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ORIGINAL PAPER

Low-temperature enzymatic hydrolysis resolution in mini-emulsion media

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A low-temperature mini-emulsion medium for the enzymatic resolution of 1-phenylethanol is described for the first time. The enzymatic hydrolysis resolution of 1-phenylethyl esters with different chain-lengths in the presence of *Candida antarctica* lipase B in mini-emulsion media was shown to be significantly controlled by temperature. In this system, the direct effect of temperature on the mini-emulsion size was observed. For the longer 1-phenylethyl ester, 1-phenylethyl dodecanoate, the enzymatic resolution was promoted exclusively at low temperatures. The preparative mini-emulsion enzymatic reaction of 1-phenylethyl dodecanoate at 4 °C afforded the isolation of (*R*)-phenylethanol with a yield of 36 % with an *ee* of 99 %. (*S*)-Phenylethanol was isolated with a 51 % yield with an *ee* of 79 %.

Keywords: mini-emulsion, enzymatic kinetic resolution, secondary alcohols, low temperature

Introduction

The enzymatic kinetic resolution (EKR) of secondary alcohols via transesterification or hydrolysis is a technique used extensively in the preparation of enantiomerically pure secondary alcohols (Yun et al., 2003; Lourenço & Afonso, 2007; de Souza et al., 2009). Since lipases are active in a non-aqueous medium, attention has been focused on transesterification as an alternative to hydrolysis (Zaks & Klibanov, 1988). This is clearly associated with the insolubility of most organic substrates in aqueous media. The use of organic solvents represents a major advantage for homogenisation of the reaction mixture and consequent outcomes. Today, the need for more sustainable and "green" synthetic pathways is not compatible with the use of large amounts of organic solvents (Dunn, 2012). As a consequence, reactions in water have attracted the attention of different researchers due to water being a cheap safe, and environmentally benign solvent (Lindström, 2007; Simon & Li, 2012). A number of examples relating to the use of water as a solvent have been reported for different organic reactions. In line with the use of water as a reaction medium, mini-emulsion systems have recently been exploited. Mini-emulsions are characterised by a two-phase system in which stable nanodroplets, between 20 nm and 500 nm in size, of an organic phase are dispersed in a second continuous aqueous phase (Landfester, 2006; Solans et al., 2005). These nanodroplets are stabilised against coalescence by the addition of appropriate surfactants, which provide either electrostatic or steric stabilisation (Landfester, 2006). These features render mini-emulsions attractive media for different chemical reactions, e.g. dehydration reactions (Manabe et al., 2001; de Barros et al., 2010; Aschenbrenner et al., 2009). Kobayashi and co-workers showed that direct esterification in water in a mini-emulsion medium was possible using *p*-dodecylbenzenesulphonic acid as a surfactant-type Brønsted acid (Manabe et al., 2001). With reference to enantioselective enzymatic reactions in mini-emulsion systems, to the best of our knowledge, only one approach has been developed to date. Landfester and co-workers described the enzymatic

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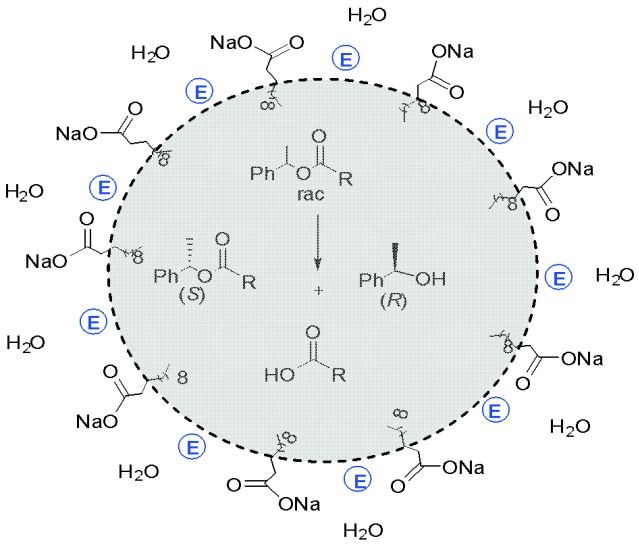

Fig. 1. Mini-emulsion methodology for enzymatic resolution of 1-phenylethanol.

hydrolysis of amino esters under a mini-emulsion system stabilised by the use of Lutensol AT50 as surfactant (Gröger et al., 2006). This method permitted the preparation of α-amino acids, as well as β-amino acids in high substrate concentrations from 500 to less than 800 g L^{-1} with enantiomeric excess (*ee*) higher than 99 %.

In ongoing efforts toward developing more sustainable enzymatic resolutions methodologies (Monteiro et al., 2010; Lourenço et al., 2007, 2010; Lourenço & Afonso, 2007; Garcia et al., 2004), the attractive features of mini-emulsions were exploited for the resolution of 1-phenylethanol by enzymatic hydrolysis catalysed by *Candida antarctica* lipase B (CALB). The surfactant and the organic substrate in water form the stable nanodroplets that are in contact with lipase, which has the ability to operate at the oil-water interface (Schmid & Verger, 1998) and selectively hydrolyses one of the enantiomers (Fig. 1).

Experimental

Materials and instrumentation

Candida antarctica lipase B (liquid formulation, hydrolytic activity 0.24 U μL^{-1}) was donated by Novo Nordisk Bioindustrial (Spain). A lyophilised form of this lipase (solid, hydrolytic activity of 5.60 U mg^{-1}) was obtained from the liquid formulation. Amano lipase AK from *Pseudomonas fluorecens* (powder, hydrolytic activity of 0.86 U mg^{-1}), Amano lipase PS from *Burkholderia cepacia* (powder, hydrolytic activity of 0.62 U mg^{-1}), Amano lipase AYS from *Candida rugosa* (powder, hydrolytic activity of 0.36 U mg^{-1}) were donated by Amano Enzyme (UK). Lipase from *Candida rugosa* (powder, hydrolytic activity of 0.99 U mg^{-1}) and porcine liver esterase (powder, hydrolytic activity of 24.2 U mg^{-1}) were purchased from Sigma. 1-Phenylethyl dodecanoate was purchased from Solchemar (Portugal). 1-Phenylethyl acetate (*I*) and 1-phenylethanol were purchased from Sigma–Aldrich and Fluka, respectively. All the other reagents were obtained commercially and used as received, unless stated otherwise. The mini-emulsions were prepared using a SONOPLUS HD 3200 series (Bandelin electronic, Germany). The mini-emulsion average droplet sizes were analysed using a Zetasizer Nano ZS (Malvern Instrument Co., UK). ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker AMX 400 spectrometer using tetramethylsilane as a reference. Gas liquid chromatography (GLC) was carried out on a Shimadzu GC-2010 plus instrument (chiral capillary column, CP-Chirasil-DexCB, 25 m × 0.25 mm × 0.25 μm; He as carrier gas; 250 °C injector; 250 °C detector; linear velocity: 28.1 cm s^{-1}; flow through column: 0.92 mL min^{-1}; split ratio: 100; oven programme A: 20 min at 120 °C; ramp: 10 °C min^{-1} up to 180 °C, 5 min; retention time (t_R in min): 1-phenylethyl acetate: t_R (*S*) = 7.00; t_R (*R*) = 7.83; 1-phenylethanol: t_R (*R*) = 10.13; t_R (*S*) = 11.06; 1-phenylethyl butyrate: t_R (*S*) = 15.42; t_R (*R*) = 16.10; oven program B: 50 min at 120 °C; ramp: 1 °C min^{-1} up to 180 °C, 25 min; 1-phenylethyl hexanoate: t_R (*S*) = 46.31; t_R (*R*) = 48.32; 1-phenylethyl dodecanoate: t_R (*S*) = 125.16; t_R (*R*) = 125.56.

Enzyme activity assay

The enzyme activity rates were investigated by means of a spectrophotometric assay carried out on a Hitachi U-2000 spectrophotometer. The substrate *p*-NPB (20 μL) was delivered from concentrated stock solutions in pure acetonitrile (35 mM). Activity assays were performed in 0.1 M phosphate buffer of pH 7.5 (970 μL) at 37 °C, initiated by the addition of enzyme (10 μL). The release of *p*-nitrophenol was monitored spectrophotometrically at 400 nm (ε_{400} = 18400 M^{-1} cm^{-1}). The non-enzymatic hydrolysis of the substrate was taken into account and subtracted from the enzyme-catalysed hydrolysis. Enzyme activity was linear in respect of the amount of enzyme within the range used in these assays. The assays were recorded in duplicate. Please refer to the supplementary information in electronic form.

Preparation of 1-phenylethyl butanoate (II) and 1-phenylethyl hexanoate (III)

A mixture of 1-phenylethanol (26.46 mmol, 2.5 g), Et_2O (25 mL) and pyridine (24.55 mmol, 1.98 mL) was stirred for 5 min followed by the addition of the corresponding acid anhydride (24.55 mmol) (butyric

anhydride or hexanoic anhydride for the preparation of *II* or *III*, respectively) at ambient temperature and subsequent heating under reflux for 6 h. After cooling to ambient temperature, the reaction was quenched by the addition of 1 M aqueous HCl (25 mL) and the product was extracted using Et_2O (2 × 25 mL). The combined organic layers were washed with saturated sodium bicarbonate solution (25 mL) and the aqueous phase was extracted with Et_2O (2 × 25 mL). The combined organic layers were dried with Na_2SO_4, filtered and the solvent was removed under reduced pressure. The product was purified by flash chromatography on a silica gel column using hexane/EtOAc ($\varphi_r = 9:1$) as the eluent to afford *II* (68 % yield) or *III* (62 % yield), respectively. For *II*: ^{1}H NMR ($CDCl_3$, 400 MHz), δ: 0.94 (3H, t, J = 7.4 Hz), 1.54 (3H, d, J = 6.6 Hz), 1.67 (2H, q, J = 7.4 Hz), 2.31 (2H, t, J = 6.6 Hz), 5.91 (1H, q, J = 6.6 Hz), 7.30 (5H, m); ^{13}C NMR ($CDCl_3$, 100 MHz), δ: 13.77, 18.60, 22.41, 36.65, 72.13, 126.18, 127.91, 128.59, 142.00, 173.04; for III: ^{1}H NMR ($CDCl_3$, 400 MHz), δ: 0.88 (3H, t, J = 6.8 Hz), 1.30 (4H, m), 1.53 (3H, d, J = 6.6 Hz), 1.63 (2H, m), 2.32 (2H, dt, J = 7.5 Hz, 0.8 Hz), 5.90 (1H, q, J = 6.6 Hz), 7.30 (5H, m); ^{13}C NMR ($CDCl_3$, 100 MHz), δ: 14.03, 22.40, 22.45, 24.79, 31.40, 34.73, 72.14, 126.19, 127.91, 128.60, 142.00, 173.24. These data are in accordance with those already published (Gnanaprakasam et al., 2010).

General procedure for enzymatic hydrolysis kinetic resolution of 1-phenylethyl esters in mini-emulsion

A mixture of 1-phenylethyl ester (1.64 mmol), 50 mM aqueous solution of sodium dodecanoate (pH = 8.5, 2.5 g) and hexadecane (0.11 mmol, 32.0 μL) was stirred at ambient temperature for 1 h. Next, the mixture was sonicated with an ultrasonic tip MS72 (2 min, 5 s on, 5 s off, 78 W, 52 % amplitude, three times) under cooling (ice bath). The mini-emulsion was added at different temperatures 4 ℃, 25 ℃ and 40 ℃ and the reaction initiated by adding the biocatalyst (CALB) (24 U, 100 μL), then the reaction mixture was stirred for 48 h. Aliquots of 250 μL were collected at different times, acidified with 1 M HCl and extracted using Et_2O (3 × 2 mL). The combined organic layers were dried with $MgSO_4$, filtered and analysed by GLC.

Preparative enzymatic kinetic resolution of 1-phenylethanol in mini-emulsion

A mixture of 1-phenylethyl dodecanoate (8.2 mmol, 2.5 g), 12.5 mM aqueous solution of sodium dodecanoate (12.5 g, pH = 8.5) and hexadecane (0.554 mmol, 0.162 mL) was stirred at ambient temperature for 1 h. Next, the mixture was sonicated with an ultrasonic tip (2 min, 5 s on, 5 s off, 98 W, 60 % amplitude, four times) under cooling (ice bath). The mini-emulsion was added at 4 ℃ and the reaction initiated by adding the biocatalyst (CALB L) (120 U, 500 μL). The reaction mixture was stirred for 96 h maintaining the pH at 8.2 by the addition of 0.1 M NaOH. A white precipitate of dodecanoic acid was formed during the reaction. The reaction mixture was frozen and extracted using Et_2O (3 × 30 mL), the organic layers were combined, dried with $MgSO_4$, filtered and the solvent was evaporated. (*R*)-1-Phenylethanol was distilled from the reaction mixture at 60 ℃ and 20 Pa, yielding 363.2 mg (36 %) with an *ee* > 99 %. After distillation, the reaction mixture containing (*S*)-1-phenylethyl dodecanoate was chemically hydrolysed with a 4 M non-aqueous solution of KOH in MeOH at 60 ℃ for 1 h. The reaction mixture was concentrated under reduced pressure, Et_2O (15 mL) was added and the solvent was removed from the suspension by evaporation. Et_2O (30 mL) was added to the suspension and the mixture was stirred for 10 min and filtered. The combined organic layers were washed with water (2 × 30 mL), dried with $MgSO_4$, filtered and the solvents were evaporated to afford (*S*)-1-phenylethanol (515.1 mg, 51 % yield, *ee* = 79 %).

Results and discussion

As stated above, the use of mini-emulsion media for enzymatic catalysis was motivated by the possibility of circumventing the insolubility of most organic substrates in aqueous media without the use of organic solvents. One significant aspect of the mini-emulsion formation is the surfactant selection. The addition of an appropriate surfactant, which provides either electrostatic or steric stabilisation to the droplets, is crucial to stabilising mini-emulsions against coalescence. The current work uses the well-known and benign anionic soap, sodium dodecanoate. Taking as a starting point the sodium dodecanoate critical micelle concentration (cmc) of 7.15–30 mM (Merta et al., 2000; Akhter & Al-Alawi, 2000), the investigation started by using 50 mM sodium dodecanoate at pH 8.5.

The studies began by screening the influence of 1-phenylethyl esters chain-length on enantioselective hydrolysis catalysed by *Candida antarctica* lipase B at 25 ℃ in a mini-emulsion medium. Four substrates, 1-phenylethyl acetate (*I*), 1-phenylethyl butanoate (*II*), 1-phenylethyl hexanoate (*III*) and 1-phenylethyl dodecanoate (*IV*) were investigated (Fig. 2, Table 1).

A pronounced effect of the substrates' chain-length in the enzymatic reaction was noted. The increasing chain-length led to a dramatic decrease in the conversion from 48 % to 4 % after 6 h (acetate and dodecanoate, respectively, entries 3 and 12, Table 1). With regard to the enantioselective hydrolysis, the enantiomeric excess follows the same trend and *ee* from 92 % to 5 % were obtained for (*S*)-enantiomer (entries 3 and 12, Table 1).

In addition, the enzymatic hydrolysis of 1-phenyl-

Table 1. Mini-emulsion enzymatic hydrolysis of 1-phenylethyl esters *I–IV* using CALB as biocatalyst

Entry[a]	Substrate	Time/h	Conv[b]/%	*ee* (*R*)-*V*[c]/%	*ee* (*S*)-*I–IV*[c]/%
1	*I*	1	40	> 99	67
2	*I*	3	48	> 99	91
3	*I*	6	48	> 99	92
4	*II*	1	28	> 99	40
5	*II*	3	34	> 99	52
6	*II*	6	35	> 99	53
7	*III*	1	27	> 99	36
8	*III*	3	26	> 99	36
9	*III*	6	27	> 99	37
10	*IV*	1	1	> 99	2
11	*IV*	3	2	> 99	3
12	*IV*	6	4	> 99	5

a) Mini-emulsion system was composed of 50 mM aqueous sodium dodecanoate solution (2.5 g, pH = 8.5), 1-phenylethyl ester (*I–IV*, 1.64 mmol), hexadecane (0.11 mmol, 32.4 μL) and CALB (24 U, 100 μL); *b*) Conv = conversion = $ee_s/(ee_s + ee_p)$, determined by GLC; *c*) determined by GLC; enantiomeric ratio (*E*) calculated according to Chen et al. (1982) was > 200 for all entries.

Table 2. Mini-emulsion enzymatic hydrolysis of *I* using different commercial biocatalysts

Entry[a]	Lipase	Conv/%	*ee* (*R*)-*V*/%	*ee* (*S*)-*I*/%	*E*
1	*Candida antarctica* lipase B	48	> 99	92	> 200
2	Porcine liver esterase	< 1	–	–	–
3	Amano lipase AK from *Pseudomonas fluorecens*	4	> 99	4	> 200
4	Amano lipase PS from *Burkholderia cepacia*	37	> 99	59	> 200
5	Lipase PS Amano SD from *Burkholderia cepacia*	27	> 99	37	> 200
6	Lipase from *Candida rugosa*	2	54	2	3.4
7	Amano lipase AYS from *Candida rugosa*	2	73	1	6.5
8	*Candida antarctica* lipase B (lyophilised)	48	> 99	92	> 200

a) For mini-emulsion system composition and determination of Conv, *ee* and *E*, see Table 1; hydrolysis time was 6 h for all entries.

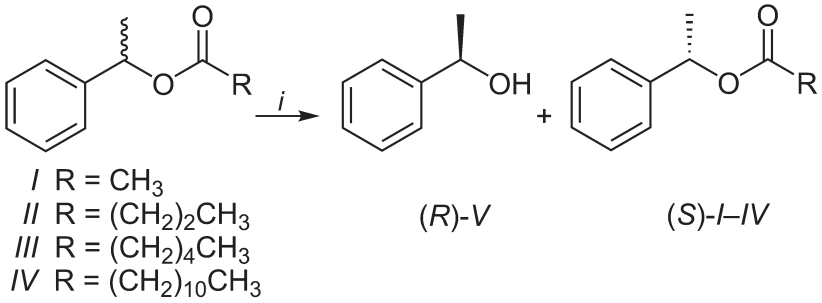

Fig. 2. Mini-emulsion enzymatic hydrolysis of 1-phenylethyl esters *I–IV*. Reaction conditions: *i*) mini-emulsion, CALB, 25 °C, 1–6 h (see Table 1); *ii*) mini-emulsion, different lipases, 25 °C, 6 h (see Table 2); *iii*) mini-emulsion, CALB, different temperature, 6 h (see Table 3); *iv*) mini-emulsion, CALB, 4 °C, different time (see Table 4).

ethyl acetate with other commercially available enzymes was performed. Of the seven enzymes screened (see Experimental), CALB was shown to be the more efficient biocatalyst (entries 1 and 8, Table 2). Either the liquid formulation or the lyophilised enzyme afforded the enzymatic resolution with 48 % conversion, > 99 % *ee* for (*R*)-enantiomer and 92 % *ee* for (*S*)-enantiomer. Porcine liver esterase, Amano lipase AYS and lipase from *Candida rugosa* afforded very low conversions (< 2 %) (entries 2, 3, 6, 7, Table 2). Amano lipase PS and lipase PS Amano SD from *Burkholderia cepacia* afforded moderate results with conversion in the range of 27–37 % (entries 4 and 5, Table 2). These results appear to indicate that *Candida antarctica* lipase B, unlike others, is suitable for use in a mini-emulsion system.

To assess the effect of temperature on the biotransformation, the reaction was performed at three different temperatures (40 °C, 25 °C and 4 °C) in the presence of CALB (Table 3). It was found that, for substrates *I–III*, the reactivity was not very sensitive to the temperature. Only minor changes were observed at different temperatures. Surprisingly, the reactivity was effectively sensitive to the temperature for *IV* and

Table 3. Effect of temperature on mini-emulsion enzymatic hydrolysis of 1-phenylethyl esters *I*–*IV* using CALB as biocatalyst

Entry[a]	Substrate	T/ ℃	Conv/%	*ee* (*R*)-*V*/%	*ee* (*S*)-*I*–*IV*/%	Mini-emulsion size[b]/nm
1	*I*	40	47	> 99	90	–
2	*I*	25	48	> 99	92	173
3	*I*	4	47	> 99	87	152
4	*II*	25	35	> 99	53	206
5	*II*	4	36	> 99	55	167
6	*III*	25	27	> 99	37	298
7	*III*	4	26	> 99	35	287
8	*IV*	40	< 1	–	–	–
9	*IV*	25	4	> 99	5	359
10	*IV*	4	29	> 99	38	302

a) For mini-emulsion system composition and determination of Conv, *ee* and *E*, see Table 1; *E* was > 200 for all entries (except for entry 8); *b*) average diameter determined prior to addition of enzyme.

Table 4. Mini-emulsion enzymatic hydrolysis of *IV* using CALB as biocatalyst at 4 ℃

Entry[a]	Surfactant/mM	Time/h	Conv/%	*ee* (*R*)-*V*/%	*ee* (*S*)-*IV*/%	*E*
1	50	24	37 (29)	> 99 (> 99)	58 (42)	> 200
2	50	48	40 (29)	> 99 (> 99)	66 (41)	> 200
3	12.5	24	41	> 99	71	> 200
4	12.5	48	44	> 99	78	> 200

a) Mini-emulsion system was composed of 50 mM or 12.5 mM aqueous sodium dodecanoate solution (2.5 g, pH = 8.5), 1-phenylethyl ester *IV* (1.64 mmol), hexadecane (0.11 mmol, 32.4 μL) and CALB (24 U, 100 μL) with 0.1 M NaOH titration (values obtained under the same conditions without 0.1 M NaOH titration are given in brackets); for determination of Conv, *ee* and *E*, see Table 1.

conversion increased to 29 % at 4 ℃ (from 4 % at 25 ℃ and 1 % 40 ℃) after 6 h (entries 10, 9 and 8, respectively, Table 3). These results clearly show that the reaction is driven by the decrease in temperature.

It is known that CALB comprises a small, five-residues α-helix located close to the active centre that has been identified as a candidate for anchoring the lipase at the oil–water interface (Martinelle et al., 1995). However, the ability to operate at the oil–water interface is directly related to access to the substrate. It is apparent that only low temperatures effectively afford the access of 1-phenylethyl dodecanoate to the lipase.

With the aim of understanding this effect, the mini-emulsion size was evaluated and correlated to the reaction extension. The first observation was that mini-emulsion size increased with the use of longer-chain 1-phenylethyl esters. Independently of the temperature, for the same 1-phenylethyl esters concentration, a two-fold increase in the mini-emulsion size was noted from *I* to *IV* (Table 3). The second observation was that, by decreasing the temperature, the size of the mini-emulsion was slightly reduced. For the system composed of *IV*, the mini-emulsion size reduction is crucial to allow the enzymatic reaction to proceed. The results indicate that the reactivity can be controlled by the size of the mini-emulsion and, consequently, by the temperature. To the best of our knowledge, this is the first time that a enzymatic reaction has been recorded as proceeding exclusively at low temperatures.

In addition to the reactivity observed at low temperature for the mini-emulsion system containing *IV*, the highest conversion obtained proved to be far from ideal (entries 1 and 2, values in brackets, Table 4). Longer reaction times, together with the 0.1 M NaOH titration of the dodecanoic acid that is released during the reaction, were essential for extending the conversion up to 40 % after 48 h, with an *ee* > 99 % for (*R*)-*V* (entry 2, Table 4).

Seeking more practical reaction conditions, the amount of surfactant was reduced to 12.5 mM, which is the value close to sodium laurate cmc (Akhter & Alawi, 2000) thus reducing the high amounts of 0.1 M NaOH needed to titrate the excess dodecanoic acid. Under these conditions, 44 % of (*R*)-*V* was converted with an *ee* > 99 % after 48 h (*E* > 200) (entry 4, Table 4).

The excellent outcome of this reaction system prompted an attempt to scale up the reaction. A 2.5 grams substrate scale reaction was performed (Fig. 3).

The conversion of (*R*)-*V* was 44 % with an *ee* of 99 % and (*S*)-*IV* with an *ee* of 77 % after 96 h. One of the main problems encountered in the use of mini-emulsions on an industrial scale is separation of the surfactant from the organic phase. Since mini-emulsions are extremely stable systems, their destabilisation is often a difficult process. Usually, alcohols, salts or acids are used to destabilise the mini-emulsion system. However, the addition of additives entails economic and environmental costs, as well as contamination of the surfactants and/or organic sol-

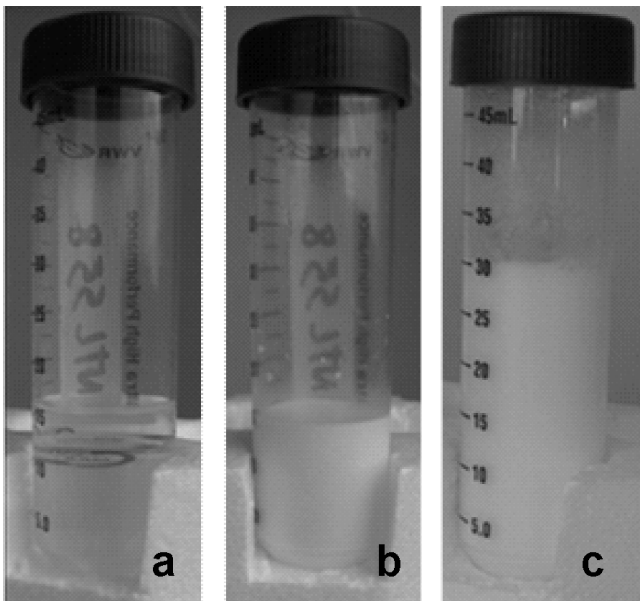

Fig. 3. Mini-emulsion reaction system for preparative enzymatic resolution of *IV*: (a) prior formation of mini-emulsion, two distinct phases (upper – organic phase and lower – aqueous phase); (b) after mini-emulsion formation (single phase – mini-emulsion); (c) at the end of reaction (two distinct phases: liquid phase – mini-emulsion and solid phase – sodium dodecanoate).

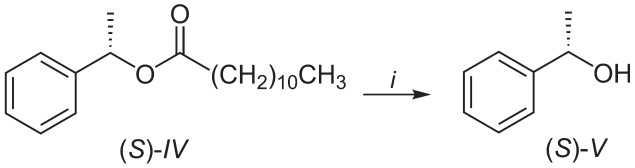

Fig. 4. Chemical hydrolysis of ester (*S*)-*IV*. Reaction conditions: *i*) 4 M solution of KOH in MeOH, 60 °C, 1 h.

vents. A way to avoid the use of additives in this work was based on simply freezing the mini-emulsion. The products were readily removed from the mini-emulsion system by first freezing the mini-emulsion then washing the organic layer (upper phase) with diethyl ether. Accordingly, taking advantage of the different boiling points of the products, the free alcohol was isolated by distillation. (*R*)-*V* was isolated with an overall yield of 36 % with an $ee > 99$ %. In order to recover the other free enantiomer, the reaction mixture containing (*S*)-*IV* was chemically hydrolysed in a 4 M non-aqueous solution of KOH in methanol at 60 °C (ter Halle et al., 2004). Under these conditions (*S*)-*V* was isolated with an overall yield of 51 % with unaltered optical purity (Fig. 4).

Conclusions

In conclusion, the enzymatic kinetic resolution of 1-phenylethanol at low temperature in a mini-emulsion system was demonstrated for the first time. The mini-emulsion temperature control and, consequently, the mini-emulsion size were shown to be crucial for the enzymatic resolution development. This preparative methodology afforded the isolation of both 1-phenylethanol enantiomers with good yields and enantiomeric excess. This methodology is currently being applied to other substrates, with the possibility of recycling the biocatalyst being addressed.

The proposed methodology can contribute to new opportunities on matters of enzymatic resolution, using water as a solvent.

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Supplementary data

Supplementary data associated with this article can be found in the online version of this paper (DOI: 10.1515/chempap-2015-0032).

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