Process Biochemistry 47 (2012) 2081–2089

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Evaluation of deep eutectic solvents as new media for *Candida antarctica* B lipase catalyzed reactions

E. Durand [a], J. Lecomte [a], B. Baréa [a], G. Piombo [a], E. Dubreucq [b], P. Villeneuve [a,*]

[a] *CIRAD, UMR IATE, F-34398 Montpellier, France*
[b] *Montpellier SupAgro, UMR 1208 IATE, Montpellier, France*

ARTICLE INFO

Article history:
Received 18 June 2012
Accepted 20 July 2012
Available online 27 July 2012

Keywords:
Deep eutectic solvent
Lipase
Candida antarctica
Biocatalysis
Transesterification

ABSTRACT

This study aimed at analyzing the advantages and limitations of several deep eutectic solvents (DESs) as 'green solvents' for biotransformation using immobilized *Candida antarctica* lipase B as catalyst. The transesterification of vinyl laurate was chosen as model reaction and the influence of substrate polarity was assessed using alcohols of various chain lengths. Results showed that grinding of immobilized lipase was essential parameters for good lipase activity. Moreover, in our model reaction some hydrogen-bond donor component from the DES can compete with the alcoholysis reaction. Indeed, side reactions were observed with DES based on dicarboxylic acid or ethylene glycol, leading to some limitations of their use. However, the results showed that other DESs such as choline chloride:urea and choline chloride:glycerol could exhibit high activity and selectivity making them promising solvents for lipase-catalyzed reactions. Finally, the best DES's specific activity – and stability up to five days incubation time – were analyzed and compared with conventional organic solvents. Experiments revealed that iCALB is less influenced by the chain length of alcohol in DES than organic solvents and it is preserves its activity with minimally destructive to protein structure.

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3), are serine hydrolases that are able to act on long-chain carboxylic ester bonds. Not only are they used for their natural hydrolyzing function but they can also catalyze ester synthesis, interesterification and transesterification reactions in non-aqueous media. Lipases are used in a wide range of industries, from food (oleochemical process) to cosmetic products (development of fragrances), pharmaceutical industry (resolution of racemic mixtures), surfactant synthesis and preparation of biodiesel [1–6]. Most lipases preserve a good activity in organic solvents and their ability to catalyze alcoholysis reactions in organic media is well known and documented [7]. However, most organic solvents can be damaging or toxic for the environment. Therefore, in a context of green chemistry a number of studies have attempted to reduce this environmental impact and tried to carry out lipase-catalyzed reactions in green solvents.

Ionic liquids were the first potentially good alternative to organic solvents for biotransformation [8–13]; due to their non volatility, their thermal stability and their high solvation properties. Nevertheless, the limitations of ionic liquids are their cost, their purification, their toxicity which is currently unclear (depending on the structure of the cationic species), and their need to be highly pure since the presence of impurities can seriously affect their physico-chemical properties and enzyme activity [14–22]. Deep eutectic solvents (DESs) are a new class of solvents typically formed by mixing an ammonium salt and a hydrogen-bond donor (HBD). In these solvents, the hydrogen-bond donor interacts with the anion, inducing a depression in the melting point of the mixture. One of the most explicit examples is the synthesis of the mixture between choline chloride and urea (with melting points of 247 °C and 133 °C, respectively) in 1:2 molar ratio resulting in a deep eutectic solvent having a room temperature melting point (12 °C) [23]. Just like ionic liquids, the melting point of DESs is often close to room temperature, and they have a low volatility and high thermal stability. However, unlike most ionic liquids, DESs are biodegradable, cheap and very easy to prepare. To date, very few publications have pointed out the use of DESs (alone or as co-solvent) as media for enzymatic processes. Indeed, various authors have shown that hydrolases or proteases maintained high activity in DESs based on choline or ethylammonium chloride paired with a hydrogen-bond donor such as alcohols, acids or amides [24–27]. The authors concluded that enzymes remained active despite the presence of chloride and strong hydrogen-bond donors that, most often, did not interfere with the reactions. They hypothesized that strong hydrogen bonds between DES components lowered their reactivity. They also reported the hydrolysis

* Corresponding author at: Supagro/INRA – UMR IATE 1208, 2 place Pierre Viala – Bâtiment 33, 34060 Montpellier Cedex 2 - France. Tel.: +33 04 99 61 20 29.
E-mail address: villeneuve@cirad.fr (P. Villeneuve).

1359-5113/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2012.07.027

by epoxide hydrolase and they showed that the reaction rates were faster in a water/DES mixture than in water. Moreover, by choosing different kind of DES as cosolvent the regioselectivity of the enzyme can be modulated. Another promising application of DES is the extraction of glycerol from biodiesel. Indeed, adding choline chloride to a biodiesel preparation allows removing the glycerol by-product by forming a choline chloride–glycerol DES as a new phase [28–31]. Due to their high capacity to solubilize polar, ionic or metal compounds, DES have also been used for many other applications including electrochemical process [32], metal deposition or dissolution [33,34]. Furthermore, some publications deal with their ability to serve as green catalytic solvents for chemical synthesis [35–37]. In this context, it seems very interesting to develop lipase-compatible DES from inexpensive and biodegradable ammonium salts. In one of the previous publications of Gorke et al. [24], the lipase B of *Candida antarctica* proved to be the best option to carry out biocatalysis reaction in DESs. Therefore, in order to know the potential of DESs as media for biotransformation using lipases, we investigated the reactivity of immobilized *C. antarctica* B lipase (iCALB) in some eutectic solvents described by Abbott et al. [38–40] with a focus on the resulting activity, selectivity and stability after a long incubation-time of the selected lipase in DESs.

2. Materials and methods

2.1. Materials

Hexane (99%) was obtained from Labover (Montpellier, France), 1-butanol anhydrous (99.8%), 1-octanol (anhydrous ≥99%), immobilized lipase B from *C. antarctica* (Novozym® 435) were purchased from Sigma–Aldrich (Saint Quentin, France). 1-Octadecanol (≥99%) and vinyl laurate (≥99%) were purchased from Fluka Saint Quentin, France). Glycerol (Gly), urea (U), ethylene glycol (EG), malonic acid (Ma), oxalic acid (Ox), choline chloride (ChCl) and ethylammonium chloride (EAC) were obtained from Sigma–Aldrich with purity ≥99% (except for EAC ≥98%).

2.2. 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 under Silica gel for three days before use. Depending on the molar ratio between ammonium salt and the hydrogen-bond donor – which is 1:1 or 1:2 in our case – the mixtures were prepared by weighing each compound directly into a flask and all precautions were taken for isolating the mixture from air moisture (by closing the flask during melting). Then, the eutectic mixtures were created through heating and stirring in a STUART Scientific S150 orbital shaker incubator at 60 °C until a colorless liquid was formed (typically 2 h).

2.3. Grinding of iCALB

The mixer mill used to grind iCALB is a RETSCH MM200. The lipase is introduced into stainless steel jars (25 ml) containing a ball of 20 mm diameter and subjected to horizontal oscillation and crushing during 5 min.

2.4. Water content determination

Mixtures were equilibrated with a saturated salt solution in a closed vessel for up to 26 days at 25 °C. Different salts were used to achieve various thermodynamic water activities: LiCl (a_w = 0.11), CH_3COOK (a_w = 0.22), K_2CO_3 (a_w = 0.44), NaCl (a_w = 0.75). The moisture uptake of each eutectic mixture was then measured with Karl Fisher titration at 20 °C using Hydranal® Coulomat AG as analyte.

2.5. General conditions for transesterification assay

These reactions were performed at 40, 50 or 60 °C in hermetically sealed flasks containing 1 ml of DES and 1 mg of crushed iCALB whose water activity (a_w) was set at 0.44 beforehand. After 30 min incubation time, 40 μmol (9.05 mg) of vinyl laurate and 240 μmol of corresponding alcohols (butanol 17.8 mg, or octanol 31.25 mg, or octadecanol 64.8 mg) were added to the suspension and the mixture was stirred using magnetic stirring. At the chosen time, the reaction was stopped by adding 1 ml of HCl solution (1 M) and all reaction products were extracted with hexane (4 ml) and analyzed by UV spectrophotometry and gas chromatography and were identified by mass spectrometry. The initial specific activity was determined during the first 5 min of the reaction. Each assay was repeated three times for each alcohol. Alternatively, incubations were performed during 16 h without addition of any alcohol in order to determine the reactivity of DES components.

2.6. UV spectrophotometry

Thanks to the high absorption capacity of the vinyl ester group, it was possible to estimate the vinyl laurate conversion simply by measuring the absorbance at 195 nm [41], thus allowing an easy and fast monitoring of reaction kinetics. A 20 μl aliquot from the reaction medium was added to 1.98 ml of hexane and the absorbance was monitored with a Perkin-Elmer LAMBDA 25 UV/Visible spectrophotometer in a quartz cuvette thermostated at 25 °C and homogene.

2.7. Gas chromatography

The selectivity of the reaction was assessed by GC analysis (Agilent 6890 series, Agilent Technologies, USA) using a capillary column (SUPELCOWAX: length 30 m, external diameter 0.32 mm, film thickness 0.25 μm) and a flame ionization detector (270 °C), with helium as the gas vector (2 ml min^{-1}). 1 μl of the reaction mixture (extracted with hexane in the case of DESs) was injected using a split injector (250 °C). Oven temperature programming: from 150 °C to 235 °C at 5 °C min^{-1}, then 15 min isotherm at 235 °C. With this setting, retention times were as follows: butanol (0.8 min), octanol (1.17 min), octadecanol (9.61 min), vinyl laurate (2.04 min), butyl laurate (3.19 min), octyl laurate (7.4 min), octadecyl laurate (22.4 min).

2.8. Mass spectrometry

All products were identified by gas chromatography coupled with electron ionization mass spectrometry (GC/EI–MS) using a Thermo Scientific Focus GC/DSQII MS apparatus and Xcalibur software in order to analyze the spectrum and fragmentation.

2.9. Lipase stability in DESs

1 mg of crushed iCALB was pre-incubated in the appropriate solvent (1 ml) under orbital stirring at 50 °C in a sealed flask. After a given time, the substrates were added to the reaction medium: 40 μmol of vinyl laurate and 240 μmol of the corresponding alcohol. The reaction was kept at 50 °C and the initial specific activity was compared with that of a mixture without pre-incubation of the lipase. The progress of the reaction was determined by UV spectrophotometry and gas chromatography according to the method previously described.

3. Results and discussion

In order to determine whether DESs could be a suitable medium for *C. antarctica* B lipase-catalyzed reactions, a model reaction was used: the alcoholysis of vinyl laurate with alcohols of different chain lengths (butanol, octanol, octadecanol). Indeed, in such reactions (Fig. 1), one of the most important parameters is the polarity of the nucleophilic substrate which can be a hindrance to the use of DES as media for biocatalytic processes. The choice of this model reaction was also made on the basis that using vinyl ester as acyl donors is a fast and effective way to evaluate lipase initial activities. This reaction allows the displacement of the reaction equilibrium toward the synthesis since the vinyl alcohol produced during the reaction is immediately isomerized into acetaldehyde, which is spontaneously eliminated from the reaction medium due to its high volatility [41]. Moreover, the strong molar absorbance of vinyl ester allows to easily follow its disappearance during the reaction course by a UV spectophotometric method.

3.1. General conditions

Before starting any experiment it was necessary to determine all the parameters that could have an impact on our alcoholysis reaction in DESs using iCALB as biocatalyst. First, we observed that it was difficult to obtain fast kinetics and reproducible results without

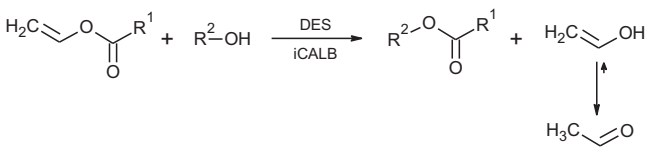

Fig. 1. General reaction of transesterification between a vinyl ester and an alcohol.

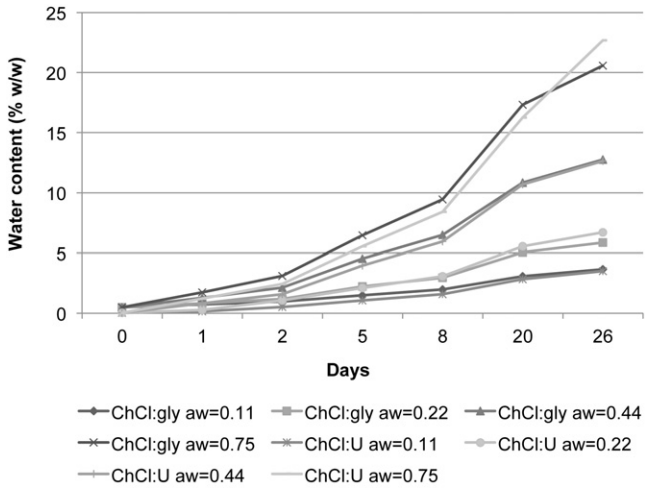

Fig. 2. Water content of ChCl:U and ChCl:Gly during 26 days of incubation time under an atmosphere of controlled moisture fixed by salts of various water activity (a_w).

a preliminary grinding of the catalyst. Indeed, reactivity measurements obtained with uncrushed iCALB were inconsistent and it was unworkable to determine initial specific activity. We know that DESs exhibit unusual solvation properties that are strongly influenced by hydrogen bonding and result in a high affinity with all protic compounds. Moreover, even if the fluidity can be adjusted, depending on the nature of the hydrogen-bond donor and the ammonium salt, the viscosity of DESs is considerably higher than that of many of conventional media (but similar to IL), introducing a limitation of mass transfer and a dispersion of immobilized enzymes. For all these reasons, it was very difficult to obtain an efficient homogenization in DESs with an uncrushed catalyst. This drawback was probably due to the nature of the inert immobilization support, a macroporous acrylic resin corresponding to hydrophobic and aprotic bead-shaped particles with a diameter ranging between 0.3 mm and 0.9 mm. Consequently, the catalyst was crushed into particles of 0.100–0.165 mm in order to enhance its dispersion among the DESs. Secondly, it was observed that the DESs were highly hygroscopic solvents therefore the reaction had to be carried out in sealed flasks in order to avoid any water uptake from air. Water is well known to have a crucial role in lipase-catalyzed synthesis reactions in water limited environments (e.g. organic solvents) where enzyme activity is obviously influenced by its hydration state that determines protein structure, flexibility and reactivity [42,43]. Moreover, in synthesis reactions such as esterification, transesterification, alcoholysis and acidolysis the water excess may induce the unwanted reverse reaction of hydrolysis. In DESs, water can decrease the viscosity and change the activity and selectivity of the enzyme. Hua Zhao et al. [25] showed that proteases in glycerol-based deep eutectic solvents have an activity and selectivity that depend on the water content of the DESs, thus a small amount of water of about 5% can increase the enzymatic activity decreasing its selectivity. On the other hand, water is the simplest hydrogen-bond donor, thus its content should be as low as possible in order to avoid any possible change of the initial eutectic composition and to prevent competitive hydrolysis reaction. For all these reasons, it seemed essential to start all transesterification assays with the same initial water content. The strategy adopted was to equilibrate the water content of solvents under an atmosphere controlled by a saturated salt solution in a closed vessel. However, attempts with two DESs (ChCl:Gly and ChCl:U) showed that the equilibrium was not yet reached after a long incubation time (26 days at 25 °C) whatever the targeted a_w (Fig. 2). Moreover, the water content of the DESs was already higher than what we expected, illustrating their very hygroscopic nature.

In other words, with this method, no equilibrium will be reached with a low water content in the DES, whatever the a_w. Consequently, we decided to limit the water content for all the DESs below 1% (w/w) that corresponds to the level obtained immediately after their synthesis (Table 1). In addition, the thermodynamic water activity (a_w) of the catalyst was adjusted to 0.44 beforehand.

3.2. Transesterification of vinyl laurate with alcohols of different chain lengths

In order to increase the knowledge on substrate reactivity in DESs, we studied the alcoholysis of vinyl laurate with aliphatic alcohols of different chain lengths in order to verify whether the use of substrates with different polarities can have an effect on the reactivity in such media. We have already explained the importance and the role of hydrogen bonding on the solubility of compounds in these solvents. Thus, assuming that an increase of the alkyl chain length of the alcohol should decrease its solubility, 1-alkanols having 4, 8 and 18 carbon atoms (1-butanol, 1-octanol and 1-octadecanol) were evaluated. All the eutectic points of the DESs used in this study were previously described in the literature (Fig. 3 and Table 2).

First, we carry out these model reaction assays in all the DESs with all the alcohols during 16 h at 60 °C with crushed iCALB. The results were compared to that obtained in toluene, a solvent of higher boiling point than hexane, which is widely used in lipase-catalyzed syntheses and particularly suitable for hydrophobic substrates conversion at high temperatures [44]. The results are listed in Table 3. In DESs ChCl:U and ChCl:Gly, the conversion

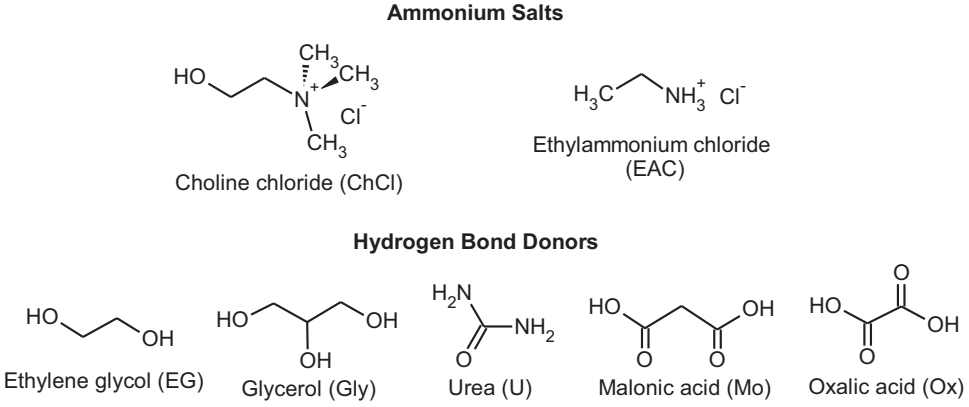

Fig. 3. Ammonium salts and hydrogen bond donors used to synthesize eutectic mixtures.

Table 1
Water content in DESs immediately after synthesis.

Solvent	ChCl:U	ChCl:Gly	ChCl:Ox	ChCl:Mo	ChCl:EG	EAC:U	EAC:Gly
Water content (% w/w)	0.04	0.14	0.75	0.18	0.08	0.27	0.31

Table 2
Composition of screened deep eutectic solvents.

DESs	Ammonium salt	Hydrogen-bond donor	Molar ratio
ChCl:U	Choline chloride	Urea	1:2
ChCl:Gly	Choline chloride	Glycerol	1:2
ChCl:Ox	Choline chloride	Oxalic acid	1:1
ChCl:Mo	Choline chloride	Malonic acid	1:1
ChCl:EG	Choline chloride	Ethylene glycol	1:2
EAC:U	Ethylammonium chloride	Urea	1:2
EAC:Gly	Ethylammonium chloride	Glycerol	1:2

of vinyl laurate into the corresponding ester was quantitative and comparable to those in toluene. In the DESs based on dicarboxylic acids, the results showed a low enzymatic activity for alcoholysis of vinyl laurate with butanol (≤10% conversion). A greater activity was found for octanol and octadecanol (from 34% to 43% conversion). In ChCl:EG and EAC:U the conversions were maximum with short-chain length alcohol (respectively 33% and 94% with butanol) and minimum with medium and long-chain ones (octanol and octadecanol). Among all the tested solvents, only one (EAC:Gly) exhibited a very low enzymatic activity with any of the alcohols. This experiment showed that the reactivity for alcoholysis in deep eutectic mixtures may depend on the nature of the nucleophilic substrate. From our results, it seems quite clear that the best eutectic mixtures to be used for achieving this type of lipase-catalyzed reaction are the DESs based on urea and glycerol as hydrogen-bond donors. Moreover, even if solvent-based EAC have a low viscosity, allowing a better mass transfer and dispersion of immobilized enzymes, the best activity was observed with the DESs containing ChCl, whatever the chain length of the alcohol used. Consequently, the DESs based on this quaternary ammonium salt appear to be the best choice for further investigation. Indeed, choline chloride is readily available as a bulk commodity chemical and almost no waste is formed during its industrial production. It is non-toxic and readily biodegradable, according to the OECD-criteria (MITI-I test; BOD measurements) reaching 93% degradation within 14 days. Consequently, we found it interesting to assess the enzymatic activity and stability in ChCl:Urea, ChCl:Gly and toluene taken as reference. The results (Fig. 4) show that the influence of the chain length on the initial specific activities is more important in toluene than in the two tested DESs. Increasing the hydrophobicity of alcohols improved the reactivity in toluene, whereas in the two DESs, especially in ChCl:Gly, the impact of the alcohol chain length was insignificant.

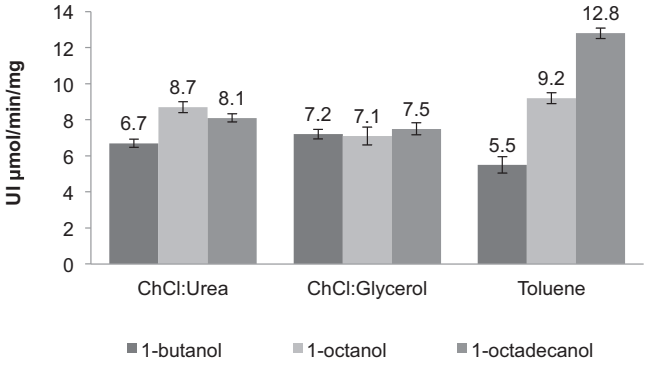

Fig. 4. Initial specific activity for the alcoholysis of vinyl laurate with alcohols of different chain lengths using crushed iCALB as biocatalyst in DESs or toluene at 60 °C.

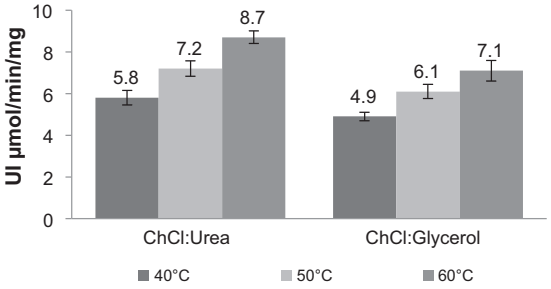

Fig. 5. Initial specific activity for transesterification of vinyl laurate with octanol by using crushed iCALB as biocatalyst in DESs at different temperatures.

Abbott et al. [23] showed that the viscosity of DESs changed significantly in function of the temperature. One of the most significant example is obtained with ChCl:U, where an increase of the temperature from 20 °C to 50 °C, decreases by 10 the viscosity of the solvent. Therefore, one of the possible ways to favor the mass transfer and dispersion of immobilized enzyme could be an increase on the reaction temperature. Fig. 5 shows an initial specific activity (μmol/min/mg) 1.5 times higher when the temperature increases by 20 °C (from 40 °C to 60 °C) in DESs based on urea and glycerol paired with choline chloride. So, one possible strategy when using highly thermostable immobilized lipases in DES would be to increase the reaction temperature up to the maximal operational stability of the selected enzyme and thus minimize the problem of the DES viscosity.

Table 3
Conversion and selectivity of vinyl laurate transesterification with three aliphatic alcohols, in different solvents at 60 °C using crushed iCALB.

Solvents	1-Butanol		1-Octanol		1-Octadecanol	
	Conversion (%)	Selectivity (%)	Conversion (%)	Selectivity (%)	Conversion (%)	Selectivity (%)
Toluene	100	>99	100	>99	100	>99
ChCl:U	100	>99	100	>99	100	>99
ChCl:Gly	100	>99	100	>99	100	>99
ChCl:Ox	10	>99[a]	43	>99[a]	41	>99[a]
ChCl:Mo	5	>99[a]	36	>99[a]	34	>99[a]
ChCl:EG	33	30.3	8	25	10	30
EAC:U	94	>99	10	>99	9	>99
EAC:Gly	3	>99	4	>99	4	>99

The transesterification between vinyl laurate (40 μmol) and alcohols (240 μmol) was catalyzed by 1 mg of crushed iCALB in 1.0 ml of solvent at 60 °C during 16 h.
[a] Side reactions were observed with the diacid component from the deep eutectic solvent.

3.3. Hydrogen-bond donor reactivity

All the DESs contain hydrogen-bond donors (HBD) which can compete with substrates in our model reaction. Indeed, in the DESs based on urea, glycerol and ethylene glycol, hydrogen-bond donors can compete with the alcohol, whereas in the DESs made of oxalic and malonic acids, these donors can compete with vinyl laurate as acyl donor. The reactivity of hydrogen-bond donors is one of the most important parameters that will allow to validate whether or not a DES can be a suitable medium for lipase-catalyzed reactions. In addition to generating by-products in the reaction, the reactivity of hydrogen-bond donors can change the initial ratio of the mixture corresponding to the eutectic point. The physico-chemical solvent properties (especially the viscosity) can be altered but this would certainly result in a modification of the activity and in possible difficulties to recover and recycle the used solvents. Although it has been proven that some DESs can reach their lowest viscosity level not necessarily at the eutectic point but in its vicinity [26,28], most of them show the lowest viscosity level at the eutectic composition, especially for DESs based on choline chloride as quaternary ammonium salt [28,38]. We observed, with glycerol and urea as components of the DES, a very low reactivity of the hydrogen-bond donor – whereas with ethylene glycol-containing DESs, the reactivity was much more important. For example, in ChCl:EG and in the absence of any 1-alkanol, 65% of the vinyl laurate were converted into ethylene glycol mono- and di-laurate after 16 h at 60 °C (Fig. 6A and B). On the other hand, the reactivity of the hydrogen-bond donor in ChCl:EG can be influenced by the presence of the substrate, thus, in the presence of 1-butanol, 1-octanol or 1-octadecanol, the conversion of vinyl laurate into ethylene glycol esters is drastically reduced. Moreover, it appears that the increase of the hydrophobicity of alcohol reduces differently the reactivity of ethylene glycol-containing DES (23%, 6% and 7% respectively). However, very low yields in alkyl laurate were observed whatever the chain length

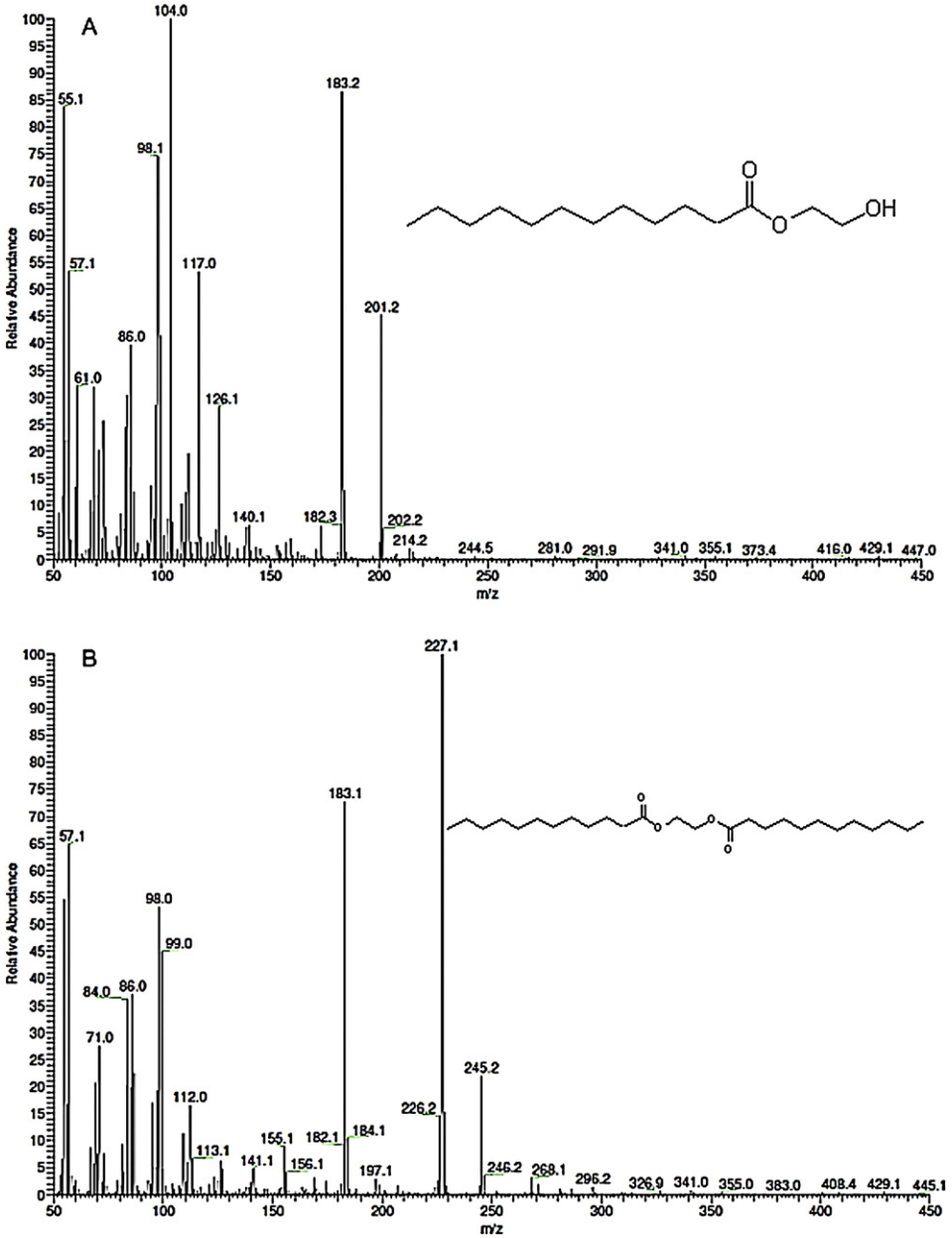

Fig. 6. Electron ionization mass spectra of the fragmentation patterns of by-products obtained from the reaction of hydrogen-bond donors components from the DESs. According to IUPAC nomenclature: (A) 2-hydroxyethyl dodecanoate; (B) 2-dodecanoyloxyethyl dodecanoate; (C) 2-octoxy-2-oxoacetic acid; (D) dioctyl oxalate; (E) 3-octoxy-3-oxopropanoic acid; (F) dioctyl propanedioate and (G) 3-octadecoxy-3-oxopropanoic acid).

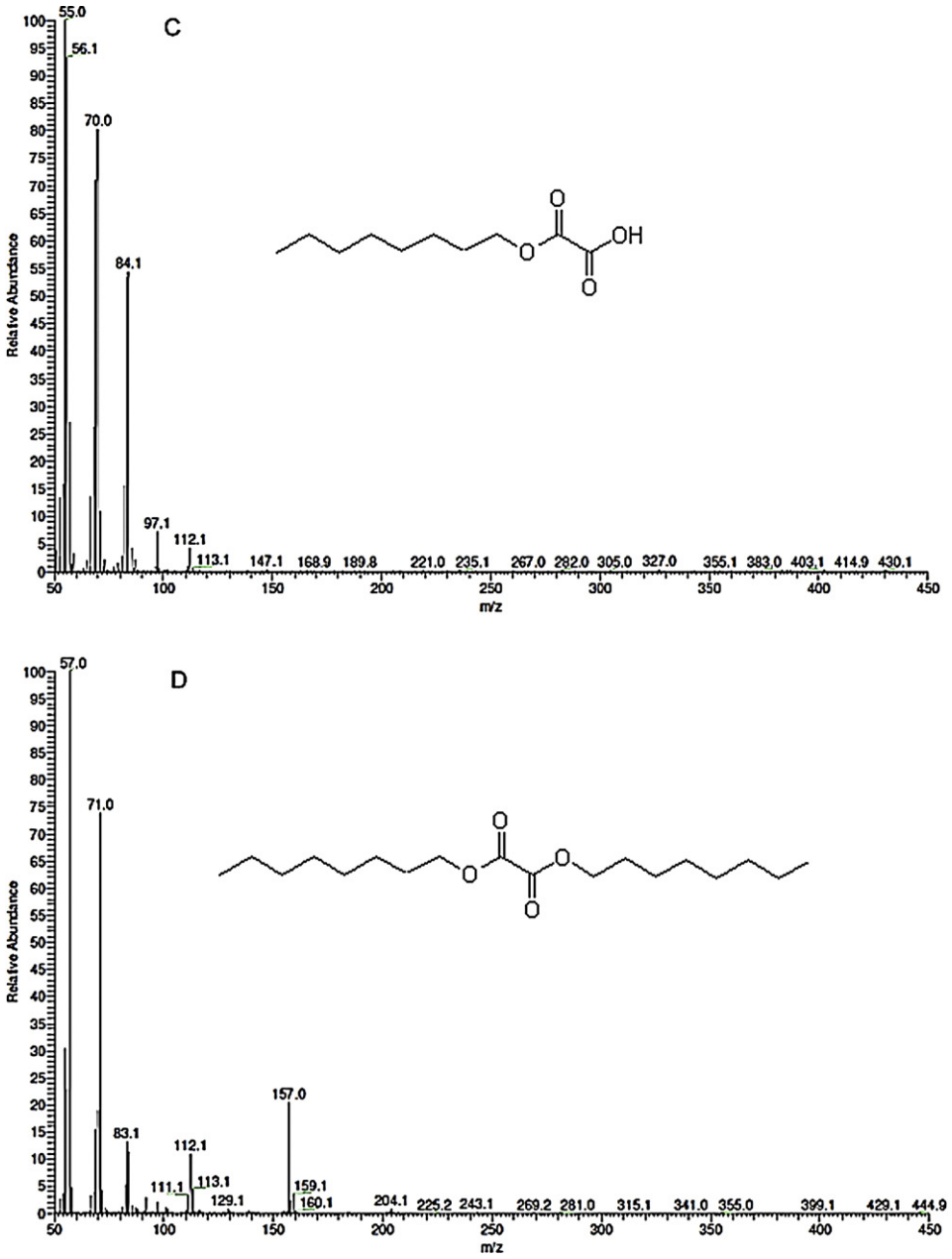

Fig. 6. (*Continued*)

of alcohol (10%, 2% and 3% respectively). In the DESs based on diacids (ChCl:Ox and ChCl:Mo), the reactivity was even more surprising. Indeed, an important formation of mono- and di-ester with the diacids components from the DESs was observed, especially with the medium-chain alcohols. Indeed, the reactivity between diacids and butanol were very low while it was high with octanol (Fig. 6C–F). With long chain alcohol (octadecanol) we observed a significant production of octadecyl mono ester with malonic acid (Fig. 6G) whereas a low reactivity was observed with oxalic acid. Nevertheless, unlike the results obtained with ChCl:EG, no difference in the reaction was observed whether or not acyl donor was added. Moreover, the DESs based on carboxylic acids already have a high viscosity compared with other hydrogen-bond donors, and so their reactivity during the lipase-catalyzed reaction substantially increases this problem. Hence, the consumption of hydrogen-bond donors increases the viscosity of the mixture that leads to a decrease of the ionic mobility, which in turn affects the mass transfer among the DES. It seems clear that the use of such media for biocatalysis reactions is not a judicious choice. However, in case of DESs based on dicarboxylic acids, it could be profited from this drawback using this hydrogen-bond donor as substrate for a transesterification reaction, even if the drastic increase of the viscosity may limit that use.

In summary, these experiments show that the alcohol employed for an alcoholysis reaction in the DESs can have a paramount effect over the activity and selectivity of iCALB.

3.4. Stability of crushed iCALB in DESs

Another limitation to the use of DESs as a medium for lipase-catalyzed reactions could be the stability of enzymes in such solvents. Therefore, we carried out a long-term stability evaluation of iCALB in ChCl:U, ChCl:Gly and toluene, the latter being taken as organic solvent reference (Fig. 7). The lipase was incubated in each

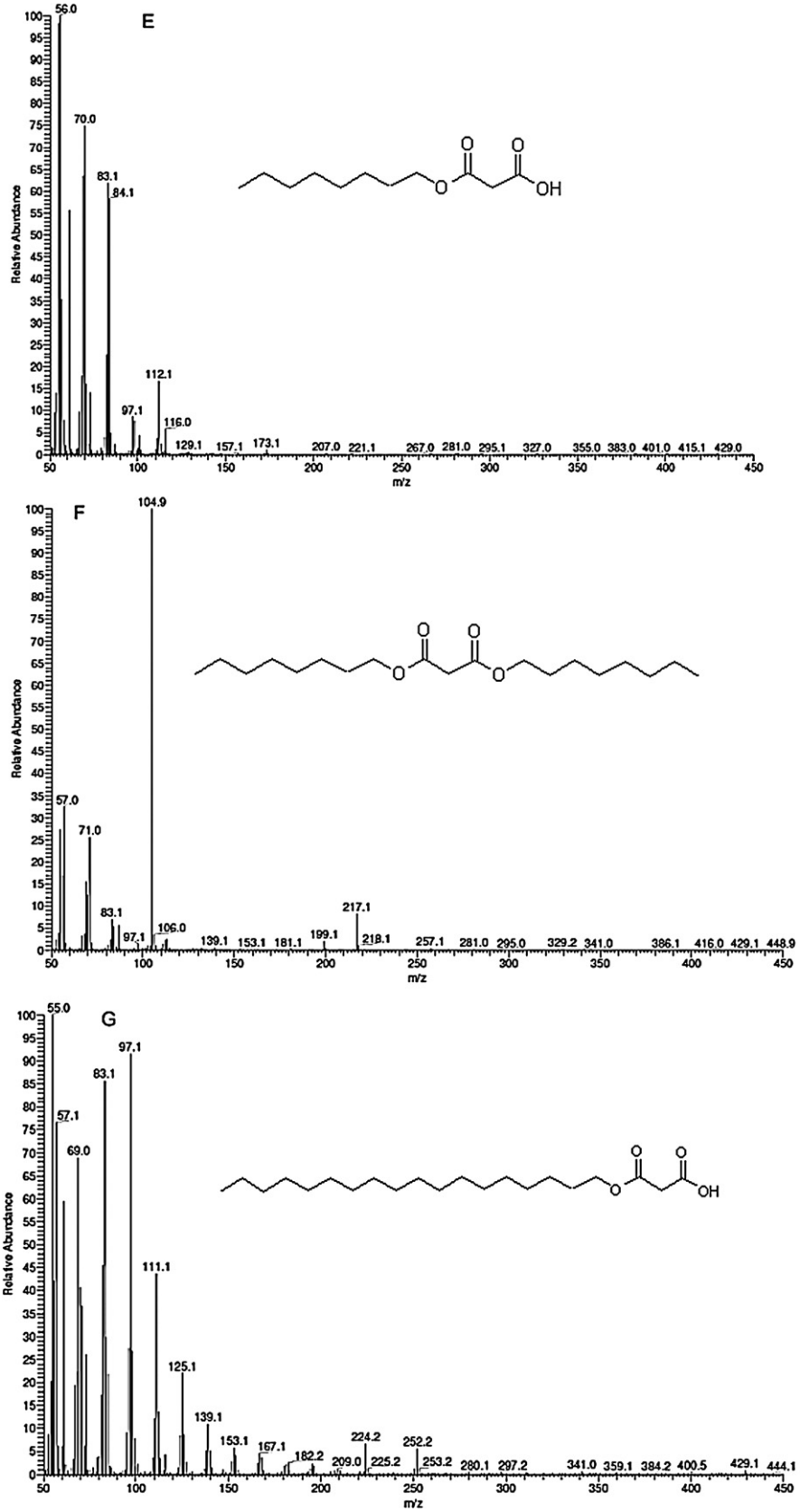

Fig. 6. (*Continued*)

solvent for 1, 2, 3, and 5 days at 50 °C prior to determine the initial specific activity of alcoholysis of vinyl laurate with octanol. In toluene, only a 15% activity loss was observed after 5 days in comparison with the reaction rate with the non-incubated lipase. In ChCl:Gly DES, a 70% activity decrease was measured after one day incubation (65% after only 5 h) but extending the incubation time to 5 days did not result in any additional enzyme activity loss. It is assumed that the enzyme needs a few hours to adopt its final

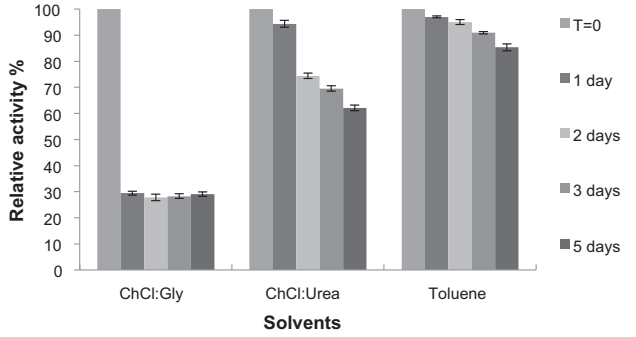

Fig. 7. Relative initial specific activity for transesterification of vinyl laurate with octanol at 50 °C using crushed iCALB after different pre-incubation times of the catalyst in DESs or toluene.

structure in glycerol-containing DES, which leads to a decrease of activity during this period. Indeed, glycerol is likely to have a negative effect on the lipase activity and stability by being adsorbed over the catalyst which reduces the diffusion of the hydrophobic substrate toward the active site of the lipase [45,46]. In this case, comparing the activity after 5 h and after 5 days incubation time shows that iCALB is maintaining the same rate and thus preserves its activity without any apparent denaturation. In ChCl:U, iCALB loses only 5% after one day incubation and less than 38% after 5 days in comparison with the rate before incubation. Consequently, the stability of iCALB in these two solvents should allow to carry out long and more complex reactions with a minimal modification of the protein structure.

4. Conclusion

It was already reported that iCALB was active in DESs composed of choline chloride or ethylammonium chloride paired with hydrogen-bond donors such as amide, hydroxyl or acid [24,26]. However, in the present study we showed that some conditions are necessary to promote biocatalysis in DESs media and that not all eutectic mixtures can be used as efficient media for lipase-catalyzed reactions. First, in the specific case of immobilized *C. antarctica* B lipase, we noticed that a preliminary grinding was crucial in order to get an efficient reaction kinetic.

Secondly, some DESs such as ChCl:Mo, ChCl:Ox and ChCl:EG can react and compete with the substrates in alcoholysis reactions leading to byproduct formation and DES destruction. Moreover, in the specific case of the DESs based on dicarboxylic acids, the viscosity that is already high from the beginning, increased significantly with side reactions, making stirring and recycling difficult. However, in most cases, the strong hydrogen bonds between DES components can dramatically lower their reactivity as it was observed in DESs based on glycerol or urea. In addition, results revealed that the reactivity for alcoholysis in deep eutectic mixtures may depend on the polarity of the nucleophilic substrate. The DESs composed of glycerol or urea combined with choline chloride as quaternary ammonium salt allowed the best initial specific activity of the lipase, comparable to those in conventional organic media. Moreover, in these DESs, the activity of iCALB seems to be less influenced by the alcohol chain length in alcoholysis reaction with vinyl laurate compared to its activity in toluene. Although we know that immobilized *C. antarctica* lipase B denaturates in solutions of urea, it did not denaturate quickly in DESs containing urea or glycerol and the stability in these DESs is sufficient to allow the reaction for several days. It is difficult to extrapolate these results to other lipases and DES combinations since the reactivity and stability of the enzymes in DESs are influenced by the components of the DES itself. The network formed by hydrogen-bonds can vary and lead to different behaviors of compounds. However, this study provides valuable information about the most widely used enzyme in industrial biocatalysis (lipase B from *C. antarctica*) in these new "green" solvents.

Acknowledgements

The authors thank Dr. Robert Lortie, Pr. Elisée Ferré and Dr Loic Remy for helpful discussions and Emilie PEREZ for the revisions in English.

References

[1] Bornscheuer UT. Lipase-catalyzed syntheses of monoacylglycerols. Enzyme Microb Technol 1995;17:578–86.
[2] Soumanou MM, Bornscheuer UT. Lipase-catalyzed alcoholysis of vegetable oils. Eur J Lipid Sci Technol 2003;105:656–60.
[3] Gog A, Roman M, Tosa M, Paizs C, Irimie FD. Biodiesel production using enzymatic transesterification—current state and perspectives. Renew Energ 2012;39:10–6.
[4] Tarahomjoo S, Alemzadeh I. Surfactant production by an enzymatic method. Enzyme Microb Technol 2003;33:33–7.
[5] Willis WM, Marangoni AG. Enzymatic interesterification. In: Akoh CC, Min DB, editors. Food lipids: chemistry, nutrition, and biotechnology. 2002. p. 839–75.
[6] Winska K, Grudniewska A, Chojnacka A, Bialonska A, Wawrzenczyk C. Enzymatic resolution of racemic secondary cyclic allylic alcohols. Tetrahedron-Asymmetr 2010;21:670–8.
[7] Li D, Xuebing X, Haraldsson GG, Tianwei T, Fang W. Enzymatic production of alkyl esters through alcoholysis: a critical evaluation of lipases and alcohols. J Am Oil Chem Soc 2005;82:341–7.
[8] Yang Z, Pan WB. Ionic liquids: green solvents for nonaqueous biocatalysis. Enzyme Microb Technol 2005;37:19–28.
[9] Van Rantwijk F, Sheldon RA. Biocatalysis in ionic liquids. Chem Rev 2007;107:2757–85.
[10] Shi YG, Cai Y, Li JR, Chu YH. Enzyme-catalyzed regioselective synthesis of carbohydrate fatty acid esters in ionic liquids. Prog Chem 2011;23:2247–57.
[11] Park S, Viklund F, Hult K, Kazlauskas RJ. Ionic liquids create new opportunities for nonaqueous biocatalysis with polar substrates: acylation of glucose and ascorbic acid. Ion Liq Greensolv Progress Prospects 2003;856:225–38.
[12] Madeira Lau R, van Rantwijk F, Seddon KR, Sheldon RA. Lipase-catalyzed reactions in ionic liquids. Org Lett 2000;2:4189–91.
[13] Hernandez-Fernandez FJ, Rios A, Lozano-Blanco LJ, Godinez C. Biocatalytic ester synthesis in ionic liquid media. J Chem Technol Biotechnol 2010;85:1423–35.
[14] Canongia Lopes JN, Costa Gomes MF, Padua AAH. Nonpolar, polar, and associating solutes in ionic liquids. J Phys Chem B 2006;110:16816–8.
[15] Pan C, Pelzer K, Philippot K, Chaudret B, Dassenoy F, Lecante P, et al. Ligand-stabilized ruthenium nanoparticles: synthesis, organization, and dynamics. J Am Chem Soc 2001;123:7584–93.
[16] Pertici P, Simonelli G, Vitulli G, Deganello G, Sandrini P, Mantovani A. (Eta-4-cyclo-octa-1,4-diene)(eta-6-cyclo-octa-1,3,5-triene)ruthenium(0) chemistry—role of molecular-hydrogen in a new synthetic route to cyclo-olefin ruthenium complexes. J Chem Soc Chem Comm 1977:132–3.
[17] Pery T, Pelzer K, Buntkowsky G, Philippot K, Limbach HH, Chaudret B, et al. Evidence for the presence of mobile surface hydrides on ruthenium nanoparticles. Chem Phys Chem 2005;6:605–7.
[18] Triolo A, Russina O, Bleif HJ, Di Cola E. Nanoscale segregation in room temperature ionic liquids. J Phys Chem B 2007;111:4641–4.
[19] Seddon KR, Stark A, Torres MJ. Influence of chloride, water, and organic solvents on the physical properties of ionic liquids. Pure Appl Chem 2000;72:2275–87.
[20] Jastorff B, Stormann R, Ranke J, Molter K, Stock F, Oberheitmann B, et al. How hazardous are ionic liquids? Structure–activity relationships and biological testing as important elements for sustainability evaluation. Green Chem 2003;5:136–42.
[21] Ranke J, Molter K, Stock F, Bottin-Weber U, Poczobutt J, Hoffmann J, et al. Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicol Environ Saf 2004;58:396–404.
[22] Ventura SPM, Marques CS, Rosatella AA, Afonso CAM, Goncalves F, Coutinho JAP. Toxicity assessment of various ionic liquid families towards *Vibrio fischeri* marine bacteria. Ecotoxicol Environ Saf 2012;76:162–8.
[23] Abbott AP, Capper G, Davies DL, Rasheed RK, Tambyrajah V. Novel solvent properties of choline chloride/urea mixtures. Chem Commun 2003;7:0–7, 1.
[24] Gorke JT, Srienc F, Kazlauskas RJ. Hydrolase-catalyzed biotransformations in deep eutectic solvents. Chem Commun 2008;123:5–123, 7.
[25] Zhao H, Baker GA, Holmes S. Protease activation in glycerol-based deep eutectic solvents. J Mol Catal B-Enzym 2011;72:163–7.
[26] Zhao H, Baker GA, Holmes S. New eutectic ionic liquids for lipase activation and enzymatic preparation of biodiesel. Org Biomol Chem 2011;9:1908–16.
[27] Lindberg D, Revenga MD, Widersten M. Deep eutectic solvents (DESs) are viable cosolvents for enzyme-catalyzed epoxide hydrolysis. J Biotechnol 2010;147:169–71.
[28] Abbott AP, Cullis PM, Gibson MJ, Harris RC, Raven E. Extraction of glycerol from biodiesel into a eutectic based ionic liquid. Green Chem 2007;9:868–72.

[29] Hayyan M, Mjalli FS, Hashim MA, Al Nashef IM. A novel technique for separating glycerine from palm oil-based biodiesel using ionic liquids. Fuel Process Technol 2010;91:116–20.
[30] Shahbaz K, Mjalli FS, Hashim MA, Al Nashef IM. Using deep eutectic solvents based on methyl triphenyl phosphunium bromide for the removal of glycerol from palm-oil-based biodiesel. Energ Fuel 2011;25:2671–8.
[31] Shahbaz K, Mjalli FS, Hashim MA, Al Nasheff IM. Eutectic solvents for the removal of residual palm oil-based biodiesel catalyst. Sep Purif Technol 2011;81:216–22.
[32] Lloyd D, Vainikka T, Murtomaki L, Kontturi K, Ahlberg E. The kinetics of the Cu2+/Cu+ redox couple in deep eutectic solvents. Electrochim Acta 2011;56:4942–8.
[33] Abbotta AP, Barron JC, Frisch G, Rydera KS, Fernando Silva A. The effect of additives on zinc electrodeposition from deep eutectic solvents. Electrochim Acta 2011;56:5272–9.
[34] Abbott AP, Capper G, McKenzie KJ, Ryder KS. Electrodeposition of zinc–tin alloys from deep eutectic solvents based on choline chloride. J Electroanal Chem 2007;599:288–94.
[35] Chen Z, Zhu W, Zheng Z, Zou X. One-pot alpha-nucleophilic fluorination of acetophenones in a deep eutectic solvent. J Fluorine Chem 2010;131:340–4.
[36] Singh B, Lobo H, Shankarling G. Selective N-alkylation of aromatic primary amines catalyzed by bio-catalyst or deep eutectic solvent. Catal Lett 2011;141:178–82.
[37] Sonawane YA, Phadtare SB, Borse BN, Jagtap AR, Shankarling GS. Synthesis of diphenylamine-based novel fluorescent styryl colorants by knoevenagel condensation using a conventional method, biocatalyst, and deep eutectic solvent. Org Lett 2010;12:1456–9.
[38] Abbott AP, Boothby D, Capper G, Davies DL, Rasheed RK. Deep eutectic solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic liquids. J Am Chem Soc 2004;126:9142–7.
[39] Abbott AR, Capper G, Gray S. Design of improved deep eutectic solvents using hole theory. Chem Phys Chem 2006;7:803–6.
[40] Abbott AP, Harris RC, Ryder KS, D'Agostino C, Gladden LF, Mantle MD. Glycerol eutectics as sustainable solvent systems. Green Chem 2011;13:82–90.
[41] Goujard L, Villeneuve P, Barea B, Lecomte J, Pina M, Claude S, et al. A spectrophotometric transesterification-based assay for lipases in organic solvent. Anal Biochem 2009;385:161–7.
[42] Affleck R, Xu ZF, Suzawa V, Focht K, Clark DS, Dordick JS. Enzymatic catalysis and dynamics in low-water environments. Proc Natl Acad Sci USA 1992;89:1100–4.
[43] Negishi S, Shirasawa S, Arai Y, Suzuki J, Mukataka S. Activation of powdered lipase by cluster water and the use of lipase powders for commercial esterification of food oils. Enzyme Microb Technol 2003;32:66–70.
[44] Laane C, Boeren S, Vos K, Vos K, Veeger C. Rules for optimization of biocatalysis in organic-solvents. Biotechnol Bioeng 1987;30:81–7.
[45] Xu Y, Nordblad M, Nielsen PM, Brask J, Woodley JM. In situ visualization and effect of glycerol in lipase-catalyzed ethanolysis of rapeseed oil. J Mol Catal B-Enzym 2011;72:213–9.
[46] Dossat V, Combes D, Marty A. Continuous enzymatic transesterification of high oleic sunflower oil in a packed bed reactor: influence of the glycerol production. Enzyme Microb Technol 1999;25:194–200.