Green Chemistry

RSC Publishing

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c3gc40899j

Evaluation of deep eutectic solvent–water binary mixtures for lipase-catalyzed lipophilization of phenolic acids

Erwann Durand,*[a] Jérôme Lecomte,[a] Bruno Baréa,[a] Eric Dubreucq,[b] Robert Lortie[c] and Pierre Villeneuve[a]

Received 15th May 2013,
Accepted 24th June 2013

DOI: 10.1039/c3gc40899j

www.rsc.org/greenchem

This work reports the first lipase-catalyzed reactions between substrates of different polarities using deep eutectic solvents as a medium. The model reaction consisted of a lipophilization process based on the alcoholysis of phenolic esters using immobilized *Candida antarctica* lipase B as a biocatalyst. Results showed that water could dramatically improve the lipase activity and change the reactivity of phenolic substrates. Indeed, very low conversions (<2%) were observed in pure DES, whereas in DES–water binary mixtures, quantitative conversions were achieved. After investigating the role of various parameters, such as the substrate concentration and ratio, pH or thermodynamic activity of water, the effect of the presence of water in pure DES based on urea or glycerol was discussed. In this paper, we propose new perspectives for the enzymatic modification of polar substrates using this novel generation of green, inexpensive and easy-to-handle solvents.

Introduction

In recent years, ionic liquids have been subject to in-depth evaluation as potential new media for performing lipase-catalyzed reactions between substrates of different polarities.[1–5] Indeed, the advantage of these mixtures is their capacity to solubilize polar and non-polar molecules, which means that good contact can be established between them and the enzyme. However, to date, limited use has been made of ionic liquids for biocatalyzed reactions on an industrial scale, owing to their high price and operational difficulties. More recently, some publications revealed that Deep Eutectic Solvents (DES) could be a promising alternative to ionic liquids as efficient "green" media in lipase-catalyzed reactions.[6–9] These solvents, which share many characteristics with ionic liquids (nonvolatile, thermally stable up to nearly 200 °C, nonflammable *etc.*), have many other advantages: they are relatively inexpensive, environmentally benign and have a very low toxicity.[10] Moreover, unlike ionic liquids, these solvents do not require a preliminary purification step. Indeed, they result from the association of a cationic salt (ammonium or more recently phosphonium) with a hydrogen-bond donor (HBD). One of the most explicit examples is the mixing of one mole of choline chloride and two moles of urea (with melting points of 247 °C and 133 °C, respectively). This results in a deep eutectic solvent with a room temperature melting point. The strong interaction between the HBD and the anion, provided by the salt, leads to a considerable reduction in the melting point of the mixture. In addition, the strong association between the components radically decreases their reactivity, making them inert in most cases. Thus, the mixture can be used at a temperature that permits biocatalytic reactions. However, until now, all biotransformations using lipases in DES have been carried out with substrates that have little affinity to the solvent. However, once the strong hydrogen-bond network is established with the DES, it could dramatically reduce the reactivity of the soluble substrates (as has frequently been observed with the hydrogen-bond donor itself). Therefore, it is likely that the next remarkable step will be to achieve the enzymatic modification of polar substrates using DES. Among them, phenolic acids, a large and diverse group of secondary metabolites that are naturally widespread in the plant kingdom, are of particular interest. Indeed, these products are known for their potential protective role against oxidative damage and their uses as natural antioxidants for application in various food, cosmetic or pharmaceutical matrices.[11–13] However, most phenolic acids confer their functional properties in a hydrophilic

[a]*CIRAD, UMR 1208 IATE, F-34398 Montpellier, France. E-mail: villeneuve@cirad.fr; Fax: +33 499613000; Tel: +33 499612029*
[b]*Montpellier SupAgro, UMR 1208 IATE, F-34060 Montpellier, France. E-mail: Eric.Dubreucq@supagro.inra.fr; Tel: +33 499612364*
[c]*National Research Council, 6100 Royalmount Av., Montreal (Qc) H4P 2R2, Canada. E-mail: robert.lortie@cnrc-nrc.gc.ca; Tel: +1-514-496-6158*

environment. For this reason, few applications in oil-based formulae have been developed. Thus, a practical approach to apply phenolic acids in a lipophilic formulation is to change their hydrophilic properties by increasing their hydrophobic affinity.[1] The main method consists of grafting an aliphatic chain on a carboxyl functional group of phenolic acids in order to modify their hydrophilic–lipophilic balance and then to obtain a new molecule with combined emulsifying properties and improved antioxidant activity. This type of lipophilization of phenolic antioxidants can be performed chemically or enzymatically. The second strategy offers several advantages: milder reaction conditions, reduced by-product formation and purification steps, and a more environmentally friendly process.[14–16] However, one of the difficulties involved in this biocatalyzed reaction lies in optimizing the synthesis (yields and kinetics) with two substrates of different polarities. Therefore, it is of paramount importance to select media in which both polar (*e.g.* phenolic compounds) and apolar (*e.g.* long-chain alcohols) substrates are soluble, at least partially, and where the enzyme activity can be maintained at a satisfactory level. Indeed, in non-polar solvents such as hexane, phenolic compounds have very low solubility, whereas lipases are generally active. Conversely, in polar organic environments, phenolic compounds have good solubility, but lipases are deactivated or exhibit a poor activity. Nevertheless, there are several reports dealing with the efficient esterification of phenolic acids with fatty alcohols either in an organic solvent or in a solvent free-system.[17–20] According to these studies, lipase-catalyzed reactions in organic solvents are still preferable to those using a solvent-free system because the former can increase the solubility of phenolic compounds and shift the reaction equilibrium to favor synthesis reactions. A binary organic solvent appears to be the best solution, since it allows for the fine-tuning of the solubility of the phenolic compounds while maintaining a good level of lipase activity. However, these enzymatic reactions remain difficult due to the prolonged reaction time or low productivity. In addition, most of the reactions have been conducted either using a low concentration of substrates, a high enzyme load or a high alcohol to phenolic compound molar ratio. This can cause difficulties when it comes to subsequent separation and purification of the products. Therefore, many studies have been carried out to propose new media for this kind of reaction with emphasis on environmentally friendly media. Thus, the challenge faced by this study is two-fold: the reactivity of the polar substrate in DES, and lipase-catalyzed reactions with substrates of two different polarities. In a previous work[21] the superior performance of choline chloride (ChCl) paired with urea (U) or glycerol (Gly) was demonstrated in comparison to other types of DESs in the alcoholysis of an aliphatic ester using *Candida antarctica* lipase B as a catalyst. Thus, in this study, we investigated the lipophilization reaction of methyl *p*-coumarate and methyl ferulate in DES ChCl:U and ChCl:Gly. Several parameters were examined in order to define and understand the factors that influence the lipase activity and the reactivity of the substrates in these neoteric solvents.

Experimental

Materials

All solvents (acetonitrile, acetone, ethyl acetate, and hexane) of analytical and HPLC grade were purchased from Labover (Montpellier, France). Anhydrous 1-butanol (99.8%) and 1-octanol (≥99%), immobilized lipase B from *Candida antarctica* (Novozym435) with a specific activity ≥ 5000 PLU g^{-1} (propyl laurate units per g), *p*-coumaric acid (>98%), ferulic acid (99%), Amberlite IR-120H, molecular sieves (3 Å), glycerol (Gly) ≥ 99%, urea (U) ≥ 99%, choline chloride (ChCl) ≥ 99% and water (CHROMASOLV PLUS) were purchased from Sigma-Aldrich (Saint Quentin, France).

Synthesis of DESs

All hydrogen-bond donors were dried under vacuum over silica gel and P_2O_5 prior to use. Ammonium salts were dried under vacuum at 60 °C over silica gel for 3 days before use. Firstly, the ammonium salt and the hydrogen-bond donor, in a molar ratio of 1 : 2, were directly weighed in a flask, avoiding any contact with air moisture. Then, the mixture was heated and stirred (a STUART Scientific S150 orbital shaker incubator, 250 rpm) at 60 °C until a colorless liquid was formed (typically 2 hours).

Chemical synthesis and purification of methyl esters

The chemical esterification of phenolic acid (6.58 mmol) was carried out in sealed flasks containing 75 ml of methanol. The reaction mixture was stirred at 60 °C using an orbital shaker incubator (250 rpm) until perfect dissolution of the substrates was obtained. Then, 3.015 g of the sulfonic resin Amberlite IR-120H (5% of the total weight of both substrates), previously dried at 110 °C for 48 h, was added and the water generated during the reaction was removed by absorption in molecular sieves (3 Å) added to the medium. Samples (20 μL) were regularly taken from the reaction medium and analyzed using TLC (chloroform–methanol 93 : 7, v/v, silica gel 60F254 TLC plates), and the spots were examined visually under UV light at 366 nm. After 5 days, the reaction mixture was filtered and the alcohol was evaporated. The residue was solubilized in ethyl acetate (30 ml) and washed successively with saturated sodium carbonate (2 × 20 ml) and water (2 × 20 ml) and finally dried over anhydrous sodium sulfate. After vacuum drying, the crude product was purified by flash chromatography on a Combi-Flash Companion system (Teledyne Isco Inc., Lincoln, NE, USA) using an elution gradient of hexane and ethyl acetate (from 90–10 to 0–100 in 45 min) to yield the corresponding methyl ester. Methyl *p*-coumarate was obtained in the form of a white powder (>99% HPLC) and methyl ferulate as a sticky oil (>99% HPLC).

Water content determination

The water content of DES was determined by using Karl Fisher titration at 20 °C using Hydranal Coulomat AG as the analyte.

pH measurement

The pH was measured on a 736 GP Titrino apparatus (Metrohm, Villebon Courtaboeuf, France) using TIAMO 1.2 software. The experiments were carried out at 60 °C (temperature reported from the pH stat = 58.6 °C) on a sample of freshly prepared 10 ml DES. Water was added step-by-step, and the pH value was taken 2 min after each addition.

Determining the water activity (a_w) in DES–water binary mixtures

Binary mixtures (5 ml) of DES with various water contents (0–20%, w/w) were freshly prepared and heated at 60 °C in hermetically sealed flasks. Then, the mixtures were allowed to cool down to room temperature, and the thermodynamic activity of water (a_w) was determined at 23.3 °C using an Aqualab CX-2 series 3 apparatus (Pullman, Washington, USA).

General conditions for the alcoholysis assay

Assays were performed at 60 °C under a nitrogen atmosphere in 30 ml hermetically sealed flasks containing 5 ml of DES with various water contents (0–20%, w/w), 200 μmol of methyl ester (35.6 mg for methyl *p*-coumarate or 41.6 mg for methyl ferulate) and 1-octanol (1200 μmol; 190.35 μl) in a 1 : 6 molar ratio. The reaction was initiated by adding 50 mg of Novozym435 and then stirred at 200 rpm using a STUART Scientific S150 orbital shaker. The reaction kinetics were monitored using HPLC on samples diluted in methanol–acetonitrile (1 : 2, v/v) and filtered at 0.45 μm.

Lipase stability in DES–water binary mixtures

In 5 ml of the appropriate DES–water mixture, 50 mg of Novozym435 were pre-incubated under orbital shaking at 60 °C in a sealed flask under a nitrogen atmosphere. After a given time, 200 μmol (35.65 mg) of methyl *p*-coumarate and 1200 μmol (190.35 μl) of 1-octanol were added to the reaction medium. The reaction was kept at 60 °C, and the initial activity was compared to that obtained without pre-incubating the lipase. The residual activity, expressed as a percentage, was defined as 100× the initial activity after incubation/initial activity without pre-incubation.

HPLC analysis

The selectivity of the reaction was assessed by HPLC analysis (Thermoscientific spectra system P4000, AS3000) using a NUCLEODUR HILIC column (Macherey-Nagel: length 250 mm, i.d. 4.6 mm, particle size 5 μm) connected to a UV detector. Three different wavelengths (200, 245 and 335 nm) were used to evaluate the results. The separation was conducted on a 20 μl sample at 28 °C at 0.8 ml min^{-1} using a binary elution as a mobile phase between solvent A: acetonitrile and solvent B: water and 0.05% w/w formic acid. The analyses were conducted as follows: 97A-3B (v/v) for 15 min, gradient up to 80A-20B in 3 min followed by an isocratic elution under the same conditions until perfect salt elution was obtained. With this setting, retention times were: methyl *p*-coumarate (4.4 min), octyl *p*-coumarate (4 min), methyl ferulate (4.4 min), octyl ferulate (4 min), glycerol (10.8 min), urea (14.8 min) and choline chloride (28.4 min).

Definition of calculations

$$\text{Conversion}\,(\%) = 100\frac{\text{moles of methyl ester consumed}}{\text{moles of methyl ester at } t = 0}$$

$$\text{Hydrolysis}\,(\%) = 100\frac{\text{moles of acid formed}}{\text{moles of methyl ester at } t = 0}$$

$$\text{Yield}\,(\%) = 100\frac{\text{moles of octyl ester formed}}{\text{moles of methyl ester consumed}}$$

where methyl ester refers to methyl *p*-coumarate or methyl ferulate, octyl ester refers to octyl *p*-coumarate or octyl ferulate, and acid refers to *p*-coumaric acid or ferulic acid.

Results and discussion

Alcoholysis of phenolic esters with 1-octanol

The first model reaction involved the alcoholysis of methyl *p*-coumarate (40 mM) with 1-octanol (240 mM) in a 1 : 6 molar ratio using Novozym435 as the biocatalyst at 60 °C (Fig. 1). The reaction was performed in ChCl:U and ChCl:Gly, the two DES previously described as the most efficient for lipase-catalyzed reactions.[21]

The reaction was initially carried out for 4 days in both solvents. However, almost no conversion (<2%) was observed regardless of the DES used. This unexpected result could not be explained by the absence of solubilization of the substrate because methyl *p*-coumarate was perfectly dissolved in the chosen DES before the addition of the biocatalyst. Indeed, DES exhibit unusual solvation properties that are strongly influenced by hydrogen bonding and result in a high affinity to all compounds capable of donating electrons or protons. In other words, any molecule capable of creating this type of bond will undoubtedly be dissolved in these solvents. In the case of phenolic compounds, several intermolecular hydrogen bonds can be established; thus, their solubilization in DES should not be a matter of concern. Furthermore, a possible deactivation of the biocatalyst by the substrate can be ruled out. Indeed, previous experiments described in the literature demonstrated and proved that this substrate can be converted reasonably well.[20,22] Thus, in the system we studied, it is likely that methyl *p*-coumarate is also associated with the hydrogen-bond network of the DES. However, the association is so strong that it is barely available for the reaction, as observed in most cases

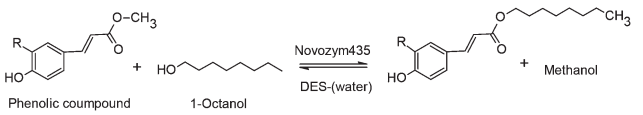

Fig. 1 Reaction scheme showing alcoholysis of phenolic esters with 1-octanol in DES using Novozym435 as a catalyst (R = H, methyl *p*-coumarate; R = OCH_3, methyl ferulate).

with the HBD. Indeed, the main consequence of the strong hydrogen bonding between choline chloride and glycerol or urea is the poor reactivity of the HBD (as competitive nucleophiles) in lipase-catalyzed transesterification.[21] In addition, preliminary studies (data not shown) have revealed how difficult it is to extract phenolic acids from DES, even by using polar immiscible solvents such as diethyl ether or acetone. The extraction of phenolic alkyl esters from DES was much easier when the alkyl chain was long. From these observations, we put forward the hypothesis that adding a protic co-solvent to the system, which creates hydrogen bonding more easily than methyl *p*-coumarate, would lessen the interaction between the DES and the substrate, making the latter more available for reacting. Our tests demonstrated that water was the best candidate for two major reasons: first, water is probably one of the best available hydrogen-bond donors, and secondly, it makes it possible to maintain a simple system, as well as the green aspect of DES. It is also important to note that water has the advantage of greatly reducing the viscosity of these mixtures, improving mass transfer, which is probably one of the main weaknesses of ionic liquids and DES.

Alcoholysis of methyl *p*-coumarate with 1-octanol in ChCl:U–water binary mixtures

When the reaction was performed with 2% (w/w) water, a slight activity was observed, corresponding to 20% conversion of the substrate after 72 h and the formation of the expected octyl *p*-coumarate product (Fig. 2). Surprisingly, adding more water to the system resulted in a significant increase in the conversion rate, which reached 100% at the apparent equilibrium (72 h) for water contents higher than 8%. As in the previous experiments, no reaction occurred in the absence of the catalyst Novozym435. These results clearly show that water is essential to the reaction. On the other hand, the eutectic mixture itself had no catalytic effect, unlike some other DES used as catalysts in chemical synthesis.[23–26] In addition, a competitive hydrolysis reaction can occur in these binary mixtures with a high water content. However, at a reaction time corresponding to the apparent equilibrium (72 h), with a water content below 6% (w/w), no hydrolysis of methyl *p*-coumarate into *p*-coumaric acid was observed (Fig. 2). With water contents of 8% and 10%, the production levels of octyl *p*-coumarate were at a maximum since conversions were very high (93% and 98%, respectively), while hydrolysis remained very low (<2%). In parallel, a multi gram scale experiment in ChCl:U with 10% water content was conducted to synthesize the lipophilized phenolic compound in high yield. The reaction was realized in the presence of 3 g of *p*-coumarate (40 mM) with 1-octanol in a 1 : 6 molar ratio, using 10 mg ml^{-1} of biocatalyst. Under these conditions, 97% of the substrate was converted after 72 h of reaction, with a yield of 93%, which confirmed the viability of this synthesis for a large scale application.

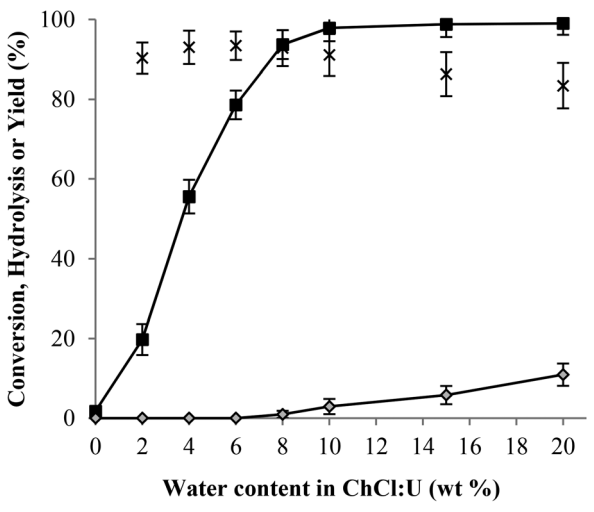

Fig. 2 Alcoholysis of methyl *p*-coumarate with 1-octanol in ChCl:U–water binary mixtures: conversion (■), yield (×) and hydrolysis (◆) rates as a function of water content after 72 h reaction time (60 °C, 40 mM methyl *p*-coumarate, 240 mM 1-octanol, 10 mg ml^{-1} Novozym435). Values are means ± SD (n = 2).

Finally, in mixtures containing greater amounts of water, *i.e.* 15% and 20% (Fig. 2), the competitive hydrolysis reaction became significant and led to a decrease in yield (86% and 83%). However, it is worth mentioning that the reduction in yield was not solely due to hydrolysis but also to the slight degradation of both the substrate and the product through side reactions.

Alcoholysis of methyl *p*-coumarate with 1-octanol in ChCl:Gly–water binary mixtures

When the reaction was performed in ChCl:Gly, the same phenomenon was observed, *i.e.* a slight activity in pure DES followed by an increase in methyl *p*-coumarate conversion with the addition of water. However, unlike the previous experiments, the reaction was much slower and failed to reach equilibrium after 725 h (30 days) reaction time, even with the highest water content of 20%. Moreover, at 725 h reaction time, both conversion and yield were lower than previous observations with ChCl:U for the same water contents. Hydrolysis remained at about the same order of magnitude (Fig. 3). As a result, the best production in octyl *p*-coumarate (62%) was obtained for the highest conversion of methyl *p*-coumarate and the highest water content in the system. However, under these reaction conditions, hydrolysis accounted for almost 10%.

Alcoholysis of methyl ferulate in ChCl:U–water and ChCl:Gly–water mixtures

In order to determine whether this process could be extended to other phenolic substrates, the alcoholysis of methyl ferulate (40 mM) with 1-octanol (240 mM) was tested at 60 °C. Once more, the presence of water in the system was necessary to convert the substrate (methyl ferulate) into the expected ester (octyl ferulate) (Fig. 4). As previously observed, faster reaction and higher conversion rates were obtained in ChCl:U–water mixtures, where the apparent equilibrium was reached after 168 h. However, unlike the results observed with methyl *p*-coumarate, the competitive hydrolysis reaction was drastically lower regardless of the DES and water content (<2% hydrolysis

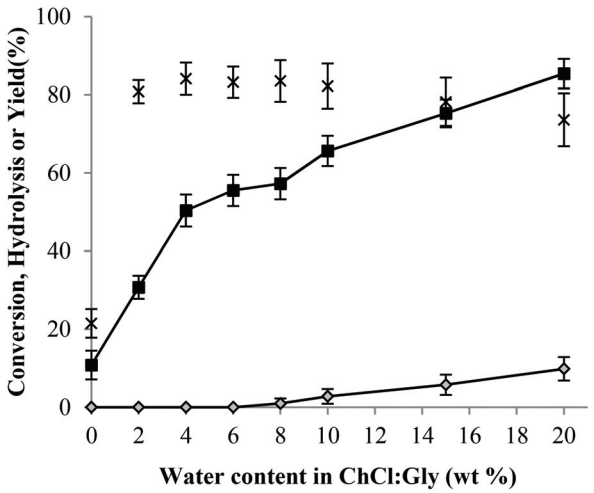

Fig. 3 Alcoholysis of methyl *p*-coumarate with 1-octanol in ChCl:Gly–water binary mixtures: conversion (■), yield (×) and hydrolysis (◆) rates as a function of water content after 725 h reaction time (60 °C, 40 mM methyl *p*-coumarate, 240 mM 1-octanol, 10 mg ml^{-1} Novozym435). Values are means ± SD (n = 2).

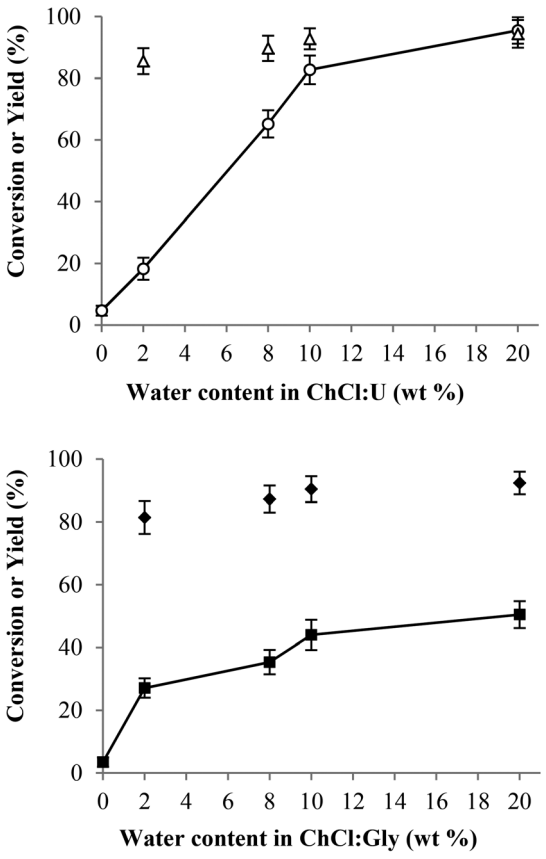

Fig. 4 Alcoholysis of methyl ferulate with 1-octanol in ChCl:Gly– and ChCl:U–water binary mixtures: conversion (○) and yield (△) in ChCl:U after 168 h; and conversion (■) and yield (◆) in ChCl:Gly after 412 h (60 °C, 40 mM methyl ferulate, 240 mM 1-octanol, 10 mg ml^{-1} Novozym435). Values are means ± SD (n = 2).

with 20% water), allowing a higher yield (≥90%) with 20% water content. Thus, while the reactivity of water seems to be drastically reduced in DES–water mixtures, it is unpredictable and depends on the nature of the substrate used.

Effect of substrate concentration

In order to assess the possible influence of octanol as a co-solvent in the different DES–water binary mixtures, we studied the effect of the phenolic ester to alcohol molar ratio on the kinetics of alcoholysis and on the final conversion. The reaction was performed for 72 h with methyl *p*-coumarate (40 mM) and different excess amounts of 1-octanol (from 1 : 1 to 1 : 12) in 8% water–ChCl:U (corresponding to the best yield without apparent hydrolysis), 2% water–ChCl:U (corresponding to the beginning of lipase activity) and pure ChCl:U. For each given water content, the final conversion of methyl *p*-coumarate was the same (methyl *p*-coumarate conversions were <2%, 19.0 ± 1% and 91.1 ± 0.2% in ChCl:U containing 0%, 2% and 8% water, respectively) for all substrate to alcohol molar ratios, with one exception. Indeed, a lower conversion (81% *vs.* 91%) was obtained when an equimolar ratio was used in 8% water–ChCl:U. With the alcoholysis kinetics, only slight differences were observed with the highest alcohol concentration (data not shown). This is probably due to the presence of 1-octanol on the hydrophobic support of the catalyst, which decreases the diffusion of methyl *p*-coumarate towards the active site of the enzyme. Thus, even if optimal conversions are obtained using 1-octanol in excess, the alcohol concentration is not a predominant factor in determining the significant differences in enzymatic reactivity between the mixtures. Moreover, the influence of methyl *p*-coumarate concentration on the final conversion rate (equilibrium at 72 h) was studied in 4%, 8% and 20% water–ChCl:U mixtures and the octanol concentration was set at 240 mM. Methyl *p*-coumarate reacted at concentrations of 40, 80, 120 and 240 mM, corresponding to the methyl *p*-coumarate to 1-octanol molar ratios of 1 : 6, 1 : 3, 1 : 2 and 1 : 1, respectively. As shown in Fig. 5, the best conversions were

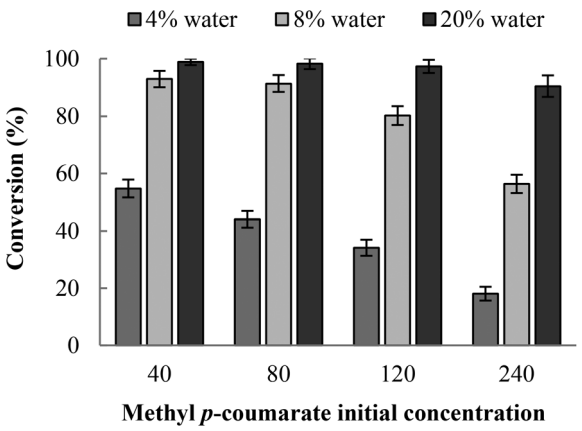

Fig. 5 Conversion of methyl *p*-coumarate at 60 °C in a ChCl:U–water binary mixture. Reactions were conducted with different % water contents (w/w) and 240 mM (1200 μmol) of 1-octanol. The enzyme load was 10 mg ml^{-1}. Values are means ± SD (n = 2).

obtained for the lowest methyl *p*-coumarate concentration, *i.e.* the highest methyl *p*-coumarate to 1-octanol molar ratio, irrespective of the water content in the DES. However, these experiments showed that the production of the desired phenolipid can be greatly improved with the concentration of water. Indeed, with 20% water content, the conversion of substrate remained almost quantitative whatever its concentration. Consequently, the production of octyl *p*-coumarate was maximized despite the presence of the competitive hydrolysis reaction (around 10%).

These results highlight the benefit of adding a large excess volume of water in the DES studied. They strengthen our initial hypothesis on the basis of the putative fact that the strong hydrogen bonding within these mixtures could decrease the thermodynamic activity of the molecules involved. In this case, it is important to note that the DES's tremendous capacity to solvate polar substrates can also be detrimental to their availability for reactions. Also, these results showed that despite the presence of excess water compared to 1-octanol (water to 1-octanol molar ratios from 5.6 to 53.7), its nucleophilic activity is substantially reduced in DES. This is probably because it is highly involved in the hydrogen-bond network and has a low thermodynamic activity. Several studies on the physical and thermodynamic properties of DES–water mixtures have revealed an increase in hydrogen bonds and the establishment of stronger interactions than in the pure eutectic mixture. Indeed, the negative values for the excess molar heat capacities (C_P: the amount of energy required to raise one mole of the substance by one degree) or molar volume (V_M: volume occupied by one mole of a substance) in DES–water binary mixtures indicate a reduction in the overall volume, which could be attributed to the strong hydrogen bonding that arises between DES and water.[27–29] Finally, according to Gutierrez *et al.*,[30] a large excess of water, corresponding to almost 50% (w/w), in DES based on choline chloride and urea is necessary to break the halide-HBD supramolecular complex and to obtain a simple aqueous solution of the two individual components.

After successfully demonstrating that water has several positive effects on lipase-catalyzed reactions in this complex network, we went on to investigate how the water in the system can directly influence enzyme activity. To achieve this, we set essential parameters in the lipase-catalyzed reactions, such as media water content, water activity (a_w) and the subsequent pH.

Effect of the water content on the water activity (a_w) of DES–water binary mixtures

It is well known that catalytic activity is very sensitive to the hydration state of the enzyme, which is essentially governed by the water activity of the system. This explains why, in the biocatalysis field, a_w is often responsible for changes in the optimal activity of the chosen biocatalyst, whereas other aspects of the system remain unchanged.[31] For this reason, the effect of water thermodynamic activity should be evaluated. The water activity measured in ChCl:U, a_w(ChCl:U), and ChCl:Gly, a_w(ChCl:Gly) was strictly proportional to the water content (W%) in the system, as shown by the corresponding linear relationships (Fig. 6):

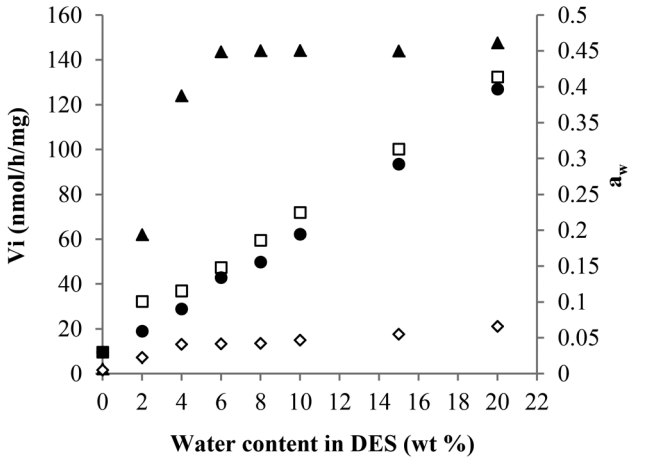

Fig. 6 Thermodynamic activity of water (a_w) and lipase initial activity (V_i, corresponds to the amount of substrate converted per hour per mg of enzyme at 60 °C) as a function of water content in ChCl:Gly and ChCl:U–water binary mixtures. V_i ChCl:Gly (◊), V_i ChCl:U (▲), a_w ChCl:Gly (□) and a_w ChCl:U (●). Values are means ± SD (n = 2).

$$a_w(\text{ChCl : U}) = 0.0183 \times \text{W\%} + 0.0204, R^2 = 0.996$$

$$a_w(\text{ChCl : Gly}) = 0.0183 \times \text{W\%} + 0.0425, R^2 = 0.993$$

Moreover, the value of the slope was identical in both cases, which indicates that choline chloride has a major effect on this parameter. As shown in Fig. 6, in urea-based DES, a significant increase in the initial enzyme activity was observed when the water content increased up to 6%, corresponding to a water activity of around 0.15. Indeed, the reaction rate in a 6% water–ChCl:U mixture ($a_w \approx 0.15$) was 67 times faster than in the pure eutectic mixture (a_w 0), while almost no difference in the initial activity was observed for higher water contents. In ChCl:Gly, a slight and gradual increase in initial lipase activity was observed when the water content was increased up to 20% and where the reaction rate was 13 times faster than in the pure DES. This result confirms the strong hydrogen-bond network that exists between water molecules and DES, which could in turn explain its poor nucleophilic activity. Indeed, a_w remains relatively low compared to the amount of water added, and below 6% water content ($a_w < 0.15$), its nucleophilic activity is particularly reduced (no hydrolysis was observed). In addition, the very low hydration state of the lipase in a pure deep eutectic solvent could explain the difficulty involved in achieving the specific reaction. However, there have been no apparent indications concerning an optimal thermodynamic activity, even though a significant enhancement in initial lipase activity was observed when a_w was increased up to 0.15.

Effect of the water content on the pH of DES–water binary mixtures

It is important to verify whether the addition of a large amount of water in the medium has a significant influence on

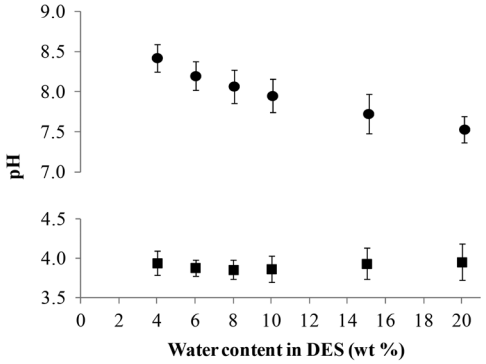

Fig. 7 pH measured in ChCl:Gly– (■) and ChCl:U–water binary mixtures (●). Values are means ± SD (n = 3).

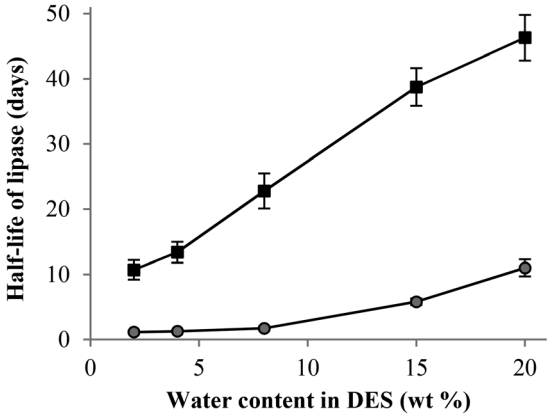

Fig. 8 Half-life of lipase (days) in alcoholysis of methyl *p*-coumarate (40 mM) with 1-octanol (240 mM) at 60 °C measured in ChCl:Gly– (■) and ChCl:U–water binary mixtures (●).

the pH and, therefore, on lipase activity. Consequently, the pH values in DES–water binary mixtures were investigated (Fig. 7). Given the difficulties encountered in assessing the pH, with a pH meter, in non-aqueous or slightly hydrated solutions, the pH of DES with water content below 4% had not been taken into account. The results obtained for both DESs revealed that adding water had little influence on pH. In ChCl:U, one unit of pH is lost (from 8.5 to 7.5) when the water content increases up to 20%. In ChCl:Gly, the pH of binary mixtures with 4% and 20% of water content is identical, although there is a significant difference in reactivity. Consequently, the pH alone cannot explain the drastic change in the enzymatic activity when water is added to the system. Nevertheless, the pH can favor lipase activity and, thus, explain its superior performance in ChCl:U. Indeed, in ChCl:U–water mixtures, the values are quite close to the optimum operating pH conditions of Novozym435 (between pH 7 and 8), compared to the acidic environment of ChCl:Gly–water mixtures.

In order to improve our understanding of the respective roles of pH and water in enzyme activity, additional experiments (60 °C, 40 mM methyl *p*-coumarate, 240 mM 1-octanol, and 10 mg ml^{-1} Novozym435) were carried out in DESs based on choline chloride and two urea derivatives: *N*-methyl urea (MeU) and acetamide (Acet). The corresponding DES, ChCl:MeU and ChCl:Acet, and their mixtures with water, were supposed to have a pH similar to ChCl:U, though different solvation properties. Indeed, from the substitution of one $-NH_2$ group of urea for CH_3NH- and CH_3-, a change in both the polarity of the HBD and the hydrogen bond network of the DES was expected. In mixtures of ChCl:MeU or ChCl:Acet containing less than 8% water, no enzymatic activity was detected (data not shown), despite a pH (7.0 to 7.6) close to the optimal conditions for Novozym435. Moreover, the water content had to be increased up to 20% before a slight activity was observed.

These results show that, in the presence of water, the nature of the hydrogen-bond donor (not the pH) is probably the main factor that determines the difference in lipase activity. In these aqueous mixtures, the HBD's physico-chemical properties have a direct impact on substrate reactivity and lipase activity. Thus, the nature of the HBD is fundamental and, when combined with water, it influences the success of the reaction.

Effect of the water content on the residual activity of lipase

In order to assess lipase relative activity in DES–water mixtures, Novozym435 was incubated for several days at 60 °C prior to determining its initial activity in the alcoholysis of methyl *p*-coumarate (40 mM) with 1-octanol (240 mM). As shown in Fig. 8, the half-life of Novozym435 increases substantially with water content, whatever the DES selected. In other words, the higher the water content, the greater the catalyst's stability. For example, increasing the water content from 2% to 20% in ChCl:U allows us to multiply by 10 the residual activity of lipase, with a half-life time of 1 day and 11 days, respectively. Thus, in mixtures with lower water contents, the residual activity was substantially reduced, which could explain the lower conversion rate observed under these conditions. On the other hand, Novozym435 was found to be much more stable in water–ChCl:Gly mixtures where the half-lives of lipase were higher than 10 days regardless of which water content was added. Consequently, Novozym435's residual activities, measured in terms of the alcoholysis reaction of methyl *p*-coumarate in a glycerol-based DES, were higher than those for a urea-based DES. Thus, the nature of the hydrogen-bond donor plays an important role in the conformation, state and stability of the lipase. However, regardless of which deep eutectic solvent is selected, increasing the water content maintains the enzyme's catalytic activity for the specific reaction tested.

Conclusion

Our results show that the lipase-catalyzed reactions of dissolved substrates (such as phenolic compounds) in DES are extremely difficult to perform without the addition of water. The weak lipase activity in the pure DES solvents could be explained by the strong association of the substrate with the

DES network combined with the very low water thermodynamic activity (close to 0).

Indeed, we showed that adding large amounts of water in such media led to a significant increase in the efficiency of the reaction. Although reactions were carried out with high water content, their thermodynamic activity remained low, which can explain the low hydrolytic activity of water in that system. A possible explanation is the formation of supramolecular complexes between DES and water, involving very strong interactions through hydrogen bonding. Thus, for the specific reactions tested, the addition of a large amount of water in a deep eutectic mixture could have many advantages: (i) reduced viscosity and improved mass transfer; (ii) a significant increase in lipase's initial activity and final conversion rate; (iii) maintenance of the enzyme's catalytic activity. As far as the pH values are concerned, the acidic or basic state of the DES depends on the nature of the hydrogen-bond donor. The addition of water did not cause any significant variation. In this context, the secondary role played by pH in lipase's catalytic activity was confirmed by the major differences obtained between DES based on hydrogen-bond donors of similar chemical nature and similar pH values. Thus, the hydrogen-bond donor appears to be essential. Once it has been combined with water, a successful reaction is possible.

However, further research is needed so that this very simple, efficient and attractive system (low cost, low toxicity, biodegradable and easy to prepare with 100% atom economy) can be applied to the modification of polar substrates.

Notes and references

1 M. H. Katsoura, A. C. Polydera, L. D. Tsironis, M. P. Petraki, S. K. Rajačić, A. D. Tselepis and H. Stamatis, *New Biotechnol.*, 2009, **26**, 83–91.
2 F. Ganske and U. T. Bornscheuer, *Org. Lett.*, 2005, **7**, 3097–3098.
3 Z. Yang, Z. Guo and X. Xu, *J. Am. Oil Chem. Soc.*, 2012, **89**, 1049–1055.
4 M. H. Katsoura, A. C. Polydera, L. D. Tsironis, M. P. Petraki, S. K. Rajačić, A. D. Tselepis and H. Stamatis, *New Biotechnol.*, 2009, **26**, 83–91.
5 M. J. Kim, M. Y. Choi, J. K. Lee and Y. Ahn, *J. Mol. Catal. B: Enzym.*, 2003, **26**, 115–118.
6 J. T. Gorke, F. Srienc and R. J. Kazlauskas, *Chem. Commun.*, 2008, 1235–1237.
7 D. Lindberg, M. D. Revenga and M. Widersten, *J. Biotechnol.*, 2010, **147**, 169–171.
8 H. Zhao, G. A. Baker and S. Holmes, *J. Mol. Catal. B: Enzym.*, 2011, **72**, 163–167.
9 H. Zhao, C. Zhang and T. D. Crittle, *J. Mol. Catal. B: Enzym.*, 2013, **85–86**, 243–247.
10 M. Hayyan, M. A. Hashim, A. Hayyan, M. A. Al-Saadi, I. M. AlNashef, M. E. S. Mirghani and O. K. Saheed, *Chemosphere*, 2013, **90**, 2193–2195.
11 W. Vanden Berghe, *Pharmacol. Res.*, 2012, **65**, 565–576.
12 M. A. Soobrattee, V. S. Neergheen, A. Luximon-Ramma, O. I. Aruoma and T. Bahorun, *Mutat. Res., Fundam. Mol. Mech. Mutagen.*, 2005, **579**, 200–213.
13 W. Y. Huang, Y. Z. Cai and Y. B. Zhang, *Nutr. Cancer*, 2010, **62**, 1–20.
14 L. J. Lopez-Giraldo, M. Laguerre, J. Lecomte, M. C. Figueroa-Espinoza, M. Pina and P. Villeneuve, *OCL – Oleagineux, Corps Gras, Lipides*, 2007, **14**, 51–59.
15 M. C. Figueroa-Espinoza and P. Villeneuve, *J. Agric. Food Chem.*, 2005, **53**, 2779–2787.
16 H. Stamatis, V. Sereti and F. N. Kolisis, *J. Am. Oil Chem. Soc.*, 1999, **76**, 1505–1510.
17 Y. Zhiyong, G. Zheng and X. Xuebing, *Food Chem.*, 2012, **132**, 1311–1315.
18 G. J. H. Buisman, C. T. W. van Helteren, G. F. H. Kramer, J. W. Veldsink, J. T. P. Derksen and F. P. Cuperus, *Biotechnol. Lett.*, 1998, **20**, 131–136.
19 B. Guyot, B. Bosquette, M. Pina and J. Graille, *Biotechnol. Lett.*, 1997, **19**, 529–532.
20 H. Stamatis, V. Sereti and F. N. Kolisis, *J. Mol. Catal. B: Enzym.*, 2001, **11**, 323–328.
21 E. Durand, J. Lecomte, B. Baréa, G. Piombo, E. Dubreucq and P. Villeneuve, *Process Biochem.*, 2012, **47**, 2081–2089.
22 Z. Yang, Z. Guo and X. Xu, *Food Chem.*, 2012, **132**, 1311–1315.
23 N. Azizi and Z. Manocheri, *Res. Chem. Intermed.*, 2012, **38**, 1495–1500.
24 V. De Santi, F. Cardellini, L. Brinchi and R. Germani, *Tetrahedron Lett.*, 2012, **53**, 151–5155.
25 Y. A. Sonawane, S. B. Phadtare, B. N. Borse, A. R. Jagtap and G. S. Shankarling, *Org. Lett.*, 2010, **12**, 1456–1459.
26 H. R. Lobo, B. S. Singh and G. S. Shankarling, *Green Chem. Lett. Rev.*, 2012, **5**, 487–533.
27 R. B. Leron, A. N. Soriano and M.-H. Li, *J. Taiwan Inst. Chem. Eng.*, 2012, **43**, 551–557.
28 R. B. Leron and M.-H. Li, *Thermochim. Acta*, 2012, **530**, 52–57.
29 S.-H. Wu, A. R. Caparanga, R. B. Leron and M.-H. Li, *Thermochim. Acta*, 2012, **544**, 1–5.
30 M. C. Gutierrez, M. L. Ferrer, C. R. Mateo and F. del Monte, *Langmuir*, 2009, **25**, 5509–5515.
31 P. J. Halling, *Enzyme Microb. Technol.*, 1994, **16**, 178–206.

