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Deep Eutectic Solvents as Suitable Solvents for Lipase-Catalyzed Transesterification Reactions

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In this work, three deep eutectic mixtures (DES 1: choline chloride/urea; DES 2: choline chloride/glycerol; and DES 3: tetrabutylammonium bromide/imidazole) were investigated as mediums for the synthesis of glucose laurate and glucose acetate. Aiming to achieve a greener and more sustainable approach, the synthesis reactions were catalyzed by lipases from *Aspergillus oryzae* (LAO), *Candida rugosa* (LCR), and porcine pancreas (LPP). The hydrolytic activity of lipases against *p*-nitrophenyl hexanoate revealed no evidence of enzyme inactivation when DES were used as medium. Regarding the transesterification reactions, combining LAO or LCR with DES 3 resulted in the efficient production of glucose laurate (from glucose and vinyl laurate) (conversion >60%). The best result for LPP was observed in DES 2, with 98% of product production after 24 hours of reaction. When replacing vinyl laurate by a smaller hydrophilic substrate, vinyl acetate, a distinct behavior was observed. LCR and LPP performed better in DES 1, yielding more than 80% of glucose acetate after 48 hours of reaction. The catalytic activity of LAO was less pronounced, reaching only nearly 40% of product in DES 3. The results highlight the potential of combining biocatalysis with greener and environmentally-safer solvents, for the synthesis of differentiated chain-length sugar fatty acid esters (SFAE).

Introduction

Sugar fatty acid esters (SFAEs) are nonionic surfactants with broad applications in the cosmetic, food and pharmaceutical industries.[1] They are derived from renewable resources and own desirable properties such as nontoxicity, no irritability, tastelessness, odorless, biocompatibility, and biodegradability.[2]

At the commercial level, they are still synthesized chemically, however, recent research has shown that lipases can be used for the synthesis increasing productivity and selectivity. Apart from the regio-selectivity issues, the opposing polarity of the substrates (sugar and fatty acid/ester) requires appropriate solvent selection. Lipases are enzymes with high regio-, stereo- and enantioselectivity, able to catalyze a large number of different reactions with differentiated substrates.[3] However, their combination with an organic solvent may result in partial or complete inactivation. To address these issues, ionic liquids (ILs) and deep eutectic solvents have been investigated in recent years as organic solvent alternatives. As compared to ILs, DES have the benefit of being non-volatile, non-flammable, biodegradable, and slightly or non-toxic to the environment.[4]

The combination of lipases with deep eutectic solvents results in a truly green and environmentally friendly methodology. Nevertheless, only scarce research on SFAE synthesis under these conditions has been published. Pöhnlein and co-workers were pioneers in the biosynthesis of SFAE using deep eutectic solvents. These authors reported the synthesis of glucose-6-*O*-hexanoate in different DES mixtures, using Novozyme 435 as catalyst. Choline chloride/urea and choline chloride/glucose mixtures, showed the best performance as solvents for the transesterification reactions.[5]

More recently, Hollenbach et al., used an hydrophobic DES, composed of (−)-menthol and decanoic acid, for the synthesis of glucose monodecanoate catalyzed by immobilized CALB (Novozyme 435). The authors observed that the polarity of the solvent is crucial for the synthesis productivity.[6] Siebenhaller and co-workers prepared sugar-choline chloride deep eutectic solvents, where the saccharide from the solvent was also used as substrate. In this study, up to nine vinyl and methyl esters substances were investigated. While employing vinyl substrates, higher efficiency was obtained.[7] Hollenbach et al., used the same catalyst for the synthesis of 6-decanoyl-D-glucose with choline chloride/urea or glycerol DES. The reactions were carried out using an ultrasonic pre-treatment to enhance mass transfer and higher initial reaction velocities. A 4-fold increase was achieved compared to the conventional agitation approach.[4]

Besides the scarce data, studies have been restricted to the use of Novozyme 435 as catalyst. The potential for other lipases SFAEs synthesis has never been investigated.

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The effect of three DES (choline chloride/urea, choline chloride/glycerol, and tetrabutylammonium bromide/imidazole) on the transesterification activity of three lipases was investigated here: *Aspergillus oryzae*, *Candida rugosa*, and porcine pancreas. The usage of multiple enzymes (free forms) and comparing their efficacy amongst three distinct DES is, for the first time, explored here. Furthermore, DES composed of tetrabutylammonium bromide/imidazole, was never explored for transesterification purposes. Two vinyl substrates were also investigated to determine the influence of chain length (vinyl acetate and vinyl laurate) on the synthesis catalysis. The eutectic mixtures were characterized by ^{1}H NMR, FTIR, density, viscosity, refractive index and polarity. The transesterification reactions were followed by HPLC-RI (RI = refractive index) chromatography.

Results and Discussion

Production and characterization of DES

The search for green alternatives to organic solvents has intensified in recent years, owing mostly to environmental concerns. Deep eutectic solvents have attracted growing interest as alternatives. Herein, three DES were produced namely choline chloride/urea (DES 1), choline chloride/glycerol (DES 2) and tetrabutylammonium bromide/imidazole (DES 3). All DES are classified as type III, regarding that a quaternary ammonium salt (choline chloride and tetrabutylammonium bromide) is combined with a hydrogen bond donor, HBD (urea, glycerol, or imidazole).[8]

After production, the most important physicochemical properties of the produced DES were measured, as represented in Table 1.

All data obtained are in accordance with the literature data.[9–11] Among the different properties, all DES showed similar values. DES 1, composed of choline chloride and urea, demonstrated the highest values of density and viscosity, which may render poor enzyme mobility. One of the key constraints of DES in contrast to typical organic solvents is their high degree of viscosity. High-viscosity solvents are more difficult to handle, making industrial scale-up problematic. However, high-viscosity DES solvents have certain benefits. They may provide improved selectivity, higher mass transfer, and a more regulated response.[12]

Even though molecular sieves were added to the DES prior to usage, a residual water amount (≈2–3 %) is retained due to their high hygroscopic character (Table 1). Previous studies have demonstrated that slight hydration of choline chloride/urea DES, up to 5 %, leads to the highest level of lipase activity, which implies interfacial activation.[13] Moreover, other studies showed that the presence of small amounts of water has no effect on the enzyme's esterification/transesterification activity.[14,15]

FTIR analysis provides valuable insights into the chemical structure of the DES produced. All spectra revealed the expected profile (Figure 1). In DES 1 and 2, composed of choline chloride and urea/glycerol, between 3100–3600 cm^{-1} a broad band is depicted, corresponding to the –OH and –NH

Table 1. Water content and physico-chemical characterization of the produced DES, namely density (ρ), viscosity (η at 25 °C), refractive index (n_D) and polarity (E_{NR}).

	Water content (*w*/*w* %)	Density (ρ)	Viscosity (η, mPa s)	Refractive index (n_D)	Polarity E_{NR} kcal.mol^{-1} (λ nm)
DES 1	2.86	1.23 g mL^{-1}	1319	1.51	50.336 (568)
DES 2	1.75	1.21 g mL^{-1}	369	1.49	50.072 (571)
DES 3	2.33	1.10 g mL^{-1}	661	1.50	51.055 (560)

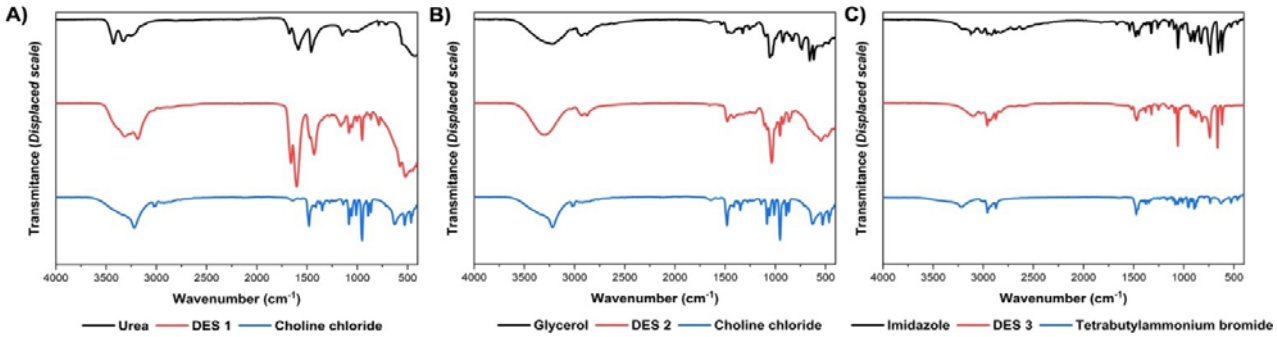


Figure 1. FTIR spectra of the produced deep eutectic solvents. A) DES 1 and their starting compounds choline chloride and urea; B) DES 2 and their starting compounds choline chloride and glycerol; C) DES 3 and their starting compounds tetrabutylammonium bromide and imidazole.

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stretching vibrations of the HBD superimposed with the –OH of choline chloride. In this area, only a small broad band at 3083 cm^{-1} is observed for DES 3, corresponding to the –NH of the imidazolium ring. The -CH of the aliphatic chains of tetrabutylammonium bromide are observed between 2771–3015 cm^{-1}. The carbonyl group (C=O) of urea on DES 1 is observed at 1654 cm^{-1}. Compared with their starting compounds, all spectra showed small deviations, proving hydrogen bond interactions upon DES formation. FTIR spectra of choline chloride-based DES 1 and 2, are in accordance with the ones reported.[16,17]

^{1}H NMR spectra of the DES showed the expected profile with no signs of impurities or side products formed through the heating process (Figure 2). The choline chloride moiety is represented by 4 different signals: a singlet at δ_H 3.16 ppm, a triplet at δ_H 3.44 ppm, a multiplet at δ_H 3.80 and a broad singlet at δ_H 5.74 ppm, corresponding to the protons d, c, b and a, respectively. ^{1}H spectrum of urea is represented by only one signal, a singlet at δ_H 5.65 ppm, resulting from the NH_2 protons (Figure 2A). Glycerol spectrum (Figure 2B) depicts the OH signals at δ_H 4.37 ppm as a triplet (protons a) and at δ_H 4.45 ppm as a doublet (proton d). Aliphatic protons b and c are detected between δ_H 3.24–3.45 ppm as multiplets.

After DES 1 formation, a chemical shift of δ_H 0.3 ppm was observed for the mobile protons (δ_H 5.5 ppm), with also small deviations for all the other signals. The same behavior was observed for DES 2 (Figure 2B), evidencing strong chemical interactions. Previous studies conducted by Delso and co-workers showed a similar NMR profile for both DES. However, the authors studied the impact of water in the DES mixtures, which resulted in the signals moving to greater chemical shift values.[18] Due to the low water content of our DES (< 3 % *w/w*), the NMR spectra revealed more resolved signals with predicted multiplicities.

^{1}H spectrum of tetrabutylammonium bromide (Figure 2C) shows 4 signals related to the butylene chains. The CH_3 are observed at δ_H 0.89 ppm as a triplet, followed by the CH_2: a sextet at δ_H 1.28 ppm (b), a quintet at δ_H 1.56 ppm (c), and finally, a multiplet at δ_H 3.21 ppm assigned to the CH_2 near the nitrogen (a). The aromatic protons of imidazole are observed as singlets at δ_H 7.05 ppm (protons b) and at δ_H 7.70 ppm (proton a). The NH can be observed at δ_H 12.14 ppm (data not shown). After DES 3 formation, only slight chemical shifts can be depicted compared with DES 1 and 2. This is due to the presence of fewer mobile protons present in the starting compounds (only one NH in imidazole). Nevertheless, all these deviations revealed the formation of hydrogen bonds between the DES components.

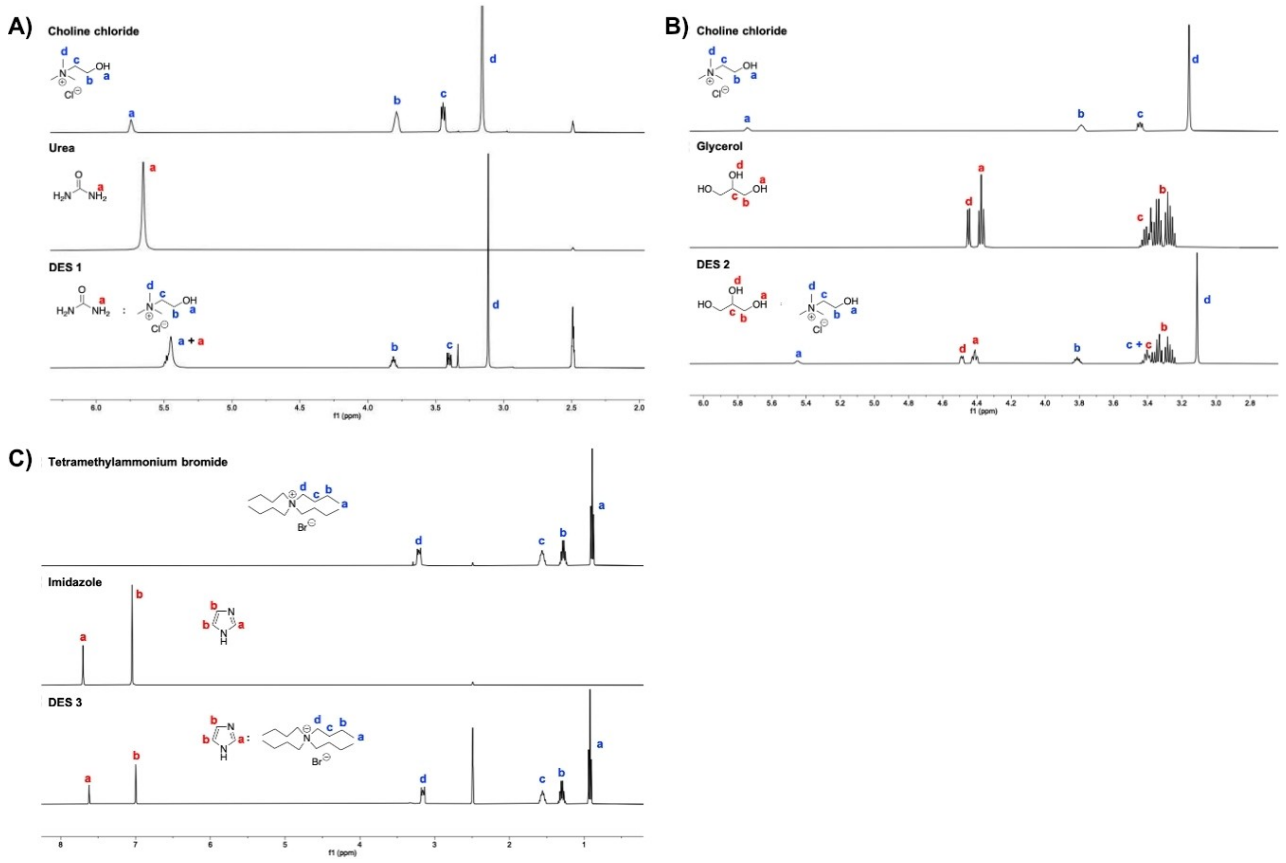


Figure 2. ^{1}H NMR of the individual components and after DES formation (DMSO-d_6, 400 MHz): A) choline chloride, urea and DES 1; B) choline chloride, glycerol and DES 2; C) imidazole, tetramethylammonium bromide and DES 3.

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Effect of DES on lipases' hydrolytic activity

Temperature, pH, salts, and, most significantly, the solvent, can all have an impact on enzyme catalytic efficiency.[19] Lipases are known to own high stability in ideal conditions of pH (6–8) and temperature (25–37 °C).[20,21] As a result, before starting with the transesterification reactions, it is critical to assess the influence of the produced deep eutectic solvents on the hydrolytic activity of the enzymes. Figure 3 compiles the results of activity obtained for all lipases (lipase from *Aspergillus oryzae*, LAO; lipase from *Candida rugosa*, LCR; lipase from porcine pancreas, LPP) when incubated from 1 h until 72 h in the presence of DES. The enzyme activity was evaluated using *p*-nitrophenyl hexanoate as substrate (Figure 3A).

From all lipases tested, we may observe that the pancreatic enzyme (LPP) showed the lowest hydrolytic performance (Figure 3B). This lipase displayed its highest activity after 1 h of incubation on DES 3, being 57% more active than the control (only buffer). After 24 h, the activity is similar to the one in the aqueous medium, retaining 76% of its initial activity after 72 h. In DES 1 and 2, LPP showed good activity after 1 h of incubation, however, in both solvents, after 24 h, the activity decreases abruptly. For LPP, we may conclude that choline-based eutectic mixtures have a more deactivating effect.

Lipase from *Aspergillus oryzae* (LAO), exhibited the most consistent activity profile in the presence of the three DES tested. In DES 1 and DES 2, after 1 h, LAO remained with similar hydrolytic activity than in the buffer. After 72 h of incubation, its activity is higher than 50% compared with its initial performance. DES 3 showed the highest deactivating effect, however, the lipase retained 46% of its activity after 72 hours.

Lipase from *Candida rugosa* (LCR) demonstrated higher activity when incubated in DES 1 and DES 3 after 1 h of incubation, compared with the aqueous medium. The same tendency was observed for DES 1, until 24 h. After 24 h, in all DES, the activity remains at least 50% of its initial activity, compared with the buffer. In DES 2, LCR demonstrated its lowest performance.

Yet, none of the generated DES demonstrated an imminent deactivation of the lipases, with similar or greater activity found after 1 hour with respect to the values obtained initially under native conditions (0 h in buffer). Ribeiro et al., showed that choline chloride-based-eutectic solvents are appropriate reactional media for lipases, with the enzymes' activity preserved or even increased.[22] So far as we know, the composition of DES 3 has never been investigated for this purpose.

Transesterification activity

The transesterification of glucose with vinyl laurate is one of the most studied enzymatic reactions, being mainly explored in organic solvents, such as 2-methyl-2-butanol, dimethylsulfoxide, dimethylformamide and pyridine.[2] However, when combined, most of these solvents may inactivate lipases for SFAE synthesis. In order to mitigate these problems, alternatives, such as ionic liquids, and deep eutectic solvents have been explored in the past few years.[2,4–7,23] Moreover, apart from being considered eco-friendly substitutes for organic solvents, these greener solvents improved the solubility of the substrate, and in some cases enhanced the lipases' performance. Notwithstanding the benefits, just a few research on their usage have been reported in the literature, and they are all almost limited to the same enzyme, Novozyme 435.

Our first approach used vinyl laurate (Figure 4), one of the most explored substrates so far for glucose transesterification.

In addition to prior research indicating improved productivity when utilizing vinyl substrates instead of methyl esters, these compounds generate ethenol/acetaldehyde as a by-product, which is readily removed from the reactional media

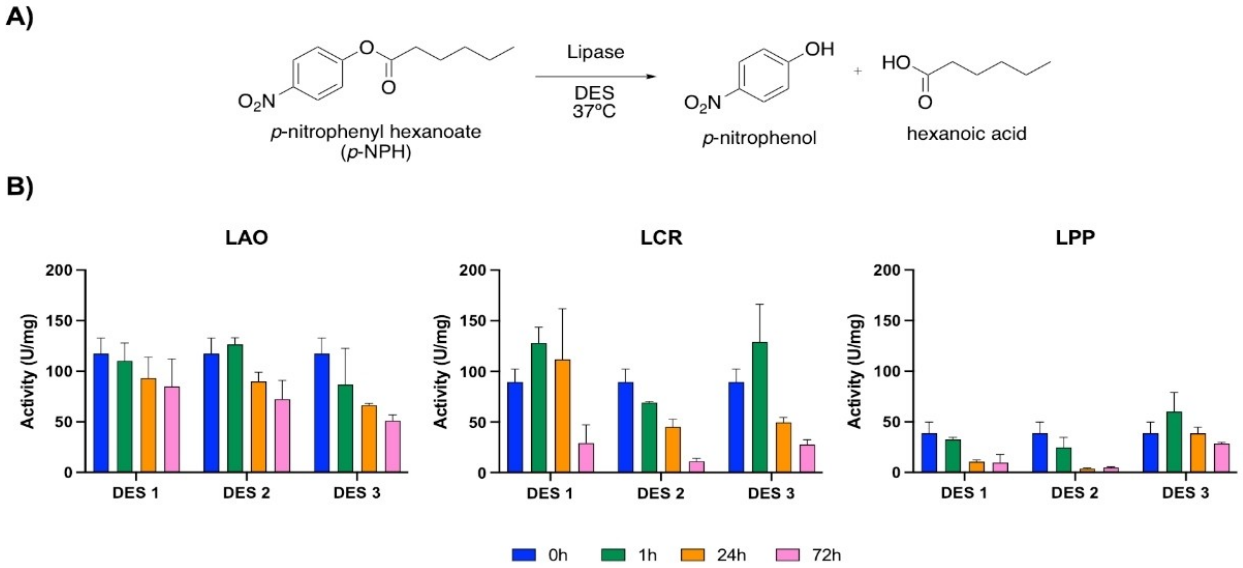


Figure 3. A) Proposed scheme for the hydrolysis of *p*-nitrophenyl hexanoate with formation of *p*-nitrophenol and hexanoic acid; B) Hydrolytic activity of lipase from *Aspergillus oryzae* (LAO); lipase from *Candida rugosa* (LCR) and lipase from *porcine pancreas* (LPP) after incubation in the presence of DES 1, 2 and 3.

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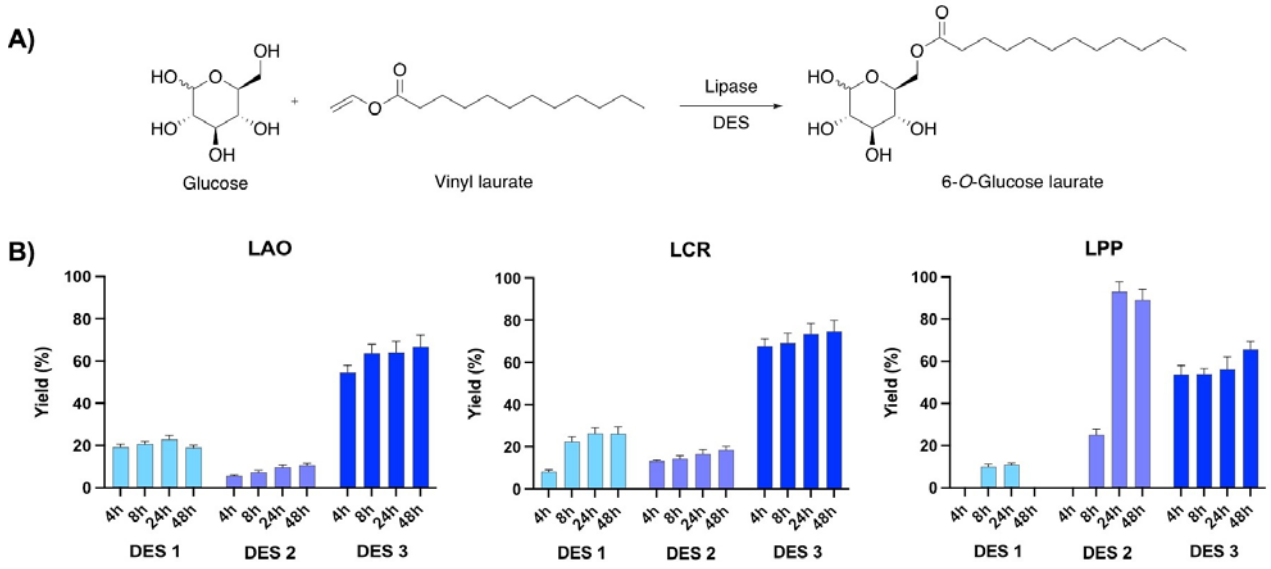


Figure 4. A) Proposed scheme for the reaction between glucose and vinyl laurate, to obtain glucose laurate; B) Yield of glucose laurate (%) obtained using lipase from *Aspergillus oryzae* (LAO), lipase from *Candida rugosa* (LCR) and lipase from porcine pancreas (LPP), respectively.

due to its low boiling point (20 °C). Using vinyl substrates, the reaction equilibrium is pulled towards the products, where the residual amount of water present in the DES may not influence the reactional outcome.

From Figure 4, we may observe that DES 3 induced the highest improvement of activity for lipases LAO and LCR. The data revealed conversions higher than 50% after 4 h of reaction, reaching up to 80% after 48 h, in both cases. The same tendency was observed for LPP in DES 3, however, higher conversions were achieved in DES 2. After 24 h, LPP was able to produce the desired product, with conversion near 98% in this medium.

In all cases, DES 1 showed the lowest productivity.

According to the literature, DES medium not only impacts enzyme stability but also has a significant role in reaction rate owing to DES-substrate interaction.[24] As a result, the data cannot be justified just by one element, but rather by a combination of numerous criteria, such as DES viscosity and polarity, substrate hydrophobicity, DES-enzyme or DES-substrate interaction. Lin and co-workers observed an higher activity of *Aspergillus oryzae* lipase using organic solvent as medium, compared to immobilized forms such as Novozym 435 and Lipozyme TLIM for the synthesis of glucose laurate.[1] The data obtained revealed the outstanding potential of DES 3 (composed of imidazole/tetrabutylammonium bromides) for SFAE synthesis with lipase-catalysis. Moreover, although LPP demonstrated limited hydrolytic activity in DES medium (Figure 3B), a distinct result is found here. This lipase is very active for the production of glucose laurate in two distinct reactional media (DES 2 and 3), with the greatest conversion in DES 2 among all reactions performed. For esterification processes, the pancreatic enzyme is widely used.[25,26] Given that this lipase is only partially soluble in aqueous environments, the eutectic solvent's improvement of its solubility may be responsible for the excellent results observed. Previous studies, combining Novozyme 435 with ionic liquids or organic solvents for the synthesis of glucose laurate, have demonstrated conversions of up to 40%.[2] Our findings highlight the potential of these lipases in the production of glucose laurate using greener and safer solvents.

In order to study the effect of the chain length of the vinyl substrates used, the transesterification reactions were also conducted using a smaller compound, vinyl acetate (Figure 5). From the data obtained, we may observe that, overall, the lipases demonstrated lower activity compared to studies with vinyl laurate. These findings are not surprising given the lower hydrophobicity of vinyl acetate, which consequently induces reduced interfacial activation. Moreover, Lee et al., reported that the affinity of lipases for substrates dextrin and acyl donor (vinyl esters) is proportional to the increase of longer-side chain acyl compounds.[27]

Apart from the low reactional outcomes obtained, LCR and LPP combined with DES 1 led to the highest conversion rates after 48 h of reaction. Conversions up to 98% were achieved for glucose acetate. The reactions, as predicted, have a delayed initial kinetic profile, taking more than 24 hours to attain significant conversions. It is critical to emphasize that an accurate choice of eutectic solvent is required for greater reactional yields. Literature data showed that LPP and LCR were unable to acylate Andrographolide in acetone, using vinyl acetate or vinyl laurate.[28] Taking this into account, while looking for optimal enzymatic performance, not only the substrate but also the solvent medium must be considered. The reactions performed on DES 2 and DES 3 generated a low amount of the predicted product, up to 40%.

The two substrates used were chosen regarding their opposite hydrophobicity. Vinyl laurate is highly hydrophobic and has been the subject of extensive studies for SFAE biosynthesis. Scarce data on vinyl acetate is found, mostly

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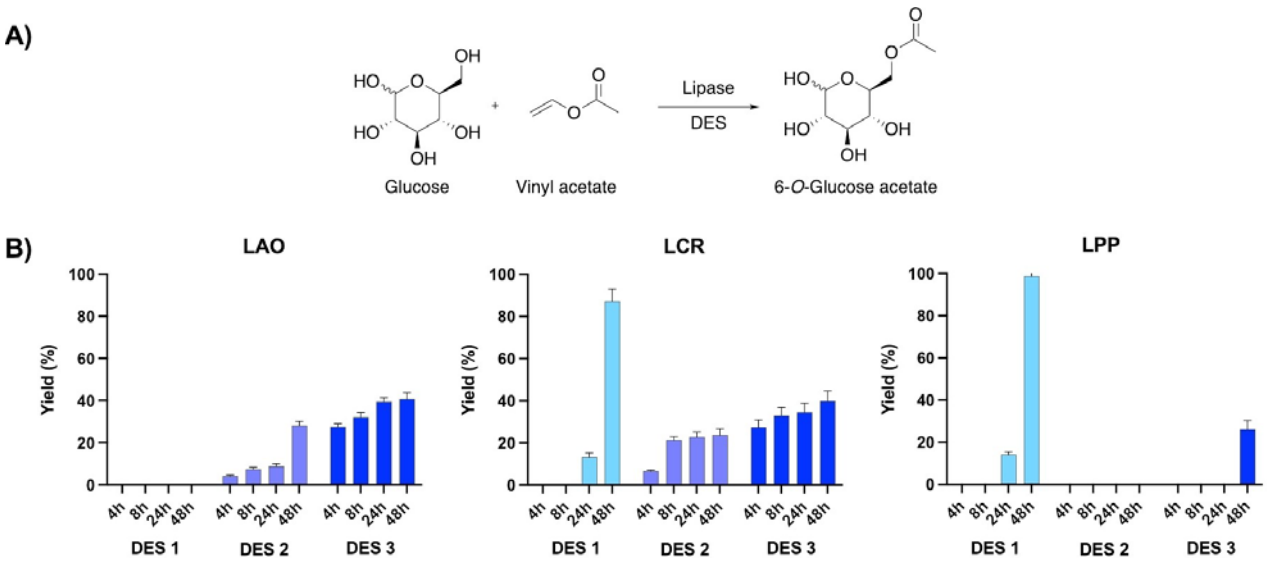


Figure 5. A) Proposed scheme for the reaction between glucose and vinyl acetate, to obtain glucose acetate; B) Yield of glucose acetate (%) obtained using lipase from *Aspergillus oryzae* (LAO), lipase from *Candida rugosa* (LCR) and lipase from porcine pancreas (LPP), respectively.

related to their higher hydrophilic character, consequently enabling lipases' interfacial activation.

As previously stated, apart from several studies demonstrating that DES are suitable mediums for lipase-catalyzed reactions, an accurate choice of DES is crucial. Molecular dynamics simulations have shown that choline chloride and urea form hydrogen bonds with lipases' superficial residues, leading to greater enzyme stability.[29]

These findings may justify the different behavior of the lipases here studied in the same DES, for both types of reactions studied: hydrolysis and transesterification. Interestingly, the simulations performed by Kovács and co-workers demonstrated that different results regarding reaction rates are related to solvent-substrates interactions, rather than enzyme-DES.[24] These findings may justify the results obtained for LPP and LCR when using vinyl acetate and DES 1, in which the reaction rate may be strongly affected by solvent-substrate interaction, besides with other criteria, such as slower interfacial activation.

Taking all of these assertions together, we can infer that numerous parameters impacted the lipase-catalyzed processes (whether for hydrolysis or transesterification) in DES medium: i) the lipase's stability in the DES (enzyme-DES interaction); ii) the solubility and interaction of the substrates with the DES; and iii) the whole system must match the favorable conditions for interfacial activation.

Conclusions

In this work, it was demonstrated the crucial importance of an accurate choice of solvent to perform enzymatic-transesterification reactions of sugar fatty acid esters (SFAE). Deep eutectic solvents were shown to play a crucial role in the selectivity and the activity of the enzymes.

Choline chloride/urea deep eutectic solvent is the most commonly explored mixture in literature, however, here we demonstrated that other mixtures, such as tetrabutylammonium bromide/imidazole have higher potential as solvents for transesterification reactions. The work here reported, showed the high efficacy of free lipases combined with DES as reaction medium, paving the way to a greener, more efficient, economical, and sustainable process.

Supporting Information

The authors have included additional figures within the Supporting Information.

Experimental Section

Materials and methods

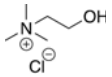
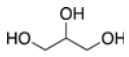
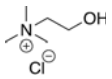
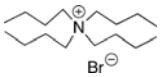
The reagents choline chloride, tetrabutylammonium bromide, imidazole, glycerol and vinyl laurate were purchased from TCI Chemicals. Glucose, vinyl acetate, pyridine, lipase from *Aspergillus oryzae* ($>100\ kU\ g^{-1}$), lipase from *Candida rugosa* ($>700\ U\ mg^{-1}$) and lipase from porcine pancreas ($46\ U\ mg^{-1}$) were acquired from Sigma-Aldrich. Choline chloride was freeze-dried 48 h prior to use. Lipase from *Aspergillus oryzae* was purified with ultracel 10 kDa ultrafiltration discs composed of regenerated cellulose (47 mm, Millipore) against ultrapure water (Milli-Q), and then freeze-dried prior to usage.

Production of deep eutectic solvents (DES)

Deep eutectic solvents were produced by mixing both components, followed by heating (80 °C) until a clear liquid solution was formed. The stoichiometric ratio used for each mixture is present in Table 2.

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Table 2. Components and molar ratio of the prepared DES.

DES	Components (HBD/HBA[a])		Molar ratio
1	 Urea	 Choline chloride	2:1
2	 Glycerol	 Choline chloride	2:1
3	 Imidazole	 Tetrabutylammonium bromide	2:1

[a] HBA: hydrogen bond acceptor.

Prior to use, molecular sieves (4 Å, Sigma-Aldrich) were added to the DES and left for at least 48 h.

Characterization of produced DES

All DES produced were characterized by ^{1}H NMR, FTIR, density, viscosity, refractive index and polarity. ^{1}H NMR (400 MHz) was performed by dissolving the products in deuterated dimethyl sulfoxide (DMSO-d_6, Cortecnet, France), and the samples were analyzed in a Bruker Advance III.

Infrared spectra were recorded on an FTIR Platinum-ATR Bruker Alpha II. The samples were analyzed over the range 400–4000 cm^{-1}, with a spectral resolution of 4 cm^{-1}. All spectra were an average of over 24 scans.

Polarity, refractive index, and density were evaluated following the procedure described by Freitas and co-workers.[30]

Water content was evaluated through ^{1}H NMR, using the methodology previously reported.[31] Briefly, 10 mg of the corresponding DES was dissolved in 1 mL of DMSO-d_6. 0.5 μL of high-purity pyridine (>99.5%) was added, and the spectra were acquired (Figure S1–S3). A blank spectrum for the quantification of the water content of the deuteride solvent was also performed.

Hydrolytic activity

The lipases (1 mg) were incubated with 100 μL of the corresponding DES (1, 2 or 3) at 37 °C for different periods of time (1 h, 24 h and 72 h). Then, 900 μL of phosphate buffer (50 mM, pH 7.8) was added and vortexed. The hydrolytic activity was measured as reported,[21,32] using *p*-nitrophenyl hexanoate as substrate.

Transesterification reactions

For each DES (1, 2 and 3), a stock solution of glucose (10 mg mL^{-1}) was first prepared. Then, 2 mL of the corresponding solution was added to a flask, followed by the addition of the vinyl substrate (1 equiv.). Afterward, 20 mg of lipase was added, and the flasks were placed at 37 °C under stirring. At different time points, aliquots of 100 μL were withdrawn and frozen until analysis by HPLC-RI.

HPLC-RI analysis

High-performance liquid chromatography analysis was performed using an HPLC Shimadzu-LC 2060 C equipped with a refractive index detector (Shimadzu RID-20A) and a 300×7.8 mm, Aminex HPX-87H column (Bio-Rad Laboratories, Inc. USA). A 10 μL sample was injected, and an aqueous solution of H_2SO_4 (5 mM) was employed as the mobile phase with a flow rate of 0.6 mL min^{-1}, operated at 60 °C. All samples (final concentration of 1 mg mL^{-1} in the mobile phase) were filtrated prior to injection using 0.2 μm Nylon filters. The yield of the product was quantified through the reduction of glucose area. Some chromatograms can be found in the Supporting Information (Figure S4–S9).

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: transesterification · lipase · deep eutectic solvent · biocatalysis · glucose esters

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