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Green bioprocesses in sponge-like ionic liquids

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ABSTRACT

Ionic liquids (ILs) are a new class of liquid solvent, whose use has led to a green chemical revolution because of their unique array of physico-chemical properties, headed by their negligible vapour pressure and their exceptional ability to stabilize biocatalysts. Hydrophobic ILs based on cations with long alkyl side-chains, *e.g.* N,N,N,N-hexadecyltrimethylammonium bis(trifluoromethylsulfonyl)imide ($[C_{16}tma][NTf_2]$), are temperature switchable ionic liquid/solid phases that behave as sponge-like systems (sponge-like ionic liquid, SLILs). Based on this new property, SLILs have been used to develop straightforward and clean approaches for producing nearly pure synthetic compounds with added value (*e.g.* geranyl acetate, anisyl acetate, methyl oleate, *etc.*) in two steps: an enzymatic synthetic step as liquid phase, and then a product separation step involving simple centrifugation as a solid phase.

1. Introduction

Green Chemistry is based on the use of safer solvents and reaction conditions, and encourages the use of environmentally benign non-aqueous solvents and efficient catalysts for chemical reactions and/or processes [1,2]. Enzymes, as catalysts of living systems, clearly constitute powerful green tools for chemical processes, since their activity and selectivity (stereo-, chemo- and region-selectivity) for catalyzed reactions are far-ranging [3]. Furthermore, solvents are key elements in chemical processes, where they act as media for mass-transport, reaction and product separation. They are responsible for a major part of the environmental impact of processes in the chemical industry and have a great impact on cost, safety and health. The search for new environmentally benign non-aqueous solvents or green solvents, being able to be easily recovered/recycled and allowing enzymes to operate efficiently in them, is a priority in the development of integral green chemical processes [4]. In this context, the recent introduction of ionic liquids (ILs) in Chemistry could lead to a green revolution in industrial processes, because of their unique array of physico-chemical properties, headed by their very low vapour pressure. Besides, ILs have recently emerged as exceptionally interesting non-aqueous reaction media for enzymatic transformations [2,5,6], which can be improved by the assistance of microwave irradiation [7,8].

The use of ILs, based on cation with short alkyl-side chains (*e.g.* 1-butyl-3-methylimidazolium), as reaction media for lipase-catalyzed biodiesel synthesis provided moderate catalytic activity because of the resulting biphasic reaction media, formed by an alcohol-IL phase immiscible with an vegetable oil phase [9,10]. In this context, among the 23 ILs tested for immobilized lipase-catalyzed biodiesel synthesis, the best yield (80%) was achieved in the 1-ethyl-3-methylimidazolium trifluoroacetate IL after 12 h at 50 °C, which did not increase at longer reaction times [9]. These results were clearly improved by using the hydrophobic IL $[Bmim][NTf_2]$ as reaction medium, which permitted to 96% biodiesel yield after 48 h at room temperature [10]. However, the efficient recovery of the IL for further reuse remains as an important problem of this approach. In this context, reaction systems based on reduced amounts of ILs is another interesting approach. By using ILs for coating heterogeneous catalysts, also named supported ionic liquid phases, SILPs, the performance of catalysts (*e.g.* activity, stability and selectivity, *etc.*) are greatly improved [6,11,12]. Biocatalysis in SILP/$scCO_2$ biphasic systems is another efficient tool for designing continuous clean biocatalytic processes

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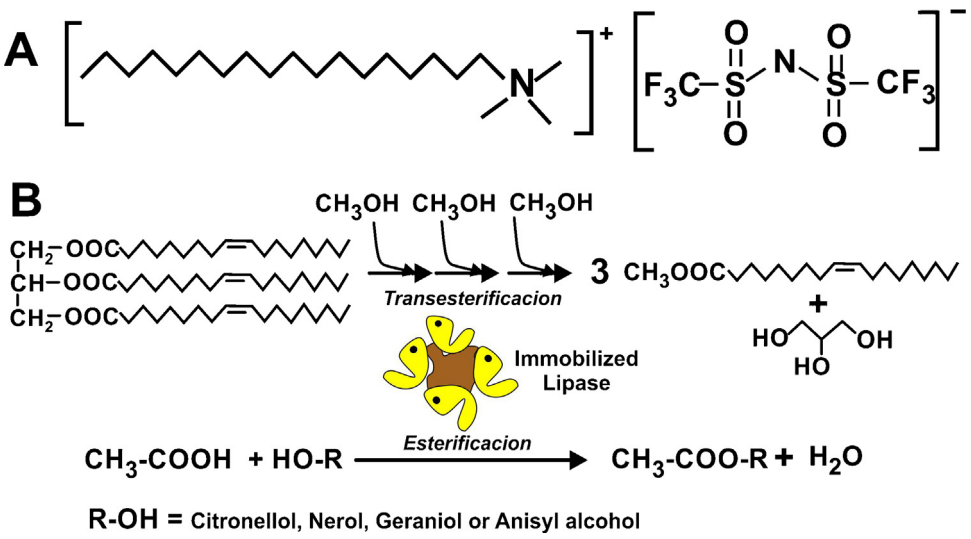

Fig. 1. (A) Structure of the IL [C_{18}tma][NTf_2], as an example of sponge-like ionic liquids (SLILs). (B) Scheme of the immobilized lipase-catalyzed synthesis of both methyl oleate by transesterification and flavour esters by esterification approaches.

that directly provide pure products [2,3]. The clean and continuous biocatalytic synthesis of biodiesel in SILP/$scCO_2$ (18 MPa and 60 °C) reached up to 95% yield by using packed bed reactors containing immobilized enzyme particles coated with ILs (*e.g.* 1.methyl-3-octadecylimidazolium bis(trifluoromethylsulfonyl)imide, *etc.*) [13].

Hydrophobic ILs based on cations with long alkyl side-chains, *e.g.* N,N,N,N-octadecyltrimethylammonium bis(trifluoromethylsulfonyl)imide [C_{18}tma][NTf_2], see Fig. 1A), are temperature switchable ionic liquid/solid phases that behave as sponge-like systems (sponge-like ionic liquid, SLILs) [14,15], which represent an interesting way for developing integral green processes. As liquid phases, these SLILs are excellent monophasic reaction media for lipase-catalyzed synthetic reactions that provided exceptional enzyme activity and stability [13,16]. As solid phases, the reaction mixture can easily be fractionated by iterative centrifugations at controlled temperatures into different phases, allowing straightforward product separation and the recovery of the SILLs [17].

The aim of this work was to demonstrate the exceptional suitability of SLILs as a new green platform for performing both the enzymatic synthesis of high added value products (*e.g.* flavour esters and methyl oleate) by means of esterification or transesterification (see Fig. 1B), and subsequent separation of the product as a nearly pure fraction using straightforward clean methodologies (*e.g.* centrifugation,). The influence of the nature of the SLILs and microwave-assistance on the biotransformation yield, as well as the use of different physical strategies for product separation, were studied.

2. Materials and methods

2.1. Materials

Immobilized *Candida antarctica* lipase B (Novozym 435, EC 3.1.1.3) was from Novozymes S.A. (Spain). The SLILs, dodecyltrimethylammonium bis(trifluoromethylsulfonyl)imide ([C_{12}tma][NTf_2], 99% purity), tetradecyltrimethylammonium bis(trifluoromethylsulfonyl)imide ([C_{14}tma][NTf_2], 99% purity), hexadecyltrimethylammonium bis(trifluoromethylsulfonyl)imide ([C_{16}tma][NTf_2], 99% purity) and octadecyltrimethylammonium bis(trifluoromethylsulfonyl)imide ([C_{18}tma][NTf_2], 99% purity) were obtained from IoLiTec GmbH (Germany). Centrifugal filters containing nylon membranes (0.2 μm pore size) were obtained from VWR Int. (Barcelona, Spain). Microwave-assisted reactions were performed in a Microwave Discover System (CEM Corporation Model 908010, USA) using high purity quartz vials (10 mL capacity).

2.2. Lipase-catalyzed synthesis of flavour esters in sponge-like ionic liquid (SLILs)

Three mmol of citronellol, geraniol, nerol or anisyl alcohol, and 1, 2 or 3 mmol of acetic acid were added to 3 mL screw-capped vials with teflon-lined septa. Then, the corresponding amount of [C_{16}tma][NTf_2] was added to reach a final IL concentration of 60 or 70% (w/w) with respect to the mass substrates. Reaction mixtures were pre-incubated at 50 °C for 10 min, resulting in fully clear monophasic systems. Then, 80 mg of MS13× per mmol of carboxylic acid were also added. The reaction was started by adding Novozym 435 (40 mg/mmol of carboxylic acid) and the reaction was incubated for 4 h at 50 °C while shaking (300 rpm), or under 4 W microwave irradiation (which provided a 50 °C constant temperature). For products analysis, aliquots (20 μL) were taken at selected times and suspended in 500 μL octane, and the resulting biphasic mixture was shaken to extract the products. The resulting mixture was centrifuged at 14,000 rpm for 10 min. Finally, 300 μL of the octane extract were added to 100 μL of a 100 mM ethyl propionate (internal standard) solution in octane, and the final solution was analyzed by CG. For full recovery of the flavour ester products at 4 h, the reaction mixtures were consecutively centrifuged four times at 14,000 rpm (15 min) and at room temperature, 21, 10 and 4 °C, which resulted in a top liquid phase of flavour ester and a bottom solid phase containing the SLIL. For the case of anisyl acetate, the reaction mixture was cooled in an ice bath for 3 h, and the resulting solid mixture was placed in a centrifugal filter, then centrifuged at 16,000 rpm (10 min) and at 0 °C. This resulted in a top SLIL solid phase, which was retained inside the filter, and a bottom liquid phase of anisyl acetate. For all cases, a sample (10 μL) of the resulting flavour ester phase was dissolved in 1 mL acetone-δ_6, then analyzed by 300 MHz ^{1}H NMR and 282 MHz ^{19}F NMR, in a Brucker AC 300E spectrometer for SLIL detection. All experiments were carried out in duplicate.

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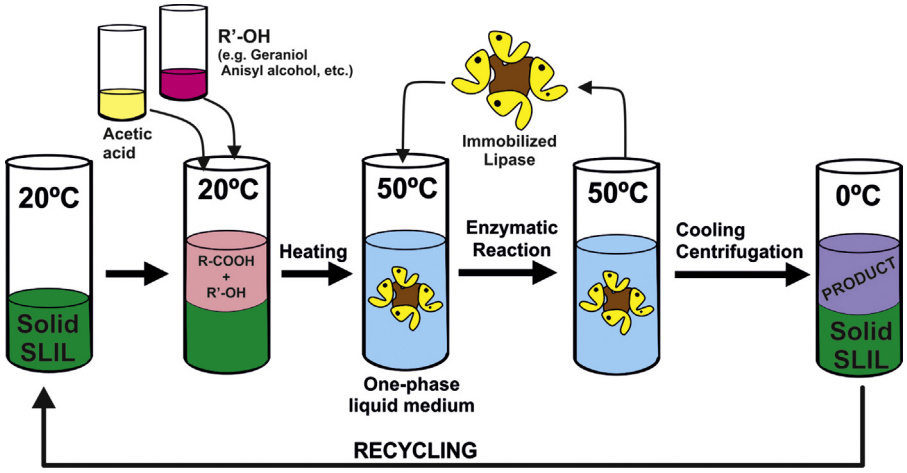

Fig. 2. Green approach for the biocatalytic synthesis of flavour esters in sponge-like ionic liquid (SLILs), and product separation by cooling and centrifugation, including full recovery and reuse of the enzyme – SLIL system.

2.3. Lipase-catalyzed methyl oleate synthesis in switchable ILs

For each IL (*i.e.* [C_{12}tma][NTf_2], [C_{14}tma][NTf_2], [C_{16}tma][NTf_2], or [C_{18}tma][NTf_2]), triolein (0.36, 0.23 or 0.11 mmol) was added into three different screw-capped vials (1.0 mL total capacity) containing SLIL and methanol (2.18, 1.37 or 0.69 mmol). The resulting mixtures at a 1/6 (mol/mol) triolein–methanol ratio gave the following SLIL/triolein/methanol ratios (w/w/w): 16.4/68.7/14.9; 47.7/43.0/9.3; 73.9/21.4/4.7, respectively. For each case, the mixture was previously incubated for 30 min at 60 °C, resulting in fully clear monophasic liquid systems. The reaction was started by adding Novozym 435 (18% w/w with respect to the amount of triolein) and the reaction mixture was maintained at 60 °C for 8 h. At selected times, 20 μL aliquots were taken and suspended in 480 mL of a dodecane/isopropanol (95/5, v/v) solution, and the resulting biphasic mixtures were shaken for 3 min, and then centrifuged at 15,000 rpm for 10 min to extract methyl oleate. Finally, 350 mL of dodecane/isopropanol extracts (upper phase) were added to 150 mL of a 100 mM ethyl decanoate and 100 mM tributyrin (internal standards) solution in dodecane/isopropanol (95/5, v/v), and the final solution was analyzed by CG. All experiments were carried out in duplicate.

2.4. GC analysis

GC analysis of the ester flavours reactions was performed with a Shimadzu GC-17A (Shimadzu Europe, Germany) equipped with an FID detector. Samples were analyzed on a SupraWax-280 capillary column (30 m × 0.32 mm × 0.5 μm, Teknockroma, Spain), using ethyl propionate as an internal standard, under the following conditions: carrier gas (He) at 1.1 mL/min; inlet split ratio, 1:20; temperature programme, 60 °C, 4 min, 10 °C/min, 240 °C, 12 min [14]. GC analysis of methyl oleate reactions was performed with a Shimadzu GC-2010 (Shimadzu Europe, Germany) equipped with an FID detector. Samples were analyzed on a TRB-BIODIESEL capillary column (10 m × 0.28 mm × 0.1 mm, Teknokroma, Spain), using both ethyl decanoate and tributyrin as internal standards, under the following conditions: carrier gas (He) at 28.6 kPa (40 mL/min total flow); temperature programme: 100 °C, 10 °C/min, 200 °C, 15 °C/min, 370 °C; variable split ratio (80:1–10:1); detector, 320 °C [16].

3. Results and discussion

3.1. Biocatalytic production of flavour esters in sponge-like ionic liquids

Flavour esters of short-chain carboxylic acids (*e.g.* geranyl acetate, *etc.*) are among the most important fragrance compounds used in the food, cosmetic and pharmaceutical industries. International legislations means that "natural" flavour substances can only be prepared either by physical processes (*e.g.* extraction) from natural sources, or by enzymatic or microbial transformation of precursors isolated from nature, without the use of organic solvents. In this context, enzyme-catalyzed direct esterification of a flavour alcohol and acetic acid in solvent-free media is the clearest way to obtain "natural" products. However, this approach provides moderate results because of the usual enzymes deactivation that occurs by direct contact with concentrated acids [18].

The suitability of the SIIL [C_{16}tma][NTf_2] as reaction medium for carrying out the enzymatic synthesis of different terpene esters (*e.g.* neryl acetate, geranyl acetate and citronellyl acetate) by direct esterification of acetic acid and the corresponding alcohol was studied at 50 °C, following the operation protocol depicted in Fig. 2. All the reactions were assayed at 60% (v/v) SLIL concentration, and at three different acetic acid/flavour alcohol molar ratios (1/3, 2/3 and 3/3 mol/mol). Product yields in the range 90–100% were obtained at all cases after 4 h reactions. Using the case of geranyl acetate as example (see Fig. 3A), it can be observed how reaction medium was fully clear monophasic systems during the enzymatic reaction at 50 °C, and then became a monophasic solid system after cooling to room temperature. Finally, the solid phase could be separated into two phases, an upper SLIL-free liquid (as determined by ^{19}F-NMR), and another bottom solid phase containing the IL by following the iterative centrifugation protocol detailed above (see Section 2). Fig. 3B shows the resulting product concentration in the upper SLIL-free phase for each assayed reaction medium, which was practically independent of the nature of the terpene alcohol in all the assayed conditions [14].

Fig. 4A depicts the time courses of the Novozym 435-catalyzed esterification of acetic acid with anisyl alcohol in 70% (w/w) [C_{16}tma][NTf_2] SLIL for a 1/1 (mol/mol) acetic acid/anisyl alcohol ratio concentration by using either MW irradiation (4 W power) or conventional heating (50 °C). The enzyme was able to synthesize anisyl acetate in both cases, reaching an up to 82% yield (with

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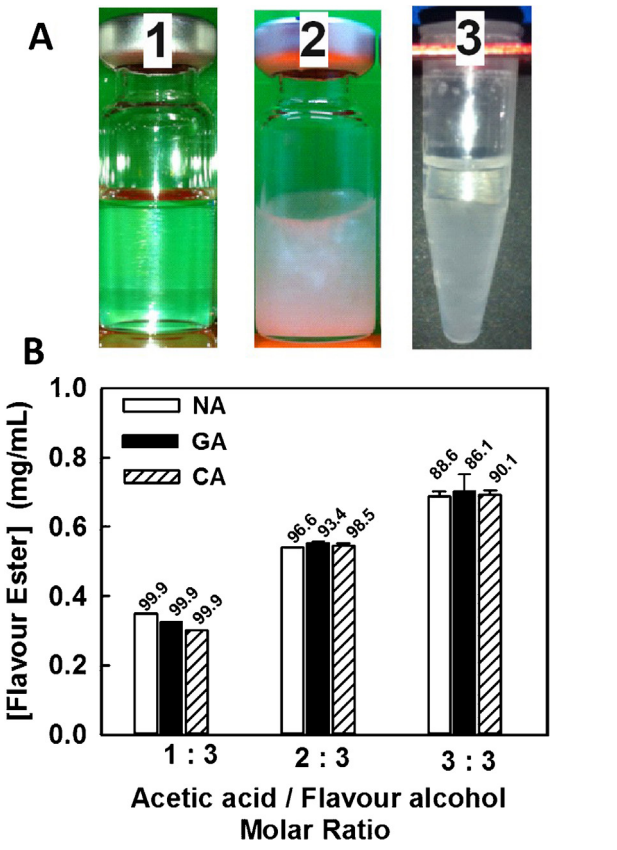

Fig. 3. Novozym 435-catalyzed synthesis of neryl acetate (NA), geranyl acetate (GA) and citronellyl acetate (CA) by direct esterification between acetic acid and the corresponding flavour alcohol in 60% (w/w) [C_{16}tma][NTf_2] SLIL at 50 °C. (A) Phase behaviour of the reaction mixture containing geranyl acetate product at 50 °C (**1**), 25 °C (**2**), and after centrifugation at 14,000 rpm (30 min) and at 10 °C (**3**). (B) Effect of the acetic acid/flavour alcohol molar ratio on the terpene ester product obtained through the esterification reaction catalyzed by Novozym 435 in 60% (w/w) [C_{16}tma][NTf_2] SLIL in a 4 h reaction at 50 °C.

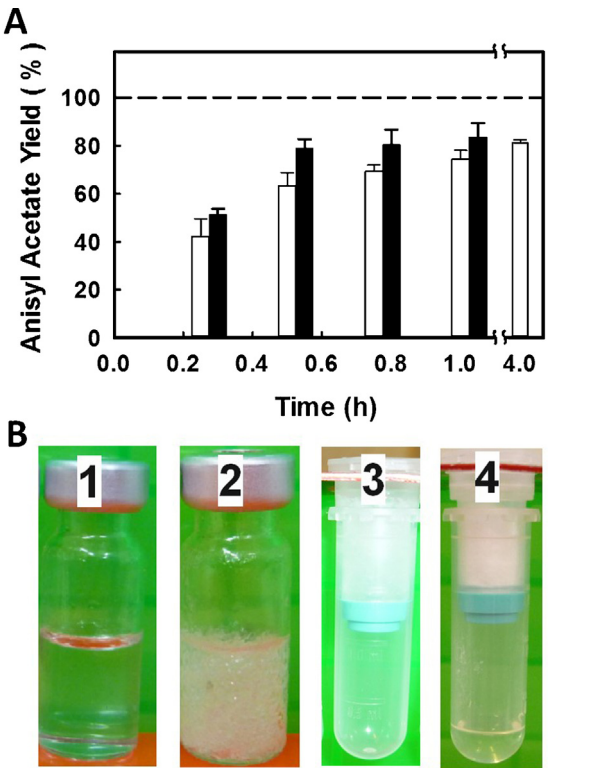

Fig. 4. Novozym 435-catalyzed synthesis of anisyl acetate by direct esterification between acetic acid and anisyl alcohol in 70% (w/w) [C_{16}tma][NTf_2] SLIL at 50 °C. (A) Time-course profiles shown by Novozym 435-catalyzed anisyl acetate synthesis by direct esterification of acetic acid with anisyl alcohol (1:1 molar ratio) in 70% (w/v) [C_{16}tma][NTf_2] SLIL at 50 °C conventional heating (white), or at 4 W microwave irradiation (black). (B) Phase behaviour of the reaction mixture containing the anisyl acetate product at 50 °C (**1**), 0 °C (**2**), 0 °C using a centrifugal filter (0.25 μm pore size), and after centrifugation at 16,000 rpm (10 min) and at 0 °C (**3**).

respect to the initial acetic acid) in 1 h under MW irradiation. The assistance with MW irradiation provided a slightly better anisyl acetate profile than conventional heating, which reached a similar product concentration as MW after 4 h. Fig. 4B shows that the final reaction mixture was a fully clear monophasic system, and once again, it became solid after cooling to room temperature. Applying the above centrifugation protocol at controlled temperatures to the solid reaction mixture did not permit the physical separation of both phases for product recovery because the solid SLIL phase rapidly re-dissolved into the anisyl acetate phase. Thus, a new fractionation approach based on the use of a centrifugal nylon filter (0.2 μm pore size) was assayed at 0 °C. Using this new approach, the SLIL solid was retained by the nylon membrane, while the liquid anisyl acetate phase crossed the membrane to the bottom tube, similarly to the way in which a wet sponge can be wringing. In this case, the synergism observed between MW irradiation and biocatalysis agreed with the results obtained for a variety of synthetic reactions, where improvements in the reaction rate have been attributed to both thermal and non-thermal effects of this irradiation [7,8].

The excellent suitability of water-immiscible ILs for carrying out biocatalytic synthetic reactions has been widely reported in the literature. This property has been attributed to the native conformation of the proteins being maintained, as demonstrated by spectroscopic techniques, thus protecting them against the usual unfolding that occurs in non-aqueous environments [2–4]. Nevertheless, the most exciting and intriguing feature of the hydrophobic ILs here considered is their ability to behave as sponge-like systems in solid phase, where products can easily separate from the IL by centrifugation. These features could be explained by reference to the solid/liquid structural organization of ILs, which possess analogous structural patterns in both solid and liquid phase, as a result of the ionic network formed by monomeric units of a cation surrounded by three anions and *vice versa*. The incorporation of molecules into this IL network leads to changes in their physicochemical properties, and even the formation of polar and non-polar regions. Thus, ILs are described as nano-structured materials, which permits neutral molecules to reside in less polar regions, while ionic or polar species undergo faster diffusion in the more polar regions [19,20]. For the case of pyrrolidinium-based ILs, it has been reported that these ILs are organized as an intricately nanostructured net, where the ILs containing cations with shorter alkyl chain substituents form alternating cation–anion monolayer structures on confinement to a thin film, whereas a cation with a longer alkyl chain substituent leads to bilayer formation [21]. In agreement with these studies, the nanostructural organization of the [C_{16}tma][NTf_2] SLIL, as temperature switchable liquid/solid phase, may be described in terms of ordered layers in which the alkyl side chains of the cations provide hydrophobic holes appropriate for "housing" flavour molecules in the solid phase. Then, centrifugation of the solid mixture across the filter allowed the "wet sponge" to be "wrung", and the product to be recovered. In this context, the ability of these SLILs to melt at temperatures compatible with enzyme catalysis permitted the development of a straightforward and green method for flavour ester production based on two-steps:

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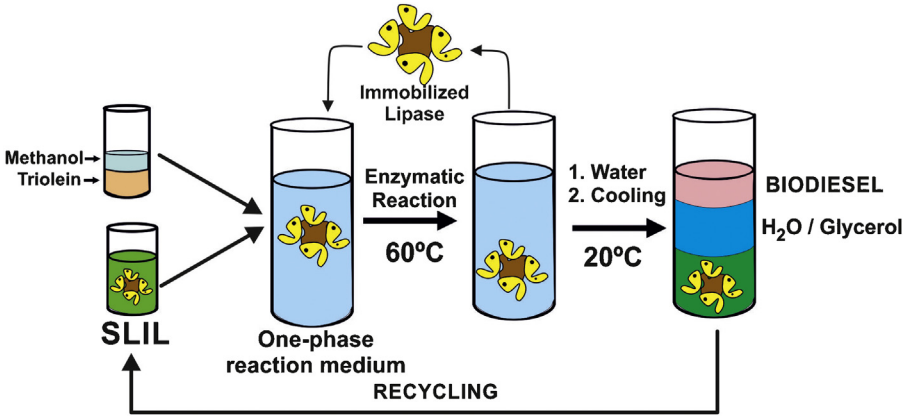

Fig. 5. Green approach for the biocatalytic synthesis of methyl oleate in sponge-like ionic liquids (SLILs), and product separation by addition of water followed by cooling and centrifugation.

(i) lipase-catalyzed direct esterification, and (ii) clean separation of the reaction product by cooling/centrifugation [14,17].

3.2. Biocatalytic production of methyl oleate in sponge-like ionic liquids

One of the most important limitations in the catalytic production of methyl oleate (biodiesel) by transesterification of triacylglycerides with methanol can be directly attributed to the non-miscibility of the substrates, leading to a two-phase system that reduces process efficiency and, for the case of biocatalysts, results in full enzyme deactivation through direct contact with methanol. It has been demonstrated how highly hydrophobic ILs based on an imidazolium cation with a long alkyl-side chain (*e.g.* $[C_{18}mim][NTf_2]$) are able to dissolve both triolein and methanol at any concentration, providing one-phase reaction media that showed excellent suitability for the biocatalytic synthesis of methyl oleate (up to 96% yield in 6 h at 60 °C). Furthermore, the excellent suitability of the enzyme-IL system for methyl oleate synthesis was also demonstrated by the total preservation of the catalytic activity for subsequent reuse [2,16]. However, any industrial application of this approach should involve a clean approach so that the synthesized methyl oleate can be extracted and the IL be recovered and reused (*e.g.* by using immobilized enzymes onto covalently attached IL phases and continuous $scCO_2$ flow) [13,22]. However, despite the greenness of supercritical fluids, their industrial application for biodiesel production may be doubtful mainly because of the excessive cost of high-pressure equipment.

In agreement with the schematic protocol of Fig. 5, the biocatalytic synthesis of methyl oleate was carried out through methanolysis of triolein, using a 1/6 triolein/methanol molar ratio as substrate, in four different $[C_n tma][NTf_2]$ (n = 12, 14, 16 or 18, respectively) SLILs as reaction media for an 8 h of reaction period at 60 °C, and at different SLIL concentrations. As can be seen in Fig. 6A, regardless of the nature of the ILs, methyl oleate yields close to 100% were obtained at SLIL concentrations higher than 40% (w/w), which could be related with the fact that a minimum IL content in the reaction medium is necessary to achieve full miscibility between the substrates as a monophasic system (see Fig. 6B) [13]. Furthermore, an increase in the alkyl chain length of cation slightly improves the catalytic efficiency of the system, and so $[C_{18}tma][NTf_2]$ was selected as the most suitable SLIL for performing methyl oleate synthesis [17].

To separate of both the methyl oleate and glycerol products from the reaction medium containing SLIL, a new cooling/centrifugation approach, based on the prior addition of hot water (60 °C), was developed (see Fig. 6B). By cooling the resulting mixture to room

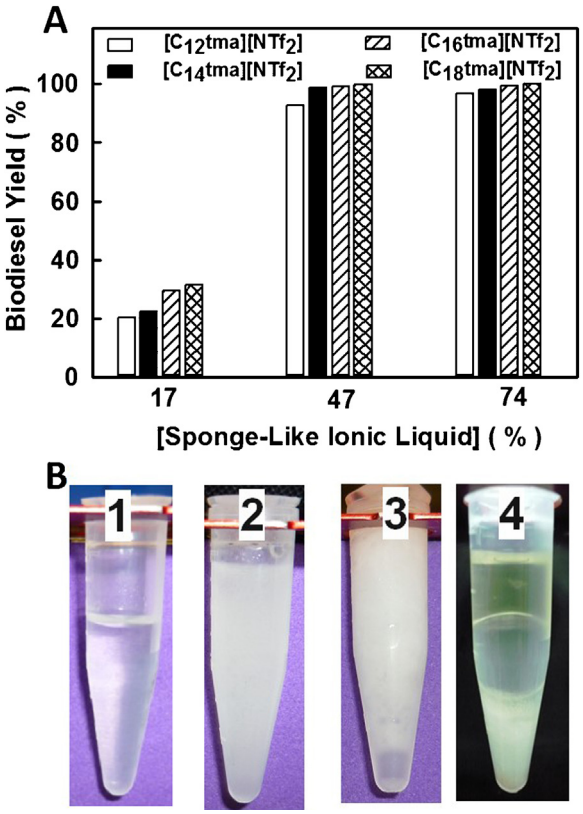

Fig. 6. (A) Effect of the alkyl chain length of the [1-alkyltrimethylammonium] cation of the SLIL on the methyl oleate yield obtained by Novozym 435-catalyzed methanolysis of triolein (1/6 triolein/methanol mol ratio) in an 8 h reaction at 60 °C and at three different SLIL concentrations. (B) Phase behaviour of the reaction mixture containing both methyl oleate and glycerol products at 60 °C (**1**), 60 °C after addition of water (**2**), 25 °C (**3**), and after three consecutive centrifugation steps at 15,000 rpm (1 h) at room temperature, 23 and 15 °C, respectively (**4**).

temperature, separation was achieved after three consecutive centrifugation steps at 15,000 rpm (15 min) at room temperature, 23 and 15 °C. This resulted in a three phases system: an upper methyl oleate phase, a middle liquid aqueous phase containing the glycerol by-product, and a bottom solid containing the SLIL (see Fig. 6B). Additionally, the presence of water, a green solvent that is not miscible with methyl oleate or SLIL, improved the separation between phases, ordered in agreement with their respective densities (methyl oleate < water < SLIL) [17]. The suitability of the proposed methodology for clean methyl oleate separation was thus

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demonstrated. Increasing the centrifugation time and/or speed of the protocol might improve the extraction yields, although the temperature at which centrifugation can be carried out is limited by the melting point of the fatty acid methyl esters present in the methyl oleate. Furthermore, this approach also includes an easy way to separate both methyl oleate and glycerol into different fractions. Other proposed approaches based on liquid–liquid extraction with organic solvents (*e.g.* hexane), or supercritical fluids are less interesting for industrial application, both from the economic and technical standpoint [2,15,17].

4. Conclusions

This work describes for the first time a straightforward and green method for efficiently extracting organic compounds (*e.g.* terpenes) dissolved in an ionic liquid by simple cooling and centrifugation. This approach is contrary to the usual heating step applied for classical separation processes in Chemical Engineering (*e.g.* distillation). Furthermore, the ability of SLILs to melt at temperatures compatible with enzyme catalysis (*e.g.* lower than 75 °C) allowed us to develop two-step protocols for producing high added value compounds (flavour esters, methyl oleate, *etc.*). The protocol involved, (i) enzyme-catalyzed transesterification or esterification reactions with a product yield close to 100%, and (ii) clean separation of the reaction products by cooling/centrifugation. Using this approach, an almost pure product was obtained, while recovery and reuse of the biocatalyst/IL system led to a very low decrease in activity because of the demonstrated ability of hydrophobic ILs to stabilize enzymes.

The unique ability of these sponge-like ionic liquids, as switchable liquid/solid phases with temperature, to "soak up" hydrophobic compounds like methyl oleate or esters flavours as a liquid phase, and then to be "wrung out" by centrifugation at the form of solid phase opens up a new sustainable platform for chemical synthesis and product separation.

Acknowledgements

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