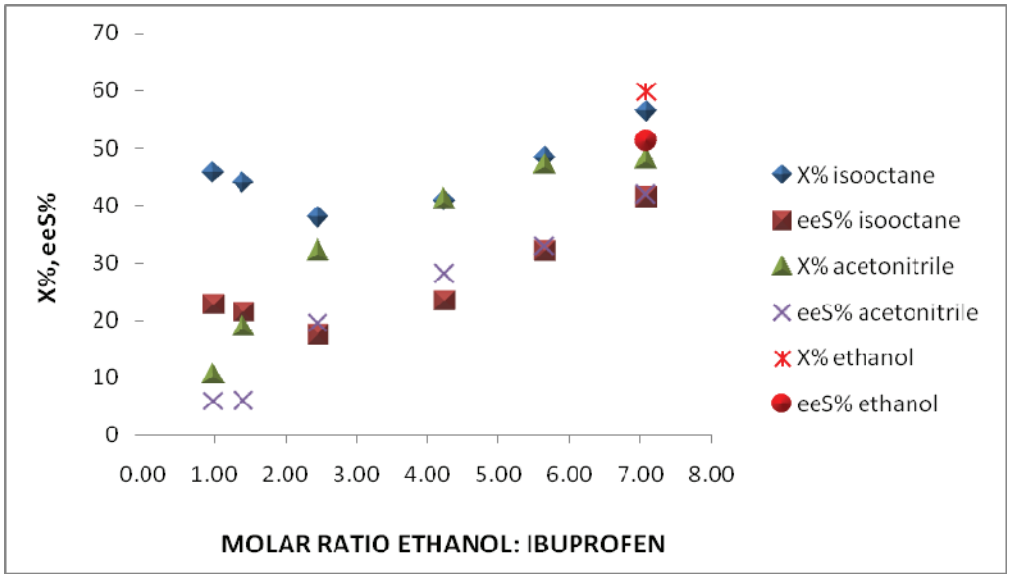

Fig. (3). Comparative results of the effect of alcohol: profen molar ratio on the conversion and enantioselectivity in the esterification of R/S-ibuprofen with ethanol using isooctane and acetonitrile as co-solvents. The values of X % and eeS % obtained with ethanol as reagent and solvent are also presented.

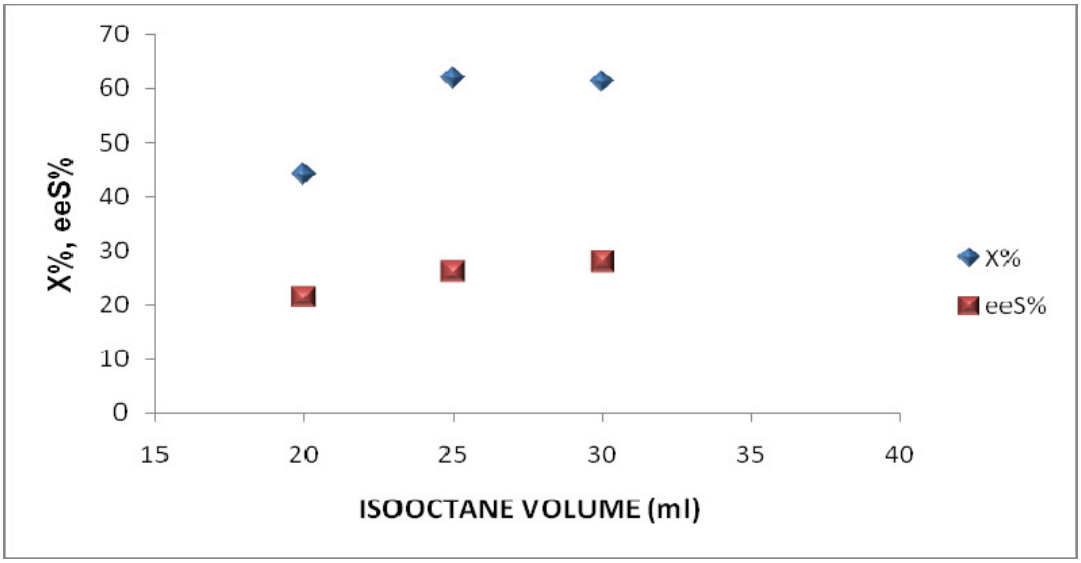

Fig. (4). Conversion (X %) and enantiomeric excess (eeS %) versus the volume of isooctane added to the reaction media when using an ethanol: ibuprofen molar ratio equal to 1.42:1.

by performing the esterification of racemic ibuprofen with ethanol catalyzed by Novozym® 435 using both a hydrophobic organic solvent (isooctane) and a hydrophilic organic solvent (acetonitrile). Various molar ratios were used, varying the volume of ethanol added to the system as described in the experimental section.

The results shown in Fig. (**3**) demonstrate that ethanol:ibuprofen molar ratios higher than or equal to 2.47 promote the conversion and enantiomeric excess, regardless of the co-solvent used. Nevertheless, the behavior of the reaction system was different depending on the chemical nature of the solvent employed when the substrates were used in stoichiometric amounts (or with a slight excess of alcohol). In this context, it is interesting to assess the esterification using various co-solvent volumes with an ethanol: ibuprofen molar ratio equal to 1.42:1. Figs. (**4** and **5**) showed the results of these experiments, demonstrating that regardless of the nature of the solvent, the enantioselectivity of the reaction is not significantly changed by increasing the amount of co-solvent above the minimum volume necessary to dissolve ibuprofen. However, the conversion of R/S-ibuprofen is favored by increasing the amount of isooctane and decreases drastically in the case of increase in the amount of acetonitrile.

Effect of the Chemical Nature of the Solvent

Based on the results shown in the previous section, the effect of various organic solvents on the conversion and

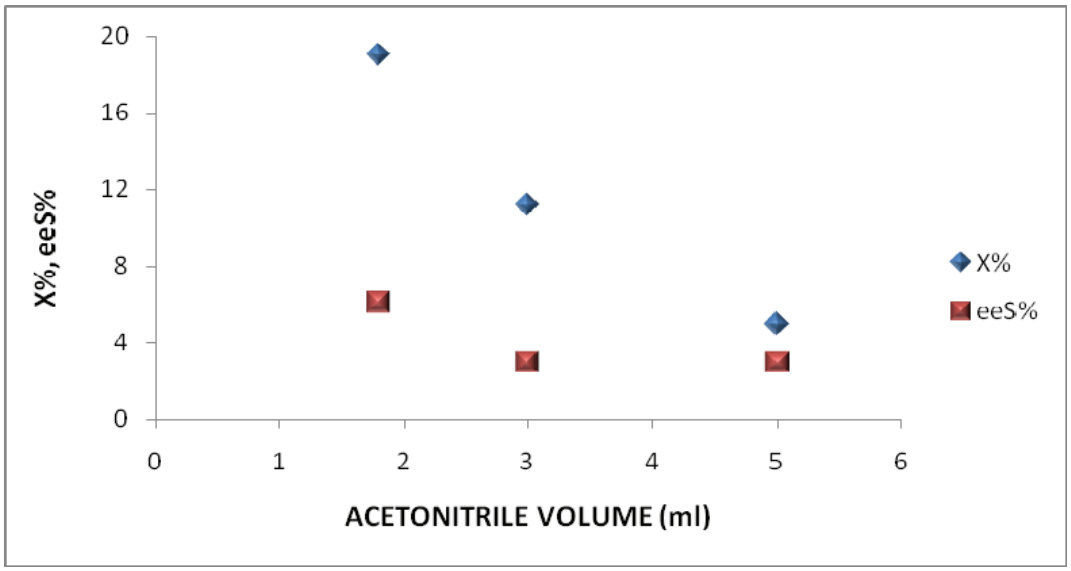

Fig. (5). Conversion (X %) and enantiomeric excess (eeS %) versus the volume of acetonitrile added to the reaction media when using an ethanol: ibuprofen molar ratio equal to 1.42:1.

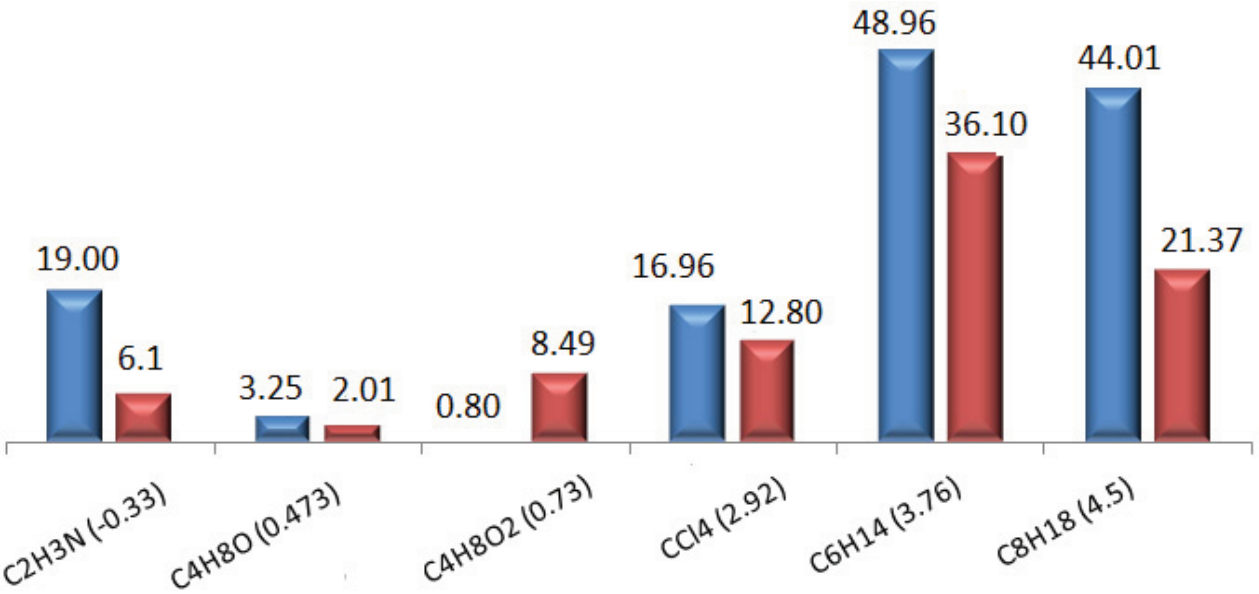

Fig. (6). Conversion (X %) and enantiomeric excess (eeS %) of the esterification of ibuprofen with ethanol (alcohol: profen ratio equals to 1.42:1) using co-solvents of increasing log P. The chemical formulas of solvents and corresponding values of log P (in parenthesis) are shown.

enantioselectivity of the esterification reaction of (R/S)-ibuprofen with ethanol was evaluated. In this context, a series of solvents with different hydrophobicity in the -0.33 to 4.5 range of log P were selected. Each solvent was used in a minimal amount as indicated in the experimental section. In these studies a fixed ethanol: ibuprofen molar ratio of 1.42:1 and 7.08:1 was used (Figs. **6** and **7**). It is important to notice that all the blank experiments showed values of conversion of 0.5 % -0.7 % with the exception of the reaction in ethyl-acetate, that showed a conversion of 1.6 %. In no case it was observed enantio-preference in the absence of catalyst. The values of X % assigned to the different systems of reaction in Figs. (**6** and **7**) were calculated as the difference between the catalyzed and non catalyzed conversion of ibuprofen.

These results agree with previous reports regarding the improvement of the enzymatic esterification when hydrophobic solvents such as isooctane are used. (see Fig. **6**). However, in conditions of significant excess of ethanol (ethanol:ibuprofen molar ratio 7.08:1) a good enzymatic performance is observed regardless of the hydrophobic or hydrophilic nature of the solvent (see Fig. **7**). In this context, it is worth noticing that solvents with intermediate values of log P (between 0.47 and 2.92) lead to lower conversions.

DISCUSSION

The effect of the nature of the alcohol in the enzymatic resolutions of profens has been largely reported in the litera-

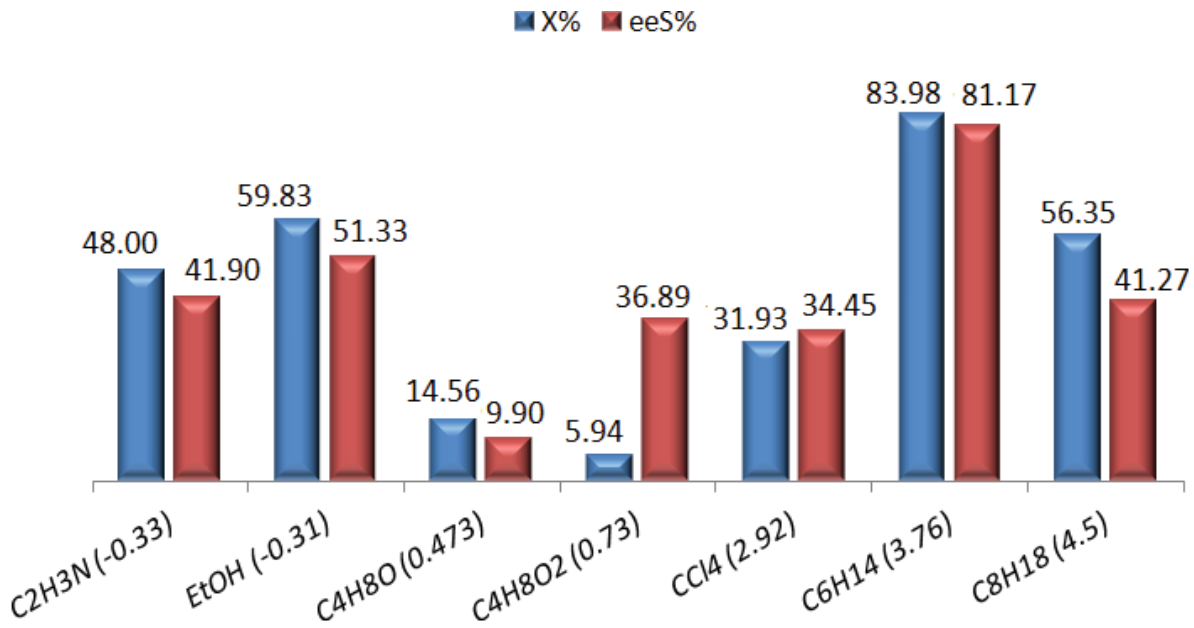

Fig. (7). Conversion (X %) and enantiomeric excess (eeS %) of the esterification of ibuprofen with ethanol (alcohol: profen ratio equals to 7.08:1) using co-solvents of increasing log P. The chemical formulas of solvents and corresponding values of log P (in parenthesis) are shown.

ture [4-11]. In this sense, many reports indicate that higher conversions in esterification catalyzed by lipases are achieved with primary alcohols, being low for secondary alcohols and null when tertiary alcohols or polyols are used. It is well known that the formation of the complex acyl-enzyme is the key step in this type of reactions; therefore, the conversion of the profen depends on the accessibility of the acyl-acceptor (the alcohol in this case) to the complex.

The behavior observed in the esterification of (R/S)-ibuprofen with primary and secondary alcohols using the commercial catalyst Novozym® 435 is in agreement with the investigation reported by Arroyo *et al.* [7, 8]. In this regard, higher enzymatic activity using primary (ethanol and 1-propanol) than a secondary alcohol (2-propanol) was evidenced in the present investigation. Previous studies reported by some of us demonstrated that the catalytic activity was low but not zero when 2-propanol was used as the acyl-acceptor in the esterification of (R/S)-ibuprofen and (R/S)-ketoprofen [12]. Somehow this observation disagrees with the investigation reported by López-Belmonte *et al.* and Liu *et al.* that observed the esterification of (R/S)-ibuprofen catalyzed with *Rhizomucor miehei* and *Candida sp* only when primary alcohols were used [4, 5]. These authors observed differences in terms of the conversion achieved in 48 h of reaction when using ethanol (59.83 %) and 1-propanol (78.5 %), without differences in the enantioselectivity (eeS % = 51.3 % and 54.4 %, respectively). The reports about the effect of the nature of the linear primary alcohols in the enzymatic esterification of profens are fairly homogeneous in terms of the trend of decrease in enzyme activity and selectivity with the increase in the length of the carbon chain of alcohol. However, few reports address the use of ethanol [4, 6, 9, 10, 15]. The effect on the progress of the reaction is attributed to the differences in the diffusion rate towards the immobilized lipase and the differences in the nucleophilicity of the alcohol that is, the longer the carbon chain, the lower the nucleophilicity of the alcohol and the steric hindrance becomes more important [10]. In this sense, the esterification of ibuprofen with ethanol (X % = 59.8 %, eeS % = 51.3 %) and 1-propanol (X % = 40.4 %, eeS % = 20.6 %) assayed in the present investigation was consistent with the behavior discussed above.

Ong *et al.* reported that the length of the carbon chain of the alcohol significantly affects the esterification reaction. The authors noted that the increase in the number of carbon atoms of alcohol decreases the alcohol:acid molar ratio required to achieve a certain level of conversion [11]. In this sense the molar ratios required to achieve 70 % of conversion in the esterification of (R/S)-ketoprofen with the lipase B of *Candida antarctica* were 4:1 for ethanol, 3:1 for n-propanol and 1:1 for n-butanol.

The investigation of the esterification of R/S-ibuprofen with ethanol catalyzed with Novozym® 435 using two solvents of opposite properties such as, isooctane (log P = 4.5, dielectric constant ε = 1.94) and acetonitrile (log P = -0.33 and ε = 36.6) showed the influence of the nature of the solvent in the reaction when it was carried out using molar ratios of ethanol: ibuprofen below 2.47:1. In fact, the characteristics of the reaction medium at higher molar ratios are defined primarily by the excess of ethanol. Nevertheless, at low excess of alcohol and stoichiometric amounts of substrates, the reaction media is greatly affected by the chemical nature of the co-solvent according to the behavior observed when the effect of the co-solvent volume was evaluated. Figs. (**4** and **5**) show that the conversion of the profen and enantiomeric excess eeS% evolve with increasing amounts of co-solvent until reaching a plateau (positive or negative depending on the solvent). This observation is consistent

with a progressive modification of the physicochemical nature of the reaction medium, reaching a point at which the reaction medium has the physicochemical characteristics of the solvent, i.e. the amount of ethanol does not determine the characteristics of the medium, but its primary role in these conditions is as a reaction substrate. The plateau region corresponds to the behavior in a medium clearly defined by the solvent added.

In conditions of slight excess of the alcohol, the results show that an increase in the volume of isooctane favors the progress thereof, the conversion increased from 44 % to 61 % by changing the volume of solvent from 20.00 mL to 30.00 mL. However, the effect on the enantioselectivity is less pronounced, increasing the eeS % from 21 % to 28 %. In contrast, the increase in the volume of acetonitrile added to the reaction medium has a negative effect on both parameters since a decrease in the conversion from 19 % to 5 % and the enantiomeric excess from 6 % to 3 % by changing the volume from 2.00 to 5.00 mL is observed.

The commercial biocatalyst Novozym® 435 was shown to be active when using acetonitrile as co-solvent in the reaction under investigation. This finding is consistent with that reported by Sinisterra *et al.* who studied the esterification of the 2-phenyl-propionic acid with 1-propanol in various organic solvents using Novozym® 435 and native CALB enzyme. The authors observed that while the log P value = 2 behaves as a "cut-off" as described above, acetonitrile and carvones were exceptions to this behavior. In fact, the authors found a lower enzyme activity than expected when using carvones and a greater activity than expected when using acetonitrile according to their values of log P [7, 8]. Based on these observations it has been proposed that parameters such as the dielectric constant (ε) and the acid/base solvent nature, should be considered when analyzing the effect of a solvent in biocatalysis. Regarding the dielectric constant, this parameter is of relevancy since most of the non-covalent interactions present in the protein are of electrostatic type, and in accordance with Coulomb's law, are inversely related to that parameter. Based on this idea, Sinisterra et al. suggest that the enzymes are more flexible in those solvents having a higher ε value. Thus, the lipase B of *Candida antarctica* may have more flexibility in acetonitrile (ε = 35.94), favoring the enzyme-substrate interaction and increasing its activity.

Fig. (**6**) shows an important effect on the nature of the solvent when ethanol: ibuprofen molar ratio equaling to 1.42:1 is used. In general, the results show that both the conversion of the profen and the enantiomeric excess to S(+)-ibuprofen increase when increasing log P values that is, hydrophobic solvents improve the enzymatic performance. This observation is in accordance with numerous reports that clearly demonstrated a dependency between the hydrophobicity of the solvent (being n-hexane and isooctane the most promising) and the progress of the reaction [4-8, 13]. As previously indicated, acetonitrile is an exception to the general behavior, in agreement with those reported Sinisterra *at al.* [7, 8]. Both the conversion and the enantiomeric excess to S(+)-ibuprofen are higher at an ethanol: ibuprofen molar ratio equal to 7.08:1 than those obtained by using a molar ratio equal to 1.42:1. Although, the increase in X% can be attributed to the displacement of the reaction by the highest concentration of one of the reactants, it does not justify the increase in selectivity (see Fig. **7**). This latter effect could be attributed to the influence of ethanol-cosolvent on the structure and flexibility of the lipase B of *Candida antarctic*a CALB. In this context, it was found that even under conditions of important excess of ethanol, the nature of the solvent influences the enzymatic performance. However, it would be necessary to establish physical and chemical characteristics of these alcohol-solvent mixtures for proper correlation with biocatalytic activity.

The present investigation is an evidence that the enzymatic kinetic resolution using ethanol without co-solvent added generates the best values of conversion and enantiomeric excess to S(+)-ibuprofen. The high value of eeS(+) % obtained using n-hexane as co-solvent is associated to the high value of conversion and not to the enantioselectivity. These results are innovative, demonstrating the feasibility of using a reagent economic and eco-compatible as ethanol in the kinetic resolution of profens, being an alcohol not considered in the study of such reactions by dehydrating properties of enzymes.

It is interesting to remark the behavior observed when using ethyl acetate as the solvent under a molar ratio of substrates equal to 7.08:1. In this particular case, the enantiomeric excess to the S(+)-ibuprofen (eeS% = 36.89 %) was much higher than the conversion value achieved (X% = 5.94 %). It is worth noticing that both the conversion and enantiomeric excess show similar values in all the other systems tested. Although the progress of the esterification is poor, the presence of ethyl acetate leads to a more selective behavior of the enzyme in terms of an improved discrimination of the enantiomers. Ongoing investigations would allow optimizing the esterification of ibuprofen using ethyl acetate as co-solvent.

CONCLUSIONS

Conclusions drawn from the results presented are the following:

- The conversion of ibuprofen and the enantioselectivity towards the isomer S(+) in the esterification catalyzed by Novozym® 435 increase with the content of ethanol in the presence of co-solvents.

- The reaction system without co-solvent added provided the best results in terms of conversion and enantiomeric excess towards S(+)-ibuprofen.

- The hydrophobic/hydrophilic and solvation properties of the solvents (which do not act as substrates) have an influence on the activity of Novozym® 435.

- Ethanol and 1-propanol can be used as substrates and solvents with yields comparable to those obtained in the presence of co-solvents.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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