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Clean enzymatic process for producing flavour esters by direct esterification in switchable ionic liquid/solid phases.

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A clean biocatalytic approach for producing flavour esters using switchable ionic liquid/solid phases as reaction/separation media has been developed. The phase behaviour of different IL/flavour acetyl esters (geranyl acetate, citronellyl acetate, neryl acetate and isoamyl acetate) mixtures was studied at several concentrations, resulting for all cases in fully homogeneous liquid media at 50ºC, and solid systems at room temperature. By using an iterative centrifugation protocol on the solid IL/flavour ester mixtures at controlled temperatures, the solid IL phase and the liquid flavour ester phase can be easily separated. The excellent suitability of immobilized *Candida antarctica* lipase B (Novozym 435) catalyst in the esterification reaction between a aliphatic carboxylic acid (acetic, propionic, butyric or valeric) with a flavour alcohol (isoamyl alcohol, nerol, citronellol or geraniol) in N,N',N",N'"-hexadecyltrimethyl-ammonium bis(trifluoromethylsulfonyl)imide ([C_{16}tma][NTf_2])IL has been demonstrated, the product yield being improved up to 100% in appropriate reaction conditions (enzyme amount, dehydrating molecular sieves, etc) at 50ºC. The enzymatic synthesis of sixteen different flavour esters was carried out in [C_{16}tma][NTf_2] by means of this approach, providing products of up to 0.757 g/mL concentration after IL separation. The residual activity of the enzyme/IL system during seven consecutive operation cycles was shown to be practically unchanged after reuse.

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1 Introduction

Flavour esters of short-chain carboxylic acid (*e.g.* isoamyl acetate, citronellyl acetate, geranyl propionate, neryl acetate, etc, see Fig. 1) are among the most important fragrance compounds used in the food, cosmetic and pharmaceutical industries. Most flavour compounds are traditionally provided by chemical synthesis or extraction from natural sources.[1] However, US[2] and European[3] legislation has meant 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. In this context, as natural flavours extracted from plant materials are often too scarce or expensive for industrial use, there is a great interest in seeking new methods for their production by means of clean industrial scale approaches.[4]

Lipases and esterases have often been used as catalysts to synthesize flavour esters by esterification or transesterification approaches in organic solvents,[5,6] ionic liquids (ILs),[7] supercritical fluids,[8] or solvent-free[9] reaction media. The use of non-natural substrates (*e.g.* acetic anhydride,[6b,9b] alkyl vinyl esters,[6c,6e,9c,9d] p-nitrophenyl acetate,[6d] etc.) in transesterification, regardless of the reaction media, is a clear breakdown point in any attempt to obtain "natural" products. However, enzyme-catalyzed direct esterification between "natural" substrates could be considered as the most straightforward way to obtain

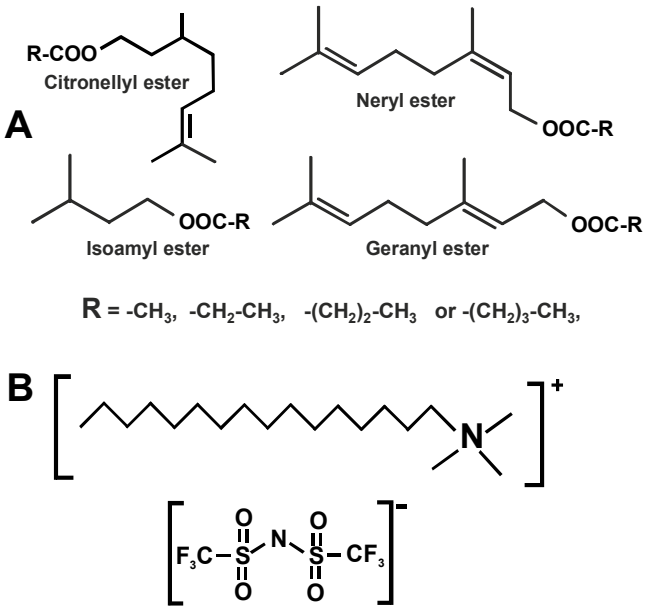

Fig. 1. **A.** Flavour esters synthesized by lipase-catalyzed esterification. **B**. The IL [C_{16}tma][[NTf_2], as an example of switchable ionic liquid/solid phase.

"natural" ester products. Furthermore, by using either supercritical fluids or solvent-free reaction media, clean processes can easily be designed. However, distinctly moderate results (*e.g.* maximum of 60-80% isoamyl acetate

yield in solvent-free,[9bc] and 80% geranyl acetate yield in supercritical ethane[8d]), the deactivation of enzyme by direct contact with acids, or cost of high-pressure equipment are also drawbacks for industrial application. Reflecting the suitability of ILs for use in enzyme-catalyzed reactions,[10] it has been reported that [Bmim][PF_6] is a suitable reaction medium for lipase-catalyzed flavour ester synthesis by esterification (*e.g.* 80% geranyl acetate yield at 311 h reaction,[7a] and up to 100% isoamyl acetate yield,[7b-d]), but product recovery by liquid-liquid extraction with organic solvents tarnish the greenest aspects of the approach. The use of high hydrostatic pressure (up to 500MPa [7d]) or pervaporation membrane systems[7b] permits isoamyl acetate separation in IL-alcohol biphasic systems.

Hydrophobic ILs based on cations with large alkyl side-chains (*e.g.* 1-methyl-3-octadecylimidazolium bis(trifluoromethylsulfonyl)imide, [C_{18}mim][NTf_2]) have been used to synthesize nickel nanoparticles with reduced diameters,[11] as anticancer agents,[12] and also as excellent reaction media for the enzymatic synthesis of biodiesel (*i.e.* up to 96% biodiesel yield in 6 h at 60ºC), because of their ability to dissolve both triolein and methanol, thus providing monophasic systems where the lipases are highly active and stable.[13]

The most interesting feature of biocatalytic processes in ILs is the possibility to design two-phase reaction systems that permit easy product recovery (*e.g.* IL/$scCO_2$ biphasic systems).[10a] The ability of hydrophobic IL with large alkyl side-chain to switch from solid to liquid phase at temperatures compatible with enzyme catalysis (*e.g.* 53ºC for [C_{18}mim][NTf_2],[13a] 43ºC for [C_{16}mim][NTf_2],[12] etc) could be used to develop easy protocols for enzymatic synthesis and separation of natural flavour ester products.

This paper demonstrates for the first time the biocatalytic synthesis of sixteen different flavour alkyl esters by direct esterification of a alkyl carboxylic acid (acetic, propionic, butyric or valeric) with a flavour alcohol (citronellol, geraniol, nerol or isoamyl alcohol) in the IL N,N',N",N"'-hexadecyltrimethylammonium bis(trifluoromethylsulfonyl) imide ([C_{16}tma][NTf_2, see Fig. 1 B) as a switchable ionic liquid/solid phase, used for the reaction and subsequent product separation by centrifugation (See Fig. 2). The influence of several reaction parameters (*e.g.* acid/alcohol ratio, IL concentration, length of the IL alkyl side-chain, enzyme amount, etc) to achieve maximal product yield is studied. A cyclic protocol for enzymatic synthesis, product separation and enzyme-IL reuse has also been developed to demonstrate the suitability of the proposed approach as a sustainable process for producing "natural" flavour ester at industrial level.

2 Experimental.

Immobilized *Candida antarctica* lipase B (Novozym® 435, EC 3.1.1.3) was from Novozymes S.A. (Spain), and was equilibrated to 0.11 Aw by over saturated LiCl solutions in a closed container at 25ºC for one week prior to use.[9d] Citronellol (95% GC), geraniol (98% GC), nerol (97% GC), isoamyl alcohol (>98% GC), citronellyl acetate (99% GC),

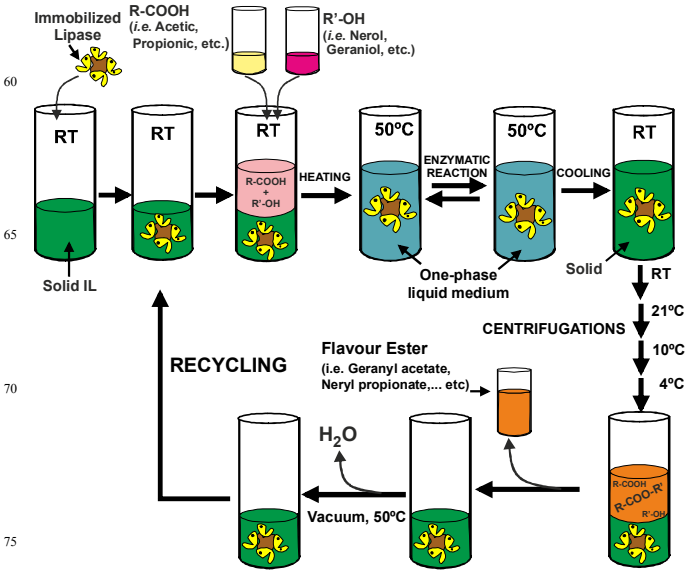

Fig. 2. Scheme of the cyclic protocol for the production of flavour esters by lipase-catalyzed direct esterification in switchable ionic liquid/solid phases, and reusing the enzyme/IL system. For details see Experimental section

geranyl acetate (>98% GC), neryl acetate (>98% GC), isoamyl acetate (>98% GC), acetic acid, propionic acid, butyric acid, valeric acid, molecular sieves 13x (MS13x; 270 mg H_2O/g adsorption capacity), solvents and other chemicals were purchased from Sigma-Aldrich-Fluka Chemical Co. The ILs, dodecyltrimethylammonium bis((trifluoromethyl)-sulfonyl)imide ([C_{12}tma][NTf_2], 99% purity), tetradecyltrimethyl-ammonium bis((trifluoromethyl) sulfonyl)imide ([C_{14}tma] [NTf_2], 99% purity), hexadecyl-trimethylammonium bis((trifluoromethyl)sulfonyl) imide ([C_{16}tma][NTf_2], 99% purity) and octadecyltrimethyl-ammonium bis((trifluoromethyl)sulfonyl)imide ([C_{18}tma] [NTf_2], 99% purity) were obtained from IoLiTec GmbH (Germany). The melting points of ILs were determined by using a Reichert Thermovar melting point apparatus equipped with a microscope, and were uncorrected. The density of solid [C_{16}tma][NTf_2] was determined by filling a 10-mL pycnometer with the IL in liquid phase at 60ºC, then cooled until 25ºC.

Phase behaviour of [C_{16}tma][NTf_2]/flavour ester mixtures.

In 1.5-mL screw-capped vials with teflon-lined septa, 0.3, 0.4 or 0.5 g of citronelly acetate, geranyl acetate, neryl acetate or isoamyl acetate were mixed with the corresponding amount of [C_{16}tma][NTf_2] to finally obtain IL concentrations of 50, 60 or 70% (w/w), respectively. All IL/flavour ester mixtures were incubated under shaking (300 rpm) for 10 min at 50ºC, which produced fully clear monophasic systems. Then, the solutions were consecutively centrifuged four times at 14,000 rpm (15 min) and at room temperature, 21, 10 and 4ºC, resulting in a top liquid phase of flavour ester, and a bottom solid phase containing IL alone. After each centrifugation step, a sample (10 µL) of the resulting top flavour ester phase was dissolved

in 1 mL acetone-d_6, then analyzed by 300 MHz ^{1}H NMR and 282 MHz ^{19}F NMR, respectively in a Brucker AC 300E spectrometer to detect the presence of the IL. See ESI.

Lipase-catalyzed flavour esters synthesis in switchable ILs.

Into 3-mL screw-capped vials with teflon-lined septa, 3 mmol of citronellol (575 µL), geraniol (537 µL), nerol (545 µL) or isoamyl alcohol (334µL), and 1, 2 or 3 mmol of acetic acid (58, 116 or 174 µL, respectively), propionic acid (75, 150 or 225 µL, respectively), butyric acid (93, 186 or 278 µL, respectively) or valeric acid (110, 220 or 330 µL, respectively) were added. Then, the corresponding amount of [C_{12}tma][NTf_2], [C_{14}tma][NTf_2], [C_{16}tma][NTf_2] or [C_{18}tma][NTf_2], respectively, was added to reach a final IL concentration of 50, 60 or 70% (w/w) with respect to the mass substrates. Reaction mixtures were pre-incubated at 50ºC for 10 min, resulting in fully clear monophasic systems, and then 80 mg MS13x per mmol of carboxylic acid were also added. The reaction was started by adding Novozym 435 (40 mg per mmol of carboxylic acid) and the reaction was incubated at 50ºC under shaking (300 rpm) for 4 h. To obtain time-course profiles, 20 µL aliquots were taken at regular intervals and suspended in 500 µL octane, and the resulting biphasic mixture was strongly shaken for 3 min to extract products. The resulting mixture was centrifuged at 14,000 rpm for 10 min. Finally, 300 µL of octane extract were added to 100 µL of 100 mM ethyl propionate (internal standard) solution in octane, and the final solution was analyzed by CG. One unit of synthetic activity was defined as the amount of enzyme that produces 1 µmol of flavour alkyl ester per min. For full recovery of the flavour ester products at 4 h, the reaction mixtures were centrifuged as described above, resulting in a fully clear liquid phase containing products and non-reacted substrates, and a solid phase containing IL, immobilized enzyme and MS13x, respectively. Samples of each liquid phase were collected and stored at -20ºC until use for product identification by GC/MS. All experiments were carried out in duplicate.

GC analysis

GC analysis was performed with a Shimadzu GC-17A (Shimadzu Europe, Germany) equipped with 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 internal standard, under the following conditions: carrier gas (He) at 1.1 mL/min; inlet split ratio, 1:20; temperature programme: 60ºC, 4min, 10ºC/min, 200ºC, 12 min. Peak retention times (min) were as follows: β-citronellol, 18.7; citronellyl acetate, 17.4; citronellyl propionate, 18.3; citronellyl butyrate, 18.8; citronellyl valerate, 21.0; geraniol, 20.0; geranyl acetate, 18.8; geranyl propionate, 19.6; geranyl butyrate, 20.9; geranyl valerate, 22.6; nerol 19.3, neryl acetate, 18.3; neryl butyrate, 20.3; neryl valerate, 22.0; isoamyl alcohol, 9.7; isoamyl propionate, 10.0; isoamyl butyrate, 11.2; isoamyl valerate, 13.0; acetic acid, 14.0; propionic acid 15.5; butyric acid, 17.1; valeric acid, 18.2; ethyl propionate, 5.1. For the neryl propionate case, temperature programme was: 60ºC, 4 min, 5ºC/min, 200ºC, 12 min. Peak retention times (min) were as follows: nerol, 28.0; neryl propionate, 27.7; propionic acid,21.1. For the isoamyl acetate propionate case, temperature programme was: 60ºC, 4 min, 4ºC/min, 140ºC, 5 min. Peak retention times (min) were as follows: isoamyl alcohol, 12.0; isoamyl acetate, 9.4; acetic acid, 20.3. See ESI.

Identification of flavour ester products by GC/MS

GC-MS analyses were carried out by using a GC-6890 (Agilent, USA) coupled with a MS-5973 (Agilent, USA) system. The GC was equipped with an HP-5MS column (30 m x 0.25 mm x 0.25 µm, Agilent, USA), used at the following conditions: carrier gas (He) at 53.9 mL/min; inlet split ratio, 1:60; temperature program: 60ºC, 10ºC/min, 200ºC, 12 min; MS source ionization energy 70 eV; the scan time was 0.5 s, covering a mass range of 40-800 amu. Each flavour alkyl ester was identified by comparison of its mass spectra with those in a computer library (NIST Library). Citronellyl acetate, retention time (Rt): 13.9 min; positive-ion (m/z) 43.2, 55.2, 69.2, 81.2, 95.2, 109.2, 123.2, 138.2. Citronellyl propionate, Rt: 15.2 min; m/z: 41.2, 57.2, 69.2, 81.2, 95.2, 109.2, 123.2, 138.2. Citronellyl butyrate, Rt: 16.2 min; m/z: 41.2, 55.2, 69.2, 81.2, 95.2, 109.2, 123.2, 138.2. Citronellyl valerate, Rt: 17.4 min; m/z: 41.2, 57.2, 69.2, 81.2, 95.2, 109.2, 123.2, 138.2. Geranyl acetace, Rt: 14.4 min; m/z: 41.2, 69.2, 93.2, 121.2, 136.2. Geranyl propionate, Rt: 15.6 min; m/z: 41.2, 57.2, 69.2, 93.2, 121.2, 136.2. Geranyl butyrate, Rt: 16.6; m/z: 41.2, 69.2, 80.2, 93.2, 121.2, 136.2. Geranyl valerate, Rt: 17.7 min; m/z: 41.2, 57.2, 69.2, 85.2, 93.2, 121.2, 136.2. Neryl acetate, Rt: 14.0 min; m/z: 41.2, 69.2, 80.2, 93.2, 121.2, 136.2. Neryl propionate, Rt: 15.3; m/z: 41.2, 57.2, 69.2, 80.2, 93.2, 121.2, 136.2. Neryl butyrate, Rt: 16.3 min; m/z: 41.2, 69.2, 80.2, 93.2, 107.2, 121.2, 136.2. Neryl valerate, Rt: 17.5; m/z: 41.2, 55.2, 69.2, 80.2, 93.2, 107.2, 121.2. Isoamyl propionate, Rt, 8.5 min; m/z: 43.2, 55.2, 70.2. Isoamyl butyrate, Rt: 9.2 min; m/z: 43.2, 55.2, 70.2, 115.2. Isoamyl valerate, Rt: 10.9 min; m/z: 43.2, 55.2, 70.2, 99.2, 117.2.

Operational stability of [C_{16}tma][NTf_2]/Novozym 435 system.

For the first operation cycle, 3 mmol of citronellol (575 µL), geraniol (537 µL) or isoamyl alcohol (334µL) and 2 mmol of acetic acid (116 µL), propionic acid (150 µL) or butyric acid (186 µL) were mixed in 3-mL screw-capped vials with the corresponding amount of [C_{16}tma][NTf_2] to reach a 60% (w/w) final IL concentration with respect mass substrates. Reaction mixtures were pre-incubated at 50ºC for 10 min, resulting in fully clear monophasic systems. The reaction was started by adding 80 mg Novozym 435, and run under shaking (300 rpm) for 14 h at 50ºC without the presence of MS13x. At this time, a 20 µL aliquot was taken and suspended in 500 µL octane for GC analysis as described above. Reaction products and non-reacted substrates were extracted from the IL media following the centrifugation protocol described above, resulting in an IL-free top liquid phase of flavour ester, and a bottom solid phase containing both the IL and immobilized enzyme. Flavour ester and non-reacted substrates were recovered by decantation, and the remaining IL/Novozym solid phase was incubated under vacuum at 50ºC for 4 h to

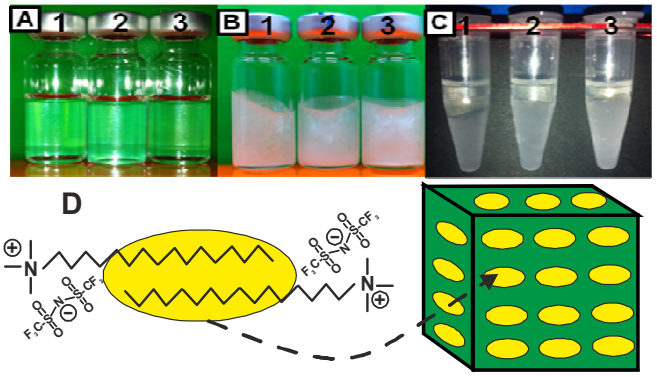

Fig. 3. Phase behaviour of 50/50 (**1**), 60/40 (**2**) and 70/30 (**3**) (w/w) [C_{16}tma][NTf_2]/geranyl acetate mixtures at 50ºC (**A**), 25ºC (**B**), and after four consecutive centrifugation steps at 14,000 rpm (15 min) and at room temperature, 21, 10 and 4ºC (**C**), respectively. **D** Schematic hypothesis of the structural organization of the solid [C_{16}tma][NTf_2] net (green) with hydrophobic holes (yellow) containing liquid geranyl acetate. See ESI.

evaporate any remaining product/substrate traces. Finally, a new biocatalytic/extraction cycle for flavour alkyl ester production was started by adding the corresponding flavour alcohol (3 mmol) and carboxylic acid (2 mmol), respectively.

3 Results and discussion.

Phase behaviour of [C_{16}tma][NTf_2] / flavour ester mixtures.

The suitability of the [C_{16}tma][NTf_2] ionic liquid to dissolve geranyl acetate, citronellyl acetate, neryl acetate or isoamyl acetate was first studied at concentration of 50, 60 and 70% IL and 50ºC, resulting in fully clear monophasic systems in all cases, even though the melting point of this IL is 64ºC (see Fig. 3A and ESI). All media became monophasic solid systems after cooling to room temperature (Fig. 3B). Furthermore, each solid phase could be separated into two phases, an upper IL-free liquid, and another bottom solid containing the IL by following an iterative centrifugation protocol of four steps (10 min, 14,000 rpm) and lowering the temperature from room (25ºC approx.) to 4ºC (Fig. 3C). The 50/50 (w/w) [C_{16}tma][NTf_2]/geranyl acetate mixture was taken as a representative example to detect any IL residual content in the flavour ester phase after each centrifugation step, based on ^{1}H and ^{19}F NMR spectra. The IL content of this top liquid phase continuously decreased, until IL-free geranyl acetate was obtained at the last step of the proposed iterative centrifugation protocol (see ESI). The absence of any peak in the ^{19}F NMR spectrum confirmed that full separation of [C_{16}tma][NTf_2] and geranyl acetate can be attained using simple and cheap methods, in this case cooling and centrifugation. Furthermore, the resulting liquid-solid biphasic systems remained stable at room temperature with time. In preliminary experiments using a unique centrifugation step at 25, 21 or 10ºC, it was observed how the resulting top liquid phase became solid by incubation in ice bath, because of the presence of residual IL content. This phenomenon was not observed when the proposed iterative cooling/centrifugation protocol was used, allowing to recover IL-free ester flavours by simple decantation at different yields (w/w), *i.e.* 51% geranyl acetate, 82% isoamyl acetate, 59% citronellyl acetate, or 60% neryl acetate. The increase in centrifugation time and/or centrifugation speed might improve these results.

These features could be explained as being a function of the solid/liquid structural organization of ILs. Although many papers have focused on the liquid phase behaviour of ILs with organic compounds,[14] no study of solid/liquid phase transitions of ILs containing dissolved substances has been published. However, as regards the structure of imidazolium ILs, Dupont reports how ILs possess analogous structural patterns in both the solid and liquid phase, as a result of an ionic network formed by monomeric units of a cation surrounded by three anions and *viceversa*.[15] The incorporation of molecules in the IL network causes changes in their physical chemical properties, and even the formation of polar and non-polar regions. Thus, ILs are described as nano-structured materials, which permit neutral molecules to reside in less polar regions, while ionic or polar species undergo faster diffusion in the more polar regions. Similarly, the phase transitions of 1-butyl-3-methylimidazolium ILs was recently studied by positron lifetime spectroscopy.[16] This paper analyzes both the occupied and free volumes of ions for four different ILs in amorphous and crystalline phases, as well as the corresponding phase transitions, and concludes that the free volume increases with temperature because the molecules requires more space to move. Zang *et al*[18] studied the tribological behaviour of 1-mehyl-3-hexadecylimidazolium tetrafluoroborate [C_{16}mim][BF_4] IL crystal as an additive of liquid paraffin, reporting how this IL (m.p. 49ºC)[13b] is transformed from solid state to liquid crystalline phase at 80ºC, the IL molecules being arranged in layers with their long molecular axes parallel to each other, and resulting in excellent tribological properties. In agreement with these results, the unique feature observed in Fig 3C might be explained as a consequence of the nano-structured organization of [C_{16}tma][NTf_2], where the large alkyl side chains of cations interact by forming hydrophobic holes suitable for the incorporation of alkyl flavour esters (see Fig 3D). Thus, geranyl acetate could be regarded as being included rather than dissolved in the liquid/solid IL phases. The decrease in free volume of the ionic net produced by cooling allowed compaction of the IL solid phase by centrifugation, and the consequent release of geranyl acetate molecules outside the net. As the density of the solid [C_{16}tma][NTf_2] (1.09 g/mL at 25ºC) is higher than that of the liquid geranyl acetate (0.92 g/mL at 25ºC), the flavour liquid phase easily shifted to the upper phase. Thus, the IL net could be considered as a nano-sponge that can switch from solid to liquid phase (and *viceversa*) as a result of temperature changes, with holes of variable volume, which are suitable for housing or releasing hydrophobic molecules as a function of their liquid or solid phase, respectively. In the same way as the pioneering work of Brennecke's group in 1999 which showed how supercritical carbon dioxide was able to extract dissolved hydrophobic compounds (*e.g.* naphthalene) into an IL phase (*e.g.* [Bmim][PF_6])[19] and the subsequent application for (bio)catalytic processes,[20] our new green approach to extract products from IL phases, based on more

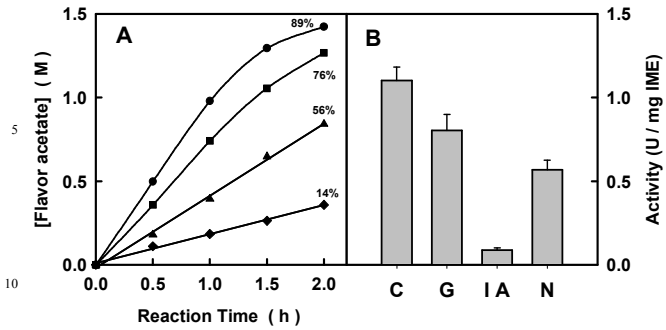

Fig. 4. Time course profiles (**A**) and enzymatic activity (**B**) shows by Novozym 435 (20 mg)-catalyzed esterification reactions of 2 mmol acetic acid with 3 mmol flavour alcohols, (●, **C**) citronellol, (■, **G**) geraniol, (▲, **N**) nerol, or (◆, **I A**) isoamyl alcohol, in 50% (w/w) [C_{16}tma][NTf_2] at 50ºC. Product yields at 2h reaction with respect initial acetic acid concentration are also included. For details, see Experimental section

straightforward technologies (*e.g.* cooling, centrifugation, etc.) can also be applied to a broad spectrum of synthetic processes, *e.g.* enzymatic reactions.

Novozym 435-catalyzed flavour esters synthesis by direct esterification in [C_{16}tma][NTf_2].

Fig. 4A depicts time courses of the Novozym 435-catalyzed esterification of citronellol, geraniol, nerol and isoamyl alcohol, respectively, with acetic acid in 50% (w/w) [C_{16}tma][NTf_2] at 50ºC, using a 2/3 (mol/mol) acetic acid/alcohol ratio concentration. As can be seen, the enzyme was able to synthesize all flavour esters, reaching up to 89% yield (with respect the initial acetic acid) for the citronellyl acetate case. However, the enzyme activity was clearly dependent on the nature of the flavour alcohol (see Fig 4B, citronellol > geraniol > nerol > isoamyl alcohol), the activity for citronellyl acetate synthesis being up to 12.5 times higher

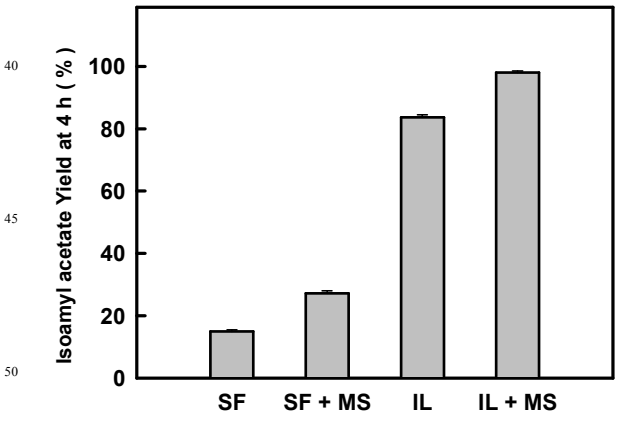

Fig. 5. Isoamyl acetate yield obtained by the esterification between acetic acid (2 mmol) and isoamyl alcohol (3 mmol) catalyzed by Novozym 435 (40 mg/mol acetic acid) after 4 h reaction at 50ºC. SF, solvent-free; SF+MS, solvent-free with molecular sieves (80 mg/mmol acetic acid); IL, 50% (w/w) [C_{16}tma][NTf_2]; IL+MS, 50% (w/w) [C_{16}tma][NTf_2] with molecular sieves (80 mg/mmol acetic acid).

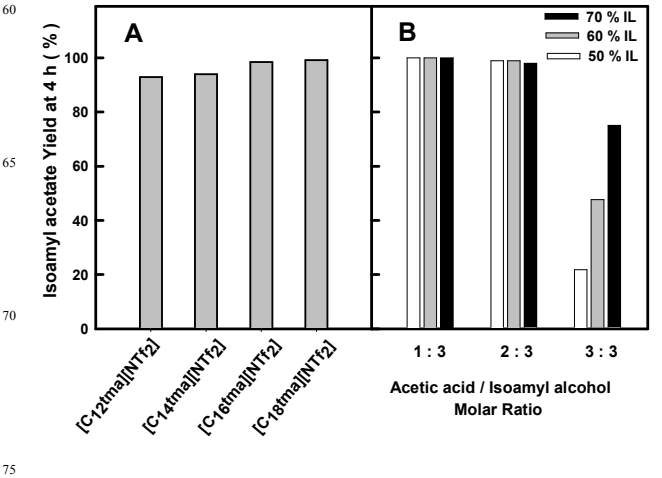

Fig. 6. **A**. Influence of the alkyl side chain length of the IL (50% w/w) on the isoamyl acetate yield obtained by the esterification between acetic acid (2 mmol) and isoamyl alcohol (3 mmol) catalyzed by Novozym 435 (40 mg/mol acetic acid) after 4 h reaction at 50ºC. **B**. Effect of the acetic acid/isoamyl alcohol molar ratio on the isoamyl acetate yield obtained by the esterification reaction catalyzed by Novozym 435 (40 mg/mol acetic acid) in different [C_{16}tma][NTf_2] concentrations after 4 h reaction at 50ºC. All reaction media contained 80 mg molecular sieves per mmol acetic acid.

than for the isoamyl acetate. To assess the influence of the [C_{16}tma][NTf_2] as reaction medium, comparative reactions for lipase-catalyzed isoamyl acetate synthesis in solvent-free and IL reaction media, with (and without) the presence of the MS13x drying agent, were carried out. Furthermore, the enzyme amount for these reactions was four-times higher than in the experiments of Fig. 4 in order to speed up synthetic processes of industrial interest. As can be seen in Fig. 5, the direct enzymatic esterification of acetic acid with isoamyl alcohol in solvent-free conditions gave a low product yield (14.9% at 4 h reaction), but was doubled in the presence of MS13x dehydrating agent because of its ability to shift the reaction equilibrium towards the synthetic product pathway by adsorbing the water by-product released. The moderate activity shown by the enzyme in these solvent-free media has been related with the high acid concentration (4.45 M for our experiments) in reaction media, which might have provoked enzyme deactivation.[9a,b] However, the isoamyl acetate yield was greatly improved when the enzymatic esterification reaction occurred in [C_{16}tma][NTf_2], reaching nearly 100% yield at 4 h reaction when MS13x was present in the reaction medium. These results agree with the excellent suitability of hydrophobic ILs for lipase-catalyzed esterification and transesterification reactions,[10,18] which has also been reported for the case of ILs based on cations with large alkyl side chains.[13] The excellent stability displayed by enzymes in water-immiscible ILs should be mentioned. This was due to the maintenance of the native conformation of the proteins as demonstrated by spectroscopic techniques (*e.g.* fluorescence, circular dichroism, FT-IR, etc.), protecting them against the usual unfolding that occurs in non-aqueous environments.[21]

The effect of the alkyl side chain length of IL cations was also studied for the Novozym 435-catalyzed synthesis of isoamyl acetate by direct esterification at 50ºC. Fig. 6A

Table 1. Production of flavour ester by Novozym 435-catalyzed esterification between a aliphatic carboxylic acid (acetic, propionic, butyric or valeric) with a flavour alcohol (isoamyl alcohol, nerol, geraniol or citronellol) in 60% (w/w) [C_{16}tma][NTf_2] after 4 h reaction at 50ºC, using 40 mg immobilized enzyme and 80 mg molecular sieves per mmol of acid substrate, respectively. Assayed substrate amounts were 3 mmol flavour alcohol and 1 (A), 2 (B) or 3 (C) mmol aliphatic carboxylic acid, respectively.

Flavour Ester	Product concentration, g/mL (yield, %)		
	A	B	C
Isoamyl acetate	0.325 (97.0 ± 0.1)	0.572 (98.1 ± 1.7)	0.369 (47.7 ± 6.4)
Isoamyl propionate	0.356 (99.9 ± 0.1)	0.601 (99.9 ± 0.1)	0.738 (94.7 ± 3.7)
Isoamyl butyrate	0.374 (99.9 ± 0.1)	0.613 (99.1 ± 0.9)	0.739 (91.1 ± 6.4)
Isoamyl valerate	0.392 (99.9 ± 0.1)	0.626 (99.8 ± 0.2)	0.741 (86.6 ± 11.5)
Neryl acetate	0.305 (99.9 ± 0.1)	0.540 (96.6 ± 0.3)	0.687 (88.6 ± 2.2)
Neryl propionate	0.314 (98.7 ± 1.3)	0.543 (95.0 ± 0.1)	0.694 (89.2 ± 2.2)
Neryl butyrate	0.330 (99.9 ± 0.1)	0.556 (95.7 ± 0.3)	0.611 (78.4 ± 0.5)
Neryl valerate	0.335 (97.8 ± 0.4)	0.559 (94.5 ± 0.6)	0.650 (85.6 ± 9.3)
Geranyl acetate	0.324 (99.9 ± 0.1)	0.552 (93.4 ± 1.1)	0.702 (86.1 ± 6.8)
Geranyl propionate	0.337 (99.6 ± 0.4)	0.570 (94.6 ± 3.4)	0.680 (83.3 ± 5.8)
Geranyl butyrate	0.350 (99.9 ± 0.1)	0.599 (97.9 ± 0.3)	0.696 (85.4 ± 7.9)
Geranyl valerate	0.355 (97,9 ± 2.1	0.593 (95.5 ± 0.3)	0.757 (93.0 ± 5.3)
Citronellyl acetate	0.301 (99.9 ± 0.1)	0.546 (98.5 ± 1.3)	0.692 (90.1 ± 1.7)
Citronellyl propionate	0.315 (99.9 ± 0.1)	0.537 (94.8 ± 0.8)	0.741 (96.0 ± 2.1)
Citronellyl butyrate	0.327 (99.9 ± 0.1)	0.558 (96.9 ± 3.1)	0.722 (93.4 ± 4.6)
Citronellyl valerate	0.339 (99.9 ± 0.1)	0.570 (97.2 ± 1.5)	0.706 (91.0 ± 9.4)

depicts the resulting isoamyl acetate yields at 4h reaction in four different [N,N',N",N"'-alkyltrimethylammonium][NTf_2] ILs at 50% (w/w) IL concentration and at a 2/3 (mol/mol) acetic acid/isoamyl alcohol molar ratio. As can be seen, the synthetic product yield increased proportionally with the length of the alkyl chain of the cation (*i.e.* [C_{18}tma] > [C_{16}min] > [C_{14}mim] > [C_{12}mim], the best results (aprox. 100% isoamyl acetate yield with respect acetic acid content) being obtained for both the [C_{16}tma][NTf_2] (m.p. 64ºC) and [C_{18}tma][NTf_2] (m.p. 74ºC) cases. The [C_{16}tma][NTf_2] IL was used in further studies because of its lower melting point. Fig. 6B shows the influence of the acetic acid/isoamyl alcohol molar ratio on Novozym 435-catalyzed isoamyl acetate synthesis by direct esterification at 50, 60 and 70% (w/w) IL concentration and 50ºC. As can be seen, the esterification of the acetic acid substrate with isoamyl alcohol was practically 100% for both the 1/3 and 2/3 (mol/mol) molar ratios at all IL concentrations. As an example, the resulting 98.1% yield for the 2/3 (mol/mol) experiment at 60% IL concentration corresponds to a 0.572 g/mL concentration of isoamyl acetate, a value higher than those reported in the literature using direct esterification.[7b-e,9a-c,e] However, increasing the acetic acid concentration up to equimolar level with respect to the isoamyl alcohol resulted in a reduction in isoamyl acetate

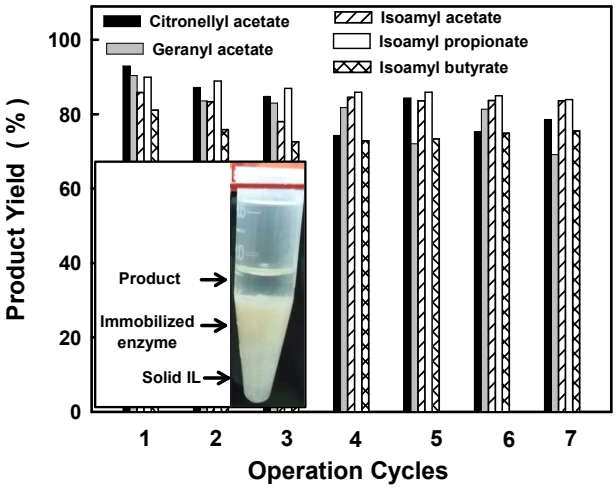

Fig. 7. Recycling protocol for Novozym 435-catalyzed flavour ester synthesis by direct esterification of acetic, propionic or butyric acids with citronellol, geraniol or isoamyl alcohol, respectively, in 60% (w/w) [C_{16}tma][NTf_2] at 50ºC. As an example, inset picture shows the phase behaviour of a reaction system after the cooling/centrifugation step carried out at the end of each catalytic cycle. See Experimental section for further details.

yield of up to 21% for the 50% (w/w) IL concentration. This was improved up to 75% isoamyl acetate yield by increasing the IL concentration to 70% (w/w).

The suitability of the proposed methodology for flavour ester production based on enzymatic synthesis by direct esterification followed by clean product separation using a cooling/centrifugation protocol, was tested for the production of sixteen different compounds, resulting from combinations of four aliphatic acids (acetic, propionic, butyric or valeric) and four flavour primary alcohols (*i.e.* isoamyl alcohol, nerol, geraniol or citronellol) (see Table 1). Enzymatic reactions were carried out at 1/3, 2/3 and 3/3 acid/alcohol molar ratios, respectively, and at 60% [C_{16}tma][NTf_2] concentration. As can be seen, the enzyme was able to synthesize all sixteen flavour esters, the product yield reaching levels higher than 90% (most close to 100%) in practically all cases, demonstrating the excellent potential of the proposed methodology. It should be emphasized that up to 0.757 g/mL concentration (*e.g.* geranyl valerate) was achieved. Furthermore, enzymatic reactions run like solvent-free systems, because nearly pure flavour products are obtained directly by the proposed cooling/centrifugation methodology. This clean approach is obviously more simpler and cheaper than those reported by other authors, such as, *e.g.* enzymatic synthesis in ILs and product separation by membrane technology,[7b-d] biocatalysis in supercritical fluids,[8] or IL/supercritical fluid biphasic systems,[18b, 20c,d] etc. Ohno and co-workers recently reported an interesting temperature-dependent phase change for tetrabutylphosphonium trifluoroacetate ([P_{4444}]CF_3COO)-type IL/water mixtures, which was successfully used to extract proteins, and suggested that an enzymatic reaction might be possible using this behaviour.[22]

Enzymatic cyclic production of flavour esters in switchable [C_{16}tma][NTf_2] solid/liquid phases

The key criteria for scaling-up any biocatalytic process for flavour ester production are the operational stability of the enzyme, and the recycling of the IL. Therefore, a cyclic laboratory protocol to reuse the biocatalyst/ [C_{16}tma][NTf_2] system for five different flavour esters was designed (see experimental section). In this approach, the phase behaviour of the flavour ester product (*e.g.* geranyl acetate) and [C_{16}tma][NTf_2] mixtures as a function of temperature was taken into account. These mixtures are homogeneous (monophasic systems) at 50ºC, but become solid below approx. 35ºC. Therefore, a cyclic protocol was established as follows: firstly the biocatalytic step was run using a 2/3 acid/alcohol molar ratio and a 60% (w/w) IL at 50ºC for 14 h, followed by cooling to room temperature (approx. 25ºC). Then, the reaction media were centrifuged four times (15 min, 14,000 rpm) at 25, 21, 10 and 4ºC, respectively, to achieve the full precipitation of the IL solid phase, thus permitting an easy separation of the flavour ester by decantation (see inset picture of Fig.7). The biocatalyst-IL solid system was then incubated under vacuum at 50ºC for 2 h to evaporate any remaining products and/or substrates, and a new cycle for flavour alkyl ester production was started by adding the corresponding substrate mixture. These reactions were carried out without MS13x to facilitate the release of remaining substrates/products during the vacuum step, thus avoiding any interference on the part of this adsorption agent, and reaction time was increased up to 14 h to compensate the absence of this dehydrating agent. As can be seen in Fig. 7, the flavour ester yield fall slightly as the operation cycles increases for all the assayed cases, *i.e.* citronellyl acetate, geranyl acetate, isoamyl acetate, isoamyl propionate and isoamyl butyrate, which may be related with a non-appropriate release of the water by-product content of the reaction media during the evaporation step. Furthermore, the attempted reduction in product yield, which could be related with the absence of MS13x, was compensated by the increase in reaction time with respect to previous experiments, reaching initial flavour ester yields higher than 80% practically all cases. Further studies to optimize the release of the water content from the biocatalyst/[C_{16}tma][NTf_2]/MS13x reaction mixture after each catalytic cycle could well improve products yields up to 100%.

The protective effect of hydrophobic ILs, based on [NTf_2] anion and alkyltrimethylammonium cations, have been reported to be related to the maintenance of the native structure of the protein.[18b,21b,c] The coating of immobilized enzyme particles with these hydrophobic ILs resulted in their excellent stabilization.[10] It is also noticeable that enzyme stabilization provided by [C_{16}tma][NTf_2] is largely better than that obtained by other enzyme stabilization approaches. For example, Karagoz *et al* reported the covalent immobilization of *R. miehei* lipase onto poly(glycidyl methacrylate-)-polystyrene diblock copolymers and application of the immobilized enzyme for isoamyl acetate synthesis by direct esterification in a solvent-free system, showing a 50% activity loss after 10 cycles reuse.[9g] In the same way, the application of adsorbed *C. rugosa* lipase onto Sepabeads® for geranyl butyrate synthesis in isooctane [5f], or the use of *S. simulans* lipase adsorbed onto Ca_2CO_3 for isoamyl acetate synthesis,[9b] by direct esterification also showed 40% residual activity after 8 cycles. However, when hydrophobic ILs (*e.g.* [Bmim][PF_6]) were used as reaction media for isoamyl acetate synthesis by enzyme-catalyzed direct esterification, excellent operational stability of the enzyme was observed. Further studies regarding the role of the alkyl side chain of [C_{16}tma][NTf_2] on the structure-function relationships of *C. antarctica* lipase B should be carried out.

Conclusions

This paper shows for the first time an easy and green method for efficiently separating a hydrophobic organic compound (*e.g.* flavours esters) from a homogeneous ionic liquid/organic compound mixture by simple cooling and centrifugation. The ability of hydrophobic ILs based on long alkyl side chain in cations (*e.g.* [C_{16}tma][NTf_2]) to melt at temperatures compatible with enzyme catalysis (*e.g.* lower than 80ºC) permitted development of a two-step protocol for flavour ester production: *i*) lipase-catalyzed direct esterification between an aliphatic acid and a flavour alcohol with product yield close to 100%, and *ii*) clean separation of the reaction product by a cooling/centrifugation method. 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 presence of a dehydrating agent (*e.g.* MS13x) in the reaction media was important for the maximum level of conversion to be reached. It should be emphasized that since substrates obtained from "natural" sources (*e.g.* acetic acid from fermentation cultures, and geraniol from plants) are used, the resulting ester product can also be considered as "natural".

The unique properties of hydrophobic ILs, based on cations with long alkyls side chains (*e.g.* [C_{16}tma][NTf_2]), to carry out combined biotransformations and clean separation steps could open up new opportunities to develop green industrial processes. Further studies on the switching from solid to liquid phases of organic compound/IL mixtures should be carried out.

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