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Research Article

Deep Eutectic Solvents for the Enzymatic Synthesis of Sugar Esters: A Generalizable Strategy?

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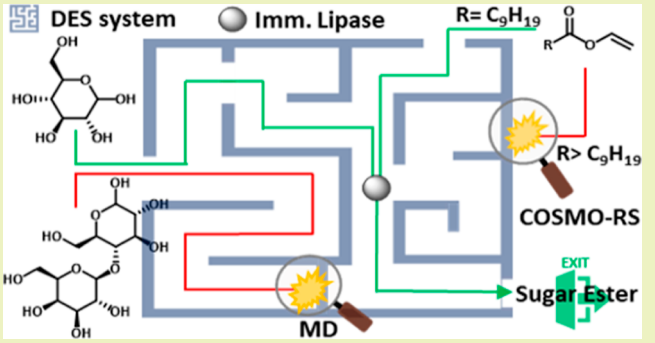
ABSTRACT: Sugar (fatty acid) esters are industrially relevant compounds, with a cumbersome production process due to the solubility issues of the substrates, which forces the use of environmentally unfriendly reaction media. Herein, deep eutectic solvents (DESs) are considered as a promising solution: several literature examples use glucose and different acyl donors to illustrate the efficient synthesis of sugar esters in classic DESs like choline chloride/urea (ChCl/U). However, this paper discloses that when sugars like lactose or other disaccharides are used, enzymes cannot efficiently perform (trans)esterifications in DESs, while the same reaction can proceed in mixtures like pyridine/tetrahydrofuran (Py/THF). This could be explained by computational solubility studies and molecular dynamics simulations of both reaction media, showing two effects: (i) on the one hand, large acyl donors (more than C10) display poor solubility in DESs and (ii) on the other hand, disaccharides interact with DES components. Thus, the DES affects the conformation of lactose (compared to the conformation observed in the Py/THF mixture), in such a way that the enzymatic reaction results impaired. Despite that classic DESs (e.g., ChCl/U) may not be useful for generalizing their use in saccharide ester syntheses, the achieved theoretical understanding of the reaction may enable the design of future DESs that can combine enzyme compatibility with eco-friendliness and efficiency in sugar chemistry.



KEYWORDS: *deep eutectic solvents, lactose esters, molecular dynamics simulation, (trans)esterification, COSMO-RS*

INTRODUCTION

Surfactants are amphiphilic compounds that partition between liquid interphases reducing surface tension. Because of this, they constitute a widely used class of chemicals across many sectors and markets.[1,2] In particular, sugar fatty acid esters (SFAEs or just "sugar esters") are non-ionic surfactants with excellent emulsifying, stabilizing, and detergent properties. Furthermore, SFAEs are non-toxic, benign to the environment, tasteless, odorless, and fully biodegradable. On the other hand, they exhibit antimicrobial activity and are skin-compatible.[3] Interestingly, these molecules, as well as other carbohydrate-based surfactants like alkyl (poly)glycosides, can be derived from renewable sources.[4,5] The chemical synthesis of SFAEs typically requires harsh reaction conditions (high temperatures, reduced pressure, and metal or alkaline catalysts) which result in high energy consumption, formation of undesirable byproducts, and low regioselectivity.[6,7] Furthermore, volatile organic solvents are frequently used even though sugar solubility is limited.[8−12] In this context, using other non-conventional (benign) media and an enzyme-based synthesis are strategies that can overcome the above-mentioned drawbacks[13] (see Scheme 1).

In this area, glucose and saccharose esters have been widely studied,[3,14,15] but research on other important sugars has been scarce hitherto. As a relevant example, the disaccharide lactose has been underinvestigated despite its large availability, being the most abundant component of cheese whey, the main waste stream of the dairy industry. This feedstock can be used as a cheap and renewable source for lactose to develop circular processes directed toward the obtainment of high-value compounds, including SFAEs.[16−19] The same may apply for many other examples of saccharides, which can be obtained from renewable resources.

The major issue in the preparation of sugar esters is the opposite solubility profile of the polar head (sugar) and the hydrophobic tail (fatty acid).[20−22] Therefore, SFAEs have been previously obtained in organic solvents, such as dimethylsulf-

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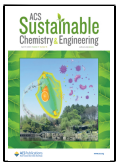
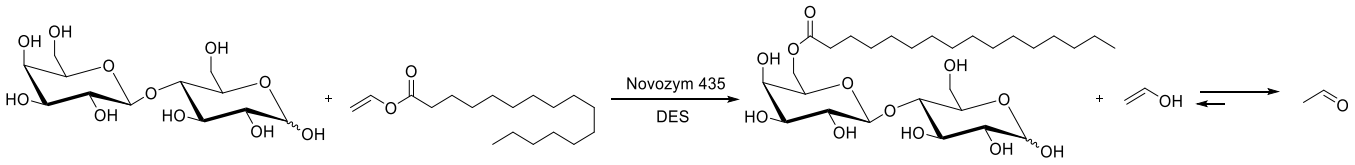


Scheme 1. General Scheme of Transesterification of Lactose with Vinyl Palmitate (VP) as an Acyl Donor



oxide (DMSO), dimethylformamide (DMF), and pyridine (Py), in which both sugars and fats are soluble.[6,23,24] These processes are efficient, but the use of toxic and non-volatile organic solvents does not match at all with the green chemistry principles.[25] The use of other, more benign, organic solvents like tertiary alcohols or ketones has been reported as well, but it generally results in low yields and/or very long reaction times due to the very low solubility of sugars (especially disaccharides as lactose) in these solvents.[11,12,26] Sugar derivatizations can be performed to enhance carbohydrate solubility in organic media (e.g., for lactose oleate[16]), yet at the cost of establishing multi-step and cumbersome processes. In this context, the use of non-conventional media like ionic liquids and deep eutectic solvents (DESs) has been proposed as a promising alternative to organic solvents.[27−29]

DESs are straightforwardly prepared by mixing hydrogen bond donors (HBDs), such as polyols, with hydrogen bond acceptors (HBAs), such as ammonium salts.[30] Because of the formation of intermolecular hydrogen bonds, the melting point of the mixture decreases sharply, and DESs are liquid and stable at room temperature. Owing to the vast variety of possible HBDs and HBAs, the possibility of changing the components' ratio, and the amount of external water added, the potential number of combinations is immense, which confers them a high degree of tunability.[31] Moreover, it is possible to appropriately select the components to prepare non-toxic and highly biodegradable DESs.[32,33] These solvents have nowadays a wide range of applications ranging from solvents for reactions, extraction, gas capture and separation, and (bio)catalysis.[34−36] The remaining challenges for DESs are mainly associated with their high viscosity, which affects the mass transfer in the reaction and adds complexity to the downstream processing. Moreover, a deep understanding about the relationship between the structure of its constituents and the solvent properties of DESs is still in its infancy.[37] For instance, it is hard to predict how DESs interact with substrates and with the tertiary structure of the enzyme. We have recently shown that the use of computational simulation tools can shed light on the complex interactions that may occur during the process.[38,39]

Several articles disclose the use of glucose and several acyl donors for enzymatic transesterifications in DESs. Pöhnlein et al.[40] reported the transesterification of glucose with vinyl hexanoate using immobilized CalB (Novozym 435) in different DESs. The product was detected when ChCl/U and ChCl/glucose DESs were used. More recently,[27] the same two hydrophilic DESs were reported for the transesterification of glucose with vinyl decanoate. The same group also explored, for the first time, the use of a hydrophobic DES (menthol/decanoic acid) as a suitable reaction medium for sugar ester production.[28] The use of fatty acid methyl esters (FAMEs) derived from single-cell oil as an acyl donor for the production of glucose fatty acid esters was also reported in a circular, integrated process fully based on lignocellulose.[4] The aforementioned examples show that, as a matter of fact, these emerging reaction media can be used for these processes. Given the industrial relevance of other SFAEs, the possibility of using DESs for disaccharides was considered in this work. To understand the system, experimental results were combined with molecular dynamics (MD) simulations that can assess the interactions between the enzyme and the reaction media. As a result, a new approach is presented in this article, which can assist in establishing structure−reactivity relationships between DESs, substrates, and enzymes, thus driving the selection and/or the design of novel benign solvents for sugar chemistry and, particularly, for biocatalytic applications.

MATERIALS AND METHODS

Materials. The immobilized *Candida antarctica* lipase B under the trade name of Novozym 435 was kindly supplied by Novozymes A/S (Denmark), vinyl palmitate (VP) was purchased from TCI Europe N.V. (Belgium), and anhydrous lactose was purchased from Fluka (Italy). All other reagents and chemicals were purchased from Merck Life Science and used as received.

Preparation of DESs. All DESs used in this work were prepared by mixing the components under magnetic stirring at 80−95 °C in a thermostat vessel until a clear liquid was obtained. In some cases, the addition of water was necessary to form the DESs or to obtain less viscous liquids.

Ultrasound Pretreatment. The corresponding fatty acid was suspended in the DES composed of choline chloride (ChCl)/urea (U) (1:2 mol/mol + 5% w/w Milli-Q H_2O) [from now onward, ChCl/U (1:2) (5% w/w water) (10 mL)], and the mixture was sonicated by a SONOPULS Ultrasonic homogenizer for 5 min (20 s pulse and 30 s pause), thus obtaining white foamy suspensions (final concentration: 100 mM). An analogous treatment was performed to prepare 10 mL suspensions containing decanoic, lauric, and palmitic acid vinyl esters, all with a concentration of 500 mM. The mixtures were kept in an ice bath during sonication.

Screening of Extraction Solvents on Commercial Saccharose Laurate. Saccharose laurate (SL, 10 mg) was incubated in 0.5 mL of DES−water mixture ChCl/U (1:2) (5% w/w water) at 55 °C and 800 rpm for 10 min. Then, the respective extraction solvent, such as ethyl acetate (EtOAc), dichloromethane (DCM), 2-methyl-2-butanol (2M2B), or 1-octanol (0.5 mL), was added, and the extraction was performed at 1200 rpm for 30 min using a termoshaker (Hettich Benelux, ProfiLab24 GmbH, Berlin, Germany). The temperature was adjusted depending on the boiling point of each studied solvent. At the endpoint, the mixtures were centrifuged (13,400 rpm, 2 min), and the organic phase was analyzed by HPLC-RID. The peak area of the extracted samples was compared with the area of a solution of SL (20 mg/mL in CH_3OH), taken as a reference value of 100%.

General Procedure for the Biocatalytic Transesterification of Sugars in DES ChCl/U (1:2) (5% w/w Water) Using Fatty Acids and Vinyl Esters (C10−C16). Reactions were performed in a total volume of 0.5 mL (using either glass vials or microcentrifuge tubes). The sugar (glucose, lactose, or saccharose) was suspended in the DES, followed by the addition of the acyl donor. The following acyl donors were evaluated: vinyl decanoate (VD, C10), lauric acid (LA, C12), vinyl laurate (VL, C12), palmitic acid (PA, C16), and vinyl palmitate (VP, C16). When ultrasound-pretreated reactions were performed, the sugar was suspended in the sonicated acyl donor suspension in a DES. These mixtures were incubated at 55, 70, or 80 °C for 10−15 min before adding the enzyme. Several sugar

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concentrations, acyl acceptor/acyl donor ratios, temperatures, and reaction times were analyzed. The conditions are summarized in Table 3.

At the endpoint, reaction mixtures were extracted with an organic solvent at 1200 rpm for 30 min. When glucose was used as an acyl acceptor, the extraction was performed with ethyl acetate (EtOAc) at 55 °C, following a previous report.[27] When disaccharides were used as acyl acceptors, the extraction was performed with 2-methyl-2-butanol (2M2B) at 80 °C. Afterward, the samples were centrifuged (13,400 rpm, 2 min), and the organic phase was analyzed by HPLC-RID, HPLC-ELSD, or HPLC-MS. Reactions were performed in duplicates, and in all cases, control and blank reactions were performed.

Biocatalytic Transesterification in a "2-in-1" DES System. For the reaction running in sugar-based DESs, in which the acyl acceptor acts as a substrate and as part of the solvent, the acyl donor VP (0.0167 mL; corresponding to a final concentration of 0.1 M) was added to the sugar-based DES (0.5 mL) and incubated at 55 °C for 10−15 min before adding the enzyme (25 mg, corresponding to 50 mg/mL). At the endpoint (48 h), the reaction mixture was extracted with 2M2B at 80 °C, 1200 rpm for 30 min before being centrifuged (13,400 rpm, 2 min) and analyzed by HPLC-RID.

For the reactions in hydrophobic DESs, in which the acyl donor acts as a substrate and as part of the solvent, the sugar (final concentration: 0.1 M) was suspended in the hydrophobic DES (0.5 mL) and incubated at 60 °C for 10−15 min before adding the enzyme (10 mg, corresponding to 20 mg/mL). For the transesterifications, the reaction mixtures were supplemented with VP (0.0167 mL; corresponding to 0.1 M), and reactions were performed at 60 °C for 96 h. At the endpoint, the mixtures were diluted with dioxane (dilution factor of 1:3) and stirred for further 30 min at 80 °C, 1200 rpm before being centrifuged (13,400 rpm, 2 min) and analyzed by HPLC-RID.

Preparative Synthesis of Sugar Fatty Acid Esters in Organic Media. The reference standard of glucose decanoate was prepared according to the literature protocol[24] with some modifications. D-Glucose (0.600 g; 3.33 mmol), vinyl decanoate (2.24 mL; 10 mmol), and Novozym 435 (0.6 g) were suspended in anhydrous Py/THF (1:1 v/v; 20 mL), and the mixture was stirred at 55 °C and 500 rpm. The round-bottom flask was equipped with a condenser to prevent the evaporation of THF. At the endpoint (48 h), the mixture was filtered under vacuum, and the immobilized enzyme was washed with CH_2Cl_2 (2 × 10 mL) and then with CH_3OH (2 × 10 mL). The filtrate was dried under vacuum, obtaining a viscous yellow crude which was purified by flash chromatography (eluent: CH_2Cl_2/CH_3OH, 90:10 v/v). Since the reaction mixture resulted insoluble in the eluent, a solid-phase sample loading was performed using CH_3OH as a solvent. The product was characterized by MS, ^{1}H NMR, and HBMC. The NMR signals are consistent with the literature.[29] The reference standard of lactose decanoate was prepared in the same way by using the immobilized lipase from *Thermomyces lanuginosus* (TLL).

HPLC Methods. All analyses were performed using a Waters C8 Symmetry column 150 × 4.6 mm, 5 μm at a flow rate of 0.5 mL/min. The injection volume was 2 μL. For HPLC-RID analyses, a 1260 Agilent Infinity equipped with a RID thermostat at 40 °C was used, whereas in the case of HPLC-ELSD analyses, a Chromaster 600 bar System, Merck Hitachi VWR, equipped with a Sedex 100 LT-ELSD was the equipment of choice. To perform HPLC-MS analyses, an Agilent 1290 infinity instrument was used. Elution conditions changed depending on the reactions and are described in the Supporting Information.

Calculation of Infinite Dilution Activity Coefficients and Solubility Using COSMO-RS. The calculations of infinite dilution activity coefficients γ_i^{∞} (IDACs) and the solubility $x_{i,S}$ of the sugars and fatty acid vinyl esters in different solvent mixtures have been performed using BIOVIA COSMOtherm 2020.[41−44] The conformer sets of all molecules have been generated using COSMOconf v3.0, and the COSMO calculations have been performed with Turbomole v6.6 using the TZVPD-FINE parameter set.

The following equation was used for calculating the solid−liquid equilibrium (SLE).[45]

$$\ln x_{i,S}\gamma_{i,S} = \frac{\Delta h_f^0}{RT}\cdot\left(\frac{T}{T_m} - 1\right) + \Delta c_p^{LS}\left(\frac{T_m}{T} - \ln\frac{T_m}{T} - 1\right) \quad (1)$$

with $x_{i,S}$ as the solubility in mole fractions, $\gamma_{i,S}$ as the activity coefficient at the solubility, T_m as the melting temperature, Δh_f^0 as the standard-state enthalpy of fusion at the temperature T_m, and Δc_p^{LS} as the difference in the standard-state heat capacity between the solid and liquid states. To solve eq 1, the solid-state data of the component of interest must be known. However, if a reference solubility $x_{i,S}^{ref}$ of component i is known, e.g., in water, eq 1 using the reference solubility $x_{i,S}^{ref}$ and the solubility of interest $x_{i,S}^{L}$ can be combined to

$$\ln x_{i,S}^{L}\gamma_{i,S}^{L} = \ln x_{i,S}^{ref}\gamma_{i,S}^{ref} \quad (2)$$

The reference solubility of lactose and glucose in water, the temperature of the reference solubility, and the melting temperatures of both components that were used within this work can be found in Table 1.

Table 1. Input Data (Reference Solubility $x_{i,S}^{ref}$ in Water and Melting Temperature T_m) for COSMOtherm Calculations of the Solubility of α-Glucose and α-Lactose in Different Solvents

sugar	reference solubility in H_2O $x_{i,S}^{ref}$ (% mol/mol)	temperature of reference solubility (°C)	melting temperature T_m (°C)
α-Glucose	58.18[46]	35	174.85[47]
α-Lactose	25.15[48]	40	223[48]

In the case of solubility of vinyl esters in different solvents, all components are liquid. Hence, solubility is calculated via the following equation for a liquid−liquid equilibrium (LLE).

$$x_{i,S}^{I}\gamma_{i,S}^{I} = x_{i,S}^{II}\gamma_{i,S}^{II} \quad (3)$$

whereby $x_{i,S}$ is the solubility and $\gamma_{i,S}$ is the activity coefficient of component i in phase I or II. In these cases, no reference solubility data is necessary.

MD Simulations of Sugars. The MD simulations of lactose and glucose in the DES ChCl/U (1:2) with and without water (5% w/w) and in the mixture of Py/THF have been performed using GROMACS *version 2019.4*.[49,50] The interactions between sugars and solvent molecules have been modeled using parameters from *CHARMM General Force Field* (CGenFF) version 4.X[51] taken from CHARMM-GUI.[52] In the case of water molecules, the CHARMM-TIP3P variant[53] was used. A cut-off radius of 1.2 nm has been introduced for the electrostatics and LJ-interactions, whereby the forces were smoothly switched between 1.0 and 1.2 nm. Long-range electrostatics were modeled with the smooth particle mesh Ewald (PME) method[54] using a Fourier spacing of 0.18 nm and a PME order of 4. The bonds with hydrogen atoms were set using SETTLE[55] for water and the LINCS[56] algorithm for all other molecules using a LINCS order of 4.

Cubic boxes including one sugar molecule—either α-glucose or α-lactose—in the respective solvent mixture have been prepared using packmol.[57] In the case of pyridine/THF mixture, 1005 molecules of pyridine and 995 molecules of THF have been added. For the DES/water mixture, 500 molecules of choline chloride, 1000 molecules of urea, and 380 molecules of water were added. An energy minimization for 5000 steps using the steepest descent algorithm has been performed. In the case of Py/THF, this is followed by an equilibration phase in the *NPT* ensemble for 10 ns. Newton's equations of motion were numerically integrated using the leapfrog algorithm[58] and a time step of 2 fs. The temperature of the system was set to 328.15 K and controlled using the velocity rescale thermostat[59] with a time constant of τ_T = 1 ps. The pressure was adjusted to 1 bar using the Berendsen

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barostat[60] and a time constant of τ_P = 2 ps. In the case of highly viscous ChCl/U (1:2) (5% w/w water), additional equilibration steps at elevated temperatures were necessary.[61,62] The equilibration is started with an *NVT* simulation at 328.15 K for 1 ns using a time step of 1 fs. Afterward, the temperature was increased to 500 K and was kept at 500 K for 20 ns to improve the equilibration of the viscous system. This is followed by a 2 ns simulation in the *NPT* ensemble using the Berendsen barostat (τ_P = 5 ps) to equilibrate the pressure to 1 bar. The equilibration phases are followed by the sampling in the *NPT* ensemble for 50 ns, switching the pressure control to Parrinello−Rahman.[63] Two replica simulations from different initial configurations and initial velocities have been prepared. The last 30 ns of the trajectory have been used to calculate the spatial distribution functions of the solvent molecules within 5 Å of the sugar molecule using GROma*ps*[64] and a spacing of 0.05 nm. Moreover, characteristic angles and dihedrals of the sugar molecules in the solvent mixtures have been determined. The MD simulations have been visualized using VMD.[65]

RESULTS AND DISCUSSION

Preparation of DESs. Different DESs were prepared, all of them resulting in clear, viscous solutions. Some of them contained choline chloride (ChCl), which was combined with urea (U) or lactose monohydrate as hydrogen bond donors (HBDs). Another DES was formed by mixing lidocaine and palmitic acid as a HBD. In some cases, water (up to 20% w/w) as a cosolvent was added[66] (Table 2). ChCl/U was used as the model DES since it is one of the most common DESs used for biocatalysis, and therefore, its thermophysical properties (as a neat solvent and/or mixed with water) have been previously investigated, also combining experimental and computational approaches.[67−69] To complement ChCl/U, a DES comprising lactose was included, as an example of "2-in-1" DES, a concept reported for other processes as well.[27,28,70−72] Herein, it was observed that the DES was obtained only if lactose monohydrate was used, whereas with anhydrous lactose, the mixture remained heterogeneous. It may be presumed that the water of crystallization of lactose monohydrate participates in the formation of the hydrogen bond network, as observed with other DESs (e.g., ChCl/levulinic acid).[73] To further explore this "2-in-1" DES concept, a hydrophobic DES containing lidocaine and the acyl donor as components was included.

Table 2. List of DESs That Were Prepared Using Different HBDs and HBAs, Molar Ratios, and Preparation Conditions

HBA, DES component 1	HBD, DES component 2	molar ratio (HBA/HBD)	H_2O (% w/w)	*T* (°C)	time (min)
ChCl	U	1:2	5	90	<30
ChCl	U	1:2	0	90	40−60
ChCl	Lac.H_2O	2.5:1	20	90	90
lidocaine	PA	1:1	0	90	60

Experimental Enzymatic Reactions with Sugars in DESs Using Different Acyl Donors. Following previous successful results reported in the literature using glucose as a sugar for transesterifications,[27,28] we aimed to extrapolate this methodology. Therefore, the first set of experiments focused on the esterification of lactose using different acyl donors, either free acids (LA, C12 and PA, C16) or vinyl esters (VD, C10; VL, C12; and VP, C16). To perform these experiments, the commonly used DES composed of ChCl/U (1:2) (5% w/w water) was selected since the preparation of glucose decanoate by enzymatic transesterification with CalB was previously reported in this solvent.[27] The results obtained are depicted in Table 3.

As can be observed in Table 3, the lactose ester formation was not detected in any of the reaction conditions. Hydrolysis of the acyl donor was observed when vinyl esters were used. Besides hydrolysis, another product was observed (see the Supporting Information, Figures S1−S4), which resulted to be the fatty acid amide. The use of urea as a source of ammonia for the chemical and enzymatic synthesis of amides has been reported previously.[74−76] Several attempts to increase product formation were carried out, including the use of vinyl esters and/or a large excess of the acyl donor, to perform irreversible reactions, and very high enzyme loadings and/or prolonged reaction time. Unfortunately, they were unsuccessful. The ultrasound pretreatment of the reaction mixture was reported to be a key step to improve the availability of fatty acid acyl donor and to increase conversions,[27] but, in this case, it did not have any effect on the reaction outcome. Since no conversions were observed with lactose, glucose was incorporated in the reaction conditions to benchmark with the literature results (Table 4).

Interestingly, the sugar ester was detected only with glucose and vinyl decanoate, C10, consistently with the literature,[27] but not with larger acyl donors, namely, C12 and C16 (with or without ultrasound pretreatment). This outcome might be ascribed to the poor solubility of large acyl donors in DESs,

Table 3. Reaction Conditions: DES−Water Mixture ChCl/U (1:2) (5% w/w Water) with Novozym 435 at 800 rpm[a]

sugar (M)	acyl donor (M)	reaction type[c]	enzyme loading (mg/mL)	temperature (°C)	time (h)	outcome
Lac (0.1)	LA (0.1)	E	20	55	48	byproduct
Lac (0.1)	VL (0.1)	T	20	55	48	hydrolysis and byproduct
Lac (0.1)	VP (0.1)	T	20	55	48	hydrolysis and byproduct
Lac (0.1)[b]	VP (1.25)	T	100	80	96	hydrolysis and byproduct
Lac (0.01)	PA (0.01)	E	50	55	48	byproduct
Lac (0.01)	VP (0.01)	T	50	55	48	hydrolysis and byproduct
Lac (0.01)	PA (0.01)	E	100	55	48	byproduct
Lac (0.01)	VP (0.01)	T	100	55	48	hydrolysis and byproduct
Lac (0.5)	VL (0.5)	T	20	55	48	hydrolysis and byproduct
Lac (0.5)	VP (0.5)	T	100	70	72	hydrolysis and byproduct
Sac (0.5)	VP (0.5)	T	100	70	72	hydrolysis and byproduct
Sac (0.1)	VP (0.1)	T	20	55	48	hydrolysis and byproduct

[a]After the stated endpoints, all reaction mixtures were extracted with 2M2B at 80 °C for 30 min at 1200 rpm and centrifuged before the analysis. [b]In this experiment, DES without the addition of water was used. [c]E: Direct esterification with fatty acid, T: Transesterification with vinyl ester.

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Table 4. Transesterification Experiments Using Glucose as a Substrate in Previously Reported Reaction Conditions,[27a]

sugar	acyl donor	outcome
glucose	VD (C10)	glucose ester detected
glucose	VL (C12)	hydrolysis and byproduct
glucose	VP (C16)	hydrolysis and byproduct
saccharose	VD (C10)	hydrolysis and byproduct
lactose	VD (C10)	hydrolysis and byproduct

[a]DES−water mixture ChCl/U (1:2) (5% w/w water), Novozym 435 (20 mg/mL), 50 °C, and 24 h. Samples were extracted with EtOAc at 55 °C (glucose) or 2M2B at 80 °C (disaccharides) and analyzed by HPLC-RID/HPLC-ELSD.

which hampers its "accessibility" to the enzyme. In fact, it was observed in the literature[27] that the distribution of vinyl decanoate in ChCl/U was a limiting factor in the synthesis of glucose decanoate when using this hydrophilic DES. In that case, the authors demonstrated that the ultrasound pretreatment reduced the mean droplet size of the vinyl ester/DES mixture, thus significantly improving the conversion. Our results suggest, however, that this pretreatment is not efficient when longer chain vinyl esters are used.

Interestingly, when disaccharides were used (saccharose or lactose), the products were not observed with C10 acyl donors either. These results suggest a different behavior of disaccharides in the DES, compared to glucose, which is not correlated with the acyl donor. Both saccharose and lactose are recognized as substrates by Novozym 435 in other solvents, as demonstrated elsewhere.[11,14,18,26] Moreover, CALB is active in diverse DESs, including ChCl/U (1:2 mol/mol), for other (trans)esterification-like reactions.[77] To confirm this, the enzyme was incubated in ChCl/U (1:2) (5% w/w water) containing lactose and vinyl palmitate (0.1 M each) for 24 h and then 0.1 M glycerol was added, thus observing the formation of glyceride monopalmitate (see the Supporting Information, Figure S5).

Considering all data, the results obtained cannot be due to enzyme inactivation but should be attributed to the interactions between substrates (acyl acceptors and donors), enzymes, and the DES components. Furthermore, effects related to sugars as acyl acceptors (glucose vs disaccharides) and to acyl donors (C10 vs C12-16) seem to play an important role. To further understand the results, the same reaction was performed in Py/THF (1:1 v/v), as it has been reported for the high-yield synthesis of several lactose esters by transesterification with vinyl esters.[24] Likewise, a commercial lipase from *T. lanuginosus* immobilized on Immobead 150 (TLL), similar to the one used in that paper, was included in the study as well (Table 5). Remarkably, lactose ester formation was detected with both enzymes in the organic medium (Table 5). Conversely, no sugar esters were observed with none of the two enzymes.

In a final attempt to find an alternative DES for lactose esterification, experiments were performed in two other DESs, namely, ChCl/Lac·H_2O (2.5:1 mol/mol) (20% w/w water) and lidocaine (Lid)/PA (1:1 mol/mol), as shown in Table 6. In both solvents, one of the substrates of the reaction acts simultaneously as a substrate and as a solvent component. This "2-in-1" DES concept approach enables the dissolution of solid substrates, making them available for an enzymatic reaction without additional solvents and has been reported for the enzymatic esterification of sugars and sugar alcohols.[27,33,40,70] Moreover, by using this "2-in-1" DES system, undesirable side reactions with the other DES components, as we observed with urea (formation of the fatty amide), could be minimized.

Fatty acids are also commonly used to prepare hydrophobic DESs, which are currently receiving increased attention thanks to their advantageous properties.[28,78] Nevertheless, also in these cases, the formation of lactose ester was not detected.

As observed (Table 6), no sugar esters were detected in any of the reaction conditions applied. Importantly, a hydrophobic DES like Lid/PA, in which the acyl donor may act both as a solvent and as a substrate, was also tested. In fact, a hydrophobic DES [menthol/decanoic acid (1:1 mol/mol)] was also reported for the synthesis of glucose decanoate.[28,29] The viscosity of this DES is lower, and it is known that lipases work very well in lipophilic media. However, no sugar esters were detected, presumably due to the low solubility of lactose. Moreover, this DES is not stable at temperatures below 60 °C and tends to solidify. In the same DES, a transesterification trial was also performed by adding vinyl palmitate (VP), but also in this case, the target product was not detected.

Computational Solvent Assessment. Overall, the experimental results suggest that the reaction between disaccharides and different acyl donors is negatively affected by the DES. Reasons related to interactions between the substrates and the DES components may be addressed, analogous to findings reported in the literature for DES.[77] Other reasons may be found in the modification of the enzyme structure and flexibility moving from an organic mixture to a DES. In any case, the assistance of computational methods became crucial to shed light on the system and find an explanation for the obtained results. For a better understanding of the substrate behavior in different reaction environments [Py/THF (1:1 v/v) and ChCl/U (1:2) (5% w/w water)], infinite dilution activity coefficients $\ln\gamma^{\infty}$ and the solubility of the sugars and fatty acid vinyl esters were calculated using the highly predictive BIOVIA COSMOtherm 2020 software.[41−44] In addition, MD simulations of a sugar molecule in these solvent mixtures were performed on the atomistic level.

Figure 1 shows the infinite dilution activity coefficients $\ln\gamma^{\infty}$ (IDACs) of α- and β-glucose/lactose as well as of vinyl decanoate, vinyl laurate, vinyl myristate, and vinyl palmitate in ChCl/U, ChCl/U (1:2) (5% w/w water), Py, THF, and Py/THF (1:1 v/v). The IDACs provide only information about

Table 5. Transesterification of Lactose with Vinyl Decanoate: Comparison between DES and Organic Medium[a]

lactose (M)	vinyl decanoate (M)	enzyme loading (mg/mL)	reaction medium	outcome
0.2	0.6	68 (Novozym® 435)	ChCl/U (1:2) (5% w/w water)	hydrolysis and byproduct
0.2	0.6	68 (TLL)	ChCl/U (1:2) (5% w/w water)	hydrolysis
0.2	0.6	68 (Novozym® 435)	Py/THF (1:1 v/v)	lactose ester detected
0.2	0.6	68 (TLL)	Py/THF (1:1 v/v)	lactose ester detected

[a]Reactions were performed at 55 °C and for 48 h.

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Table 6. Trials of Lactose (trans)Esterification in "2-in-1" DES Systems[a]

acceptor (M)	donor (M)/reaction type	enzyme loading (mg/mL)	reaction medium	T (°C)	outcome
lactose (excess)	VP (0.1)/transesterification	50	ChCl/Lac (2.5:1 mol/mol) (20% w/w H_2O)[b]	55	hydrolysis
lactose (0.1)	PA (excess)/esterification	20	Lid/PA (1:1 mol/mol)[c]	55	no conversion
lactose (0.1)	VP (0.1)/transesterification	20	Lid/PA (1:1 mol/mol)[c]	60	hydrolysis
glucose (0.5)	VD/transesterification	30	(−)-mentho/decanoic acid 1:1	50	glucose ester detected[28]
glucose (1.5)	DA/esterification	20	(−)-menthol/decanoic acid 1:1	50	glucose ester detected[28]

[a]Lactose-based DES contained 20% w/w water, and any attempt to reduce this amount resulted in no DES formation. The last two entries summarize the literature data.[28] [b]Lactose monohydrate was used to prepare the DES; reaction time: 48 h, the mixture was extracted with 2M2B at 80 °C. [c]Reactions diluted with dioxane (dilution factor of 3) and centrifuged before the analysis.

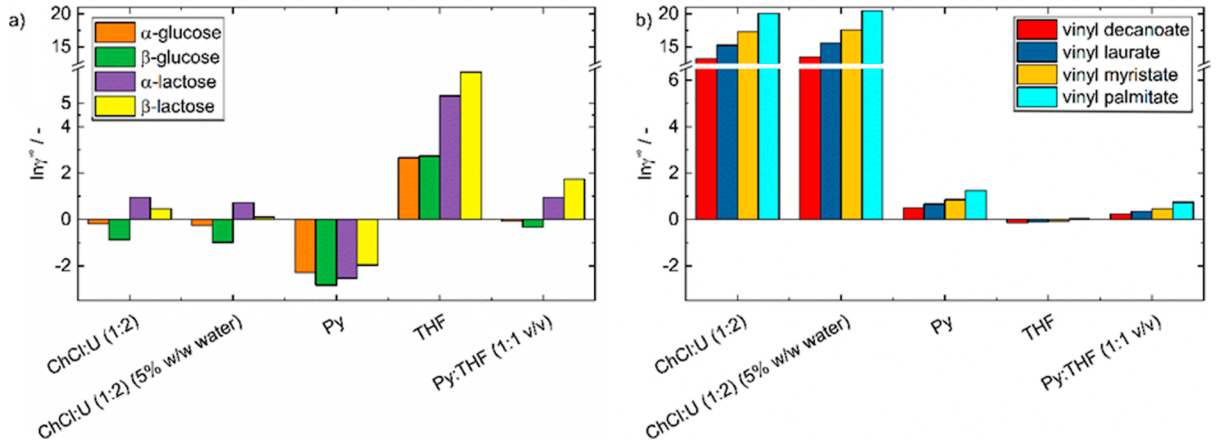

Figure 1. Infinite dilution activity coefficients $\ln\gamma^{\infty}$ of α-/β-glucose and α-/β-lactose (a) as well as vinyl decanoate, vinyl laurate, vinyl myristate, and vinyl palmitate (b) in the DES ChCl/U (1:2) with and without water (5% w/w) as well as in the mixture of Py and THF. All values have been calculated using BIOVIA COSMOtherm 2020, which has an absolute average deviation of 0.35−0.61 log units for predicting infinite dilution activity coefficients.[41−44]

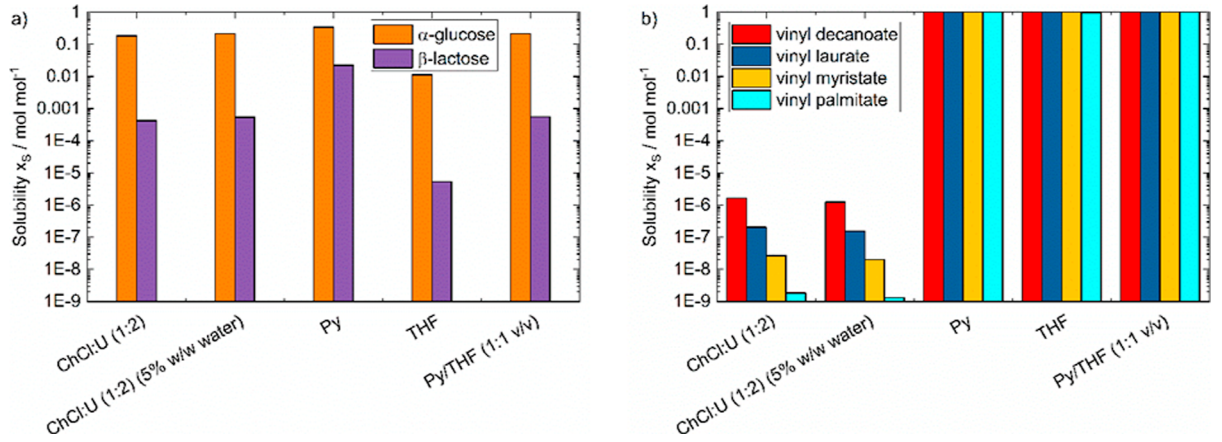

Figure 2. Solubility x_S of α-glucose and α-lactose (a) as well as vinyl decanoate, vinyl laurate, vinyl myristate, and vinyl palmitate (b) in the DES ChCl/U (1:2) with and without water as well as in the mixture of Py and THF. All values have been calculated using BIOVIA COSMOtherm 2020.[41−44] For α-glucose and α-lactose, a reference solubility and a melting temperature (Table 1) have been used for the calculations of the solid state.

the interaction of the substrate molecule with the solvent mixture. $\ln\gamma^{\infty} < 0$ signifies thereby attractive interactions between the substrate and solvent mixture, while $\ln\gamma^{\infty} > 0$ implies repulsive interactions. These values are straightforward to compute, widely used in computational solvent screening, and give reasonable solubility trends, although they are calculated at a substrate concentration of $x_{\text{Substrate}} = 0$ (Figure 1).

For glucose and lactose, the IDACs are close to zero and between −1 and 1 for the DES and DES/water mixture, whereby there is no significant influence of the water content of the DES mixtures. Moreover, the data suggest that the β-sugar variants are more soluble (smaller IDACs) than their corresponding α-sugars. It was important to analyze both the isomers of lactose because, in the experiments with ChCl/U (1:2) (5% w/w water), it was used as an isomeric mixture. Moreover, lactose is a reducing sugar, so it presents mutarotation in solution regardless of the starting form. The final isomer equilibrium of lactose in aqueous solutions is reported to be 40% α-lactose and 60% β-lactose.[79] To the best

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of our knowledge, there are no information on the mutarotation of lactose in DESs. Nevertheless, the IDACs do not confirm that the sugars are incorporated in the DESs of ChCl/U (1:2) (5% w/w water), for which a strong negative deviation from ideality ($\ln\gamma^{\infty} = 0$) would be necessary. In the case of the Py/THF (1:1 v/v) mixture, a huge difference between the interaction of the sugars with Py and THF can be observed. The IDACs suggest strong attractive interactions with Py, while the large values in THF suggest a poor solubility behavior of the sugars for lactose. In the mixture of Py and THF (1:1 v/v), the attractive and repulsive interactions balance out, resulting in IDACs between −0.5 and 2, which is in the range of ChCl/U (1:2) (5% w/w water). In the case of fatty acid vinyl esters, very high IDACs in the range of $\ln\gamma^{\infty}$ = 13−20 can be observed in ChCl/U (1:2) with and without water. This signifies a very poor solubility behavior, which even increases for longer alkyl chains. In Py, THF, and their mixture, the activity coefficients are close to zero, indicating a reasonable solubility of the vinyl esters.

Moreover, the solubility of the sugars and vinyl esters in the tested solvent mixtures can be calculated via BIOVIA COSMOtherm 2020.[41−44] However, in this case, additional information about the solid state of the sugars is necessary. Since this data is difficult to retrieve, only the solubility of the α-isomer of glucose and lactose is shown. As the pure component state of the fatty acid vinyl esters at 328 K is a liquid, no melting properties of the vinyl esters are needed for the solubility calculation. The solubility of α-glucose, α-lactose, vinyl decanoate, vinyl laurate, vinyl myristate, and vinyl palmitate in the tested solvent mixtures are summarized in Figure 2. It can be observed that α-glucose shows a good solubility in all tested solvents ranging from 18 to 34 mol % except for THF (solubility of ∼1 mol %). In the two experimentally tested solvent mixtures [Py/THF (1:1 v/v) and ChCl/U (1:2) (5% w/w water)], the solubility of α-glucose is ∼21 mol %. On the other hand, α-lactose shows in general a much lower solubility in all tested solvents compared to α-glucose with its highest solubility in pure Py and its lowest in pure THF. In the mixture of both, this evens out to ∼0.05 mol %, which is equal to the lactose solubility in the ChCl/U (1:2) (5% w/w water) DES. On the other hand, the fatty acid vinyl esters are completely miscible with Py, THF, and their 1:1 v/v mixture. They have conversely a very low solubility in the DES mixtures (10^{-9}−10^{-6} mol mol^{-1}), which becomes even lower for longer alkyl chains.

To further understand the behavior of glucose and lactose, and their interactions with the solvent mixture, MD simulations of one α-glucose/lactose molecule in ChCl/U (1:2) (5% w/w water) as well as in a mixture of Py and THF (1:1 v/v) were performed in replicas. Figure 3 shows the configuration of α-lactose in ChCl/U (1:2) (5% w/w water) (a,b) and Py/THF (1:1 v/v) (c,d) with the oxygen atom that is part of the esterification reaction highlighted as a van der Waals sphere. The spatial distribution function of the solvent molecules around α-lactose is added, which allows the identification of specific interaction sites of the solvents. As already suggested by the low IDACs of the sugars in pure pyridine, α-lactose preferably interacts with pyridine in the solvent mixtures; hence, very few interaction sites of THF around the sugars can be found (Figure 3c,d). In the case of ChCl/U (1:2) (5% w/w water) (Figure 3a,b), the interaction with α-lactose is dominated by choline (blue) with chloride (pink) forming salt bridges between choline and the OH-

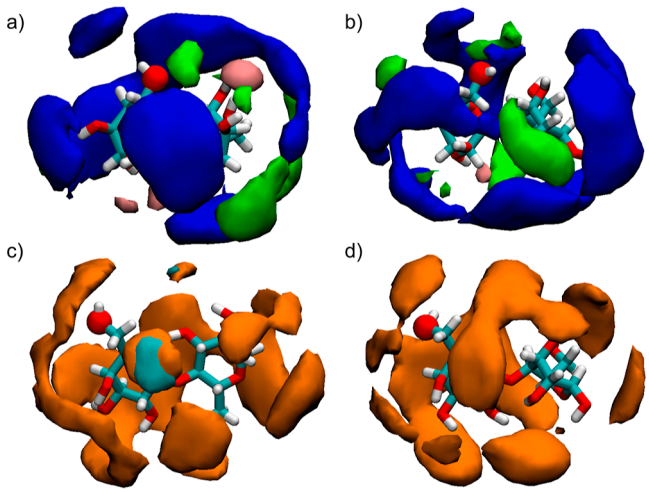

Figure 3. Snapshots of the configuration of α-lactose in the DES ChCl/U (1:2) (5% w/w water) (a,b) and in Py/THF (1:1 v/v) (c,d) from the MD simulations and two replica simulations each. α-Lactose is displayed as licorice, while the oxygen atom that is part of the esterification is highlighted as a red van der Waals sphere with a radius scaled by 0.5. Spatial distribution function of the solvents around α-lactose from the MD simulations is added to the figures. Isosurfaces of the region with high probability of a specific surface are shown in orange (Py), cyan (THF), green (urea), pink (chloride), and blue (choline).

groups of α-lactose. Nevertheless, some parts of α-lactose are also solvated by urea molecules (green) around the glucose part. Although 5% w/w of water is present in the DES mixture, they do not show any effect on the sugar solvation as already indicated by the IDACs (Figure 1). A similar pattern can also be found for α-glucose in the tested solvent mixtures (Figure S11).

As lactose has a larger conformational flexibility caused by the glycoside bond and the enzymatic catalysis of the sugar esters was not successful in the DES mixtures, its configurational changes in different solvent mixtures are studied in detail. A reference structure of α-lactose with highlighted atoms is displayed in Figure S10. In the MD simulations, α-lactose appears to be in a flat configuration with the reactive OH-group pointing outward in Py/THF (1:1 v/v), while its conformation in ChCl/U (1:2) (5% w/w water) is folded with the reactive OH-group pointing inward (Figure 4).

Interestingly, the galactose molecules are in the chair conformation in Py/THF (1:1 v/v), which causes the reactive OH-group to be pointing outward, while galactose is in the boat conformation in the DES mixtures, resulting in the folded conformation. To quantify the different configurations of α-lactose in ChCl/U (1:2) (5% w/w water) and Py/THF (1:1 v/v), characteristic angles and dihedrals within the sugar molecule are monitored from the MD simulations. Figure 5a,c displays the distance between two carbon atoms at the end of both sugar parts (C3−C9) as well as the angle between the two opposing carbons of galactose and glucose within lactose (Figure 5b,d). It can be seen that the distance C3−C9 is much larger in Py/THF (1:1 v/v) compared to the DES mixtures. While the orientation of both monosaccharides in pyridine is around 30°, α-lactose is present in a folded state in ChCl/U with the angle between both monosaccharides around 70°. Figure S12 shows a shift in the dihedrals of the glycosidic bond within α-lactose, which further highlights configurational

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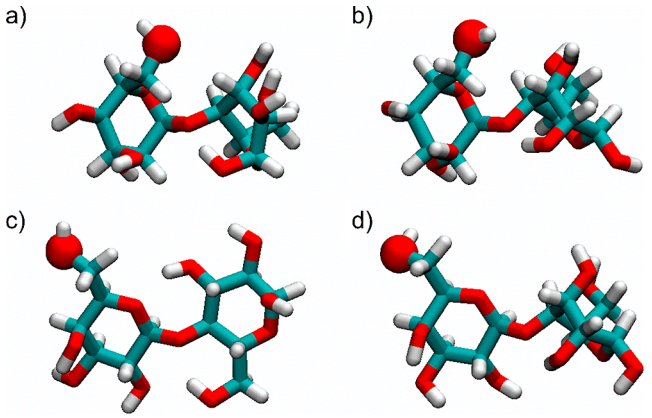

Figure 4. Conformation of α-lactose in the tested solvents ChCl/U (1:2) (5% w/w water) (a,b) and Py/THF (1:1 v/v) (c,d) from the MD simulations and two replica simulations each starting from independent initial configurations and velocities. The oxygen atom that is part of the esterification reactions is highlighted as a van der Waals sphere with a scaled radius of 0.5.

changes between both tested solvents. Moreover, the angle between the carbons C1−C2 and the glycosidic bond (C6−O2) is much larger in Py/THF (1:1 v/v) compared to the DES mixtures quantifying the chair vs boat conformation in the different environments (Figure S15). In the folded state in ChCl/U, α-lactose is more likely to have intramolecular hydrogen bonds ($d_{\text{Don-Acc}}$ < 0.35 nm and $\phi_{\text{H-Don-Acc}}$ < 30°) between the oxygen of the glycosidic bond and the OH-groups O6−H15 and O5−H14 (Figures S13 and S14); however, the active OH-group is not involved in these hydrogen bonds. Nevertheless, such an intramolecular hydrogen bond is also present in one of the simulations in Py/THF (1:1 v/v) (Figure S14).

Overall, the BIOVIA COSMOtherm calculations show that the reaction performed in ChCl/U (1:2) (5% w/w water) is solubility-limited for the vinyl esters, which becomes even worse for longer alkyl chains. On the contrary, no such limitation exists in Py, THF, and their mixture. However, this cannot explain the difference in enzyme activity between glucose and lactose in reaction with vinyl decanoate. To this end, the MD simulations revealed completely different conformations of lactose in a mixture of Py and THF and in ChCl/U (1:2) (5% w/w water), which may affect the catalytic reaction in these media.

CONCLUSIONS AND OUTLOOK

Sugar (fatty acid) esters are an important group of industrially relevant molecules. In order to address their synthetic challenges, the use of DESs as reaction media for enzymatic (trans)esterifications of disaccharides was studied. Starting from the results reported in the literature for glucose, this paper shows that the use of DESs cannot be generalized from some substrates to others. Acyl donors (fatty acids or vinyl esters) larger than C10 are not adequate substrates for the reaction with glucose due to their poor solubility in the analyzed DESs. Likewise, none of the studied acyl donors result in an effective substrate for the reaction with lactose.

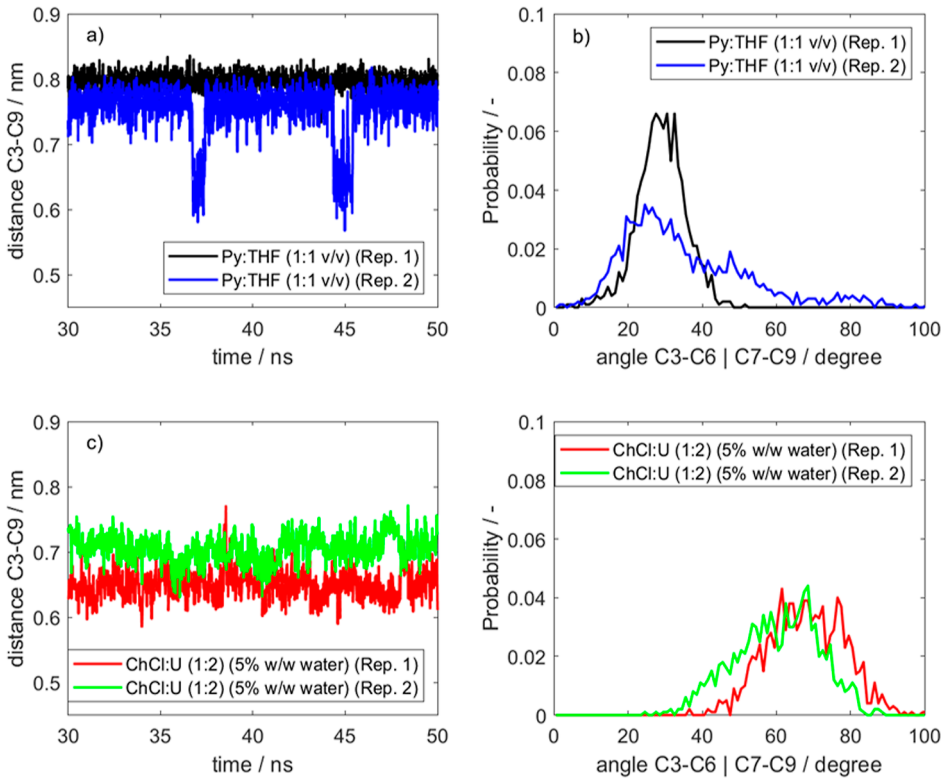

Figure 5. Characteristic angles and distances within α-lactose in the last 30 ns of the MD simulations. Distance between two opposing carbon atoms in both monosaccharides (C3−C9) in Py/THF (1:1 v/v) (a) and ChCl/U (1:2) (5% w/w water) (c) from two replica simulations each. Distribution of the angle between the two monosaccharides described by the vector of the opposing carbons of both monosaccharides (C3−C6 and C7−C9) in Py/THF 1:1 v/v (b) and ChCl/U (1:2) (5% w/w water) (d) from two replica simulations each starting from independent configurations and velocities.

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Differently from glucose, performing an ultrasound pretreatment did not lead to increased activity for lactose either. Computational methods have shown that besides the low solubility of acyl donors, there is also an orientation effect of DES on lactose that is not present in the mixture Py/THF in which only product formation was detected. Thus, a different conformation of lactose in ChCl/U (1:2) (5% w/w water), compared to Py/THF (1:1 v/v), is observed, and this may hamper the reaction, regardless of which the acyl donor used. This finding is substantiated by the fact that the formation of the fatty acid amide (from urea) was observed as a side reaction, showing that the enzyme remained active and that the esterification of lactose was largely unfavorable. Overall, the conclusion is that DESs are not innocent solvents because they interact with the substrates and the catalysts and influence the reaction outcome (or directly hamper it). Rather than being a simple "one-size-fits-all" solution, incorporating a DES in a reaction needs a profound study for each particular case, starting from the genuine motivation to use them (solubility, sustainability, etc.), considering the enzyme and substrate compatibility, and eventually the ease of a downstream unit. This paper shows that the synergy of experimental results with computational methods may shed light on the DES−substrate and DES−enzyme interactions. The same may apply for the setup of integrated, continuous "2-in-1" DES systems, starting from retrieving experimental lab data and combining them with computational tools. Using reagents as DES components may enhance the overall sustainability of the process (better use of chemicals and less waste generation), as well as the economy of the reaction. The extensive palette of options that DESs have (binary, ternary, or more complex mixtures; biodegradable components; etc.) raises expectations to envisage adapted—and hopefully generalizable—solutions for biocatalytic reactions. We hope that our work will pave the way for designing novel DESs that can broaden the use of these sustainable solvents in sugar chemistry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c07607.

HPLC methods, extraction of saccharose laurate from DES (ChCl/U 1:2, 5% w/w water), selected HPLC-RID chromatograms and MS spectra of the (trans)-esterification of lactose with VP/LA in ChCl/U 1:2 (5% w/w water), Novozym 435 inactivation test with glycerol, comparison between DES and organic solvents in the transesterification of lactose and glucose with VD (HPLC-ELSD and TLC), and structural analysis of glucose and lactose from the MD simulations (PDF)

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#R.S. and S.N.C. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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