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Reactive natural deep eutectic solvents increase selectivity and efficiency of lipase catalyzed esterification of carbohydrate polyols

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ABSTRACT

A novel approach for the enzymatic esterification of carbohydrate polyols with fatty acids was investigated, with reactive natural deep eutectic solvents (R-NADES) as reaction medium and source of reagents. Three binary hydrophilic R-NADES consisting of choline chloride (ChCl) as hydrogen bond acceptor (HBA) and sugar alcohols (D-sorbitol, xylitol, D-arabitol) as hydrogen bond donors (HBD), were prepared and characterized. The carbohydrate polyol-based R-NADES were durable viscous fluids between 40 and 80°C, the common temperature range for an enzymatic reaction. The commercially accessible lipase B from *Candida antarctica* immobilized on acrylic resins (LAR) showed significant esterification activity and exceptional thermal stability in all three tested R-NADES and successfully catalyzed the synthesis of polyol esters. Box Behnken factorial design with three levels-three variables was used for optimizing the reaction conditions. The preparative esterification of D-arabitol with lauric acid (LA) at predicted optimal values for enzyme load, (i.e., 800 U/gram D-arabitol), temperature (70 °C) and LA/D-arabitol molar ratio of 1), attained 80 mol% LA conversion after 24 h reaction time. Structural analysis based on mass, infrared and nuclear magnetic resonance spectroscopy demonstrated that the reaction product is solely the diester 1,5-dilauryl-D-arabitol (1,5-DLDA). The 1,5-DLDA product, with an NMR-purity above 99.9%, was isolated in 95% yield. Under similar conditions, 1,5-dilauryl-xylitol (1,5-DLX) and 1,6-dilauryl-D-sorbitol (1,6-DLDS) were obtained, at a LA conversion of 56 mol% and 62 mol%, respectively. A combination of docking and molecular dynamics simulations allowed to rationalize the structural stability of the lipase B from *Candida antarctica* (CalB) active site in R-NADES, as well as the selectivity of the catalyzed esterification.

1. Introduction

Biobased surfactants of biological origin or produced from renewable materials, are receiving increased attention due to their industrial applications in a myriad of products including foods, cosmetics, pharmaceuticals, toiletries, cleaners, paints, and biotechnology. Currently,

biobased surfactants account for about 25% of the global surfactant and detergent market [1], with an estimated value of USD 14.3 billion in 2023, and an anticipated growth to USD 26.2 billion by 2033 [2]. Glycolipids represent the most important sector of the biosurfactant market, as they have great potential to replace fossil fuel derived surfactants [3, 4]. Sugar derived fatty acid esters (SFAEs), a particular class of

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glycosylated lipids, are odorless, flavorless, nontoxic, possess antimicrobial activity, excellent emulsifying properties and foaming ability, exhibiting high biodegradability and biocompatibility. Therefore, they can be utilized as emulsifiers in food and personal care products, drug delivery systems, solubilizing agents, or lubricants. SFAEs are usually manufactured from renewable biobased materials such as sugars and sugar polyols which are common biorefinery feedstocks, while most of the fatty acids are derived from oilseeds or from oleochemical coproducts [5].

Traditionally, SFAEs are obtained by chemo-catalytic synthesis under harsh and energy-intensive reaction conditions, leading in most cases to undesirable byproducts, multiple substitution, and different positions of acylation, thereby reducing the productivity and affecting the biodegradability and biocompatibility of the products [1,6,7]. These main drawbacks can be solved by using biological catalysts, based on the high regioselectivity and the mild reaction conditions of enzymatic reactions. Microbial lipases from different sources, e.g., *Candida antarctica*, *Pseudomonas* species, *Candida rugosa*, *Burkholderia* species, *Rhizomucor miehei*, in native and/or immobilized form, have been already reported to catalyze the synthesis of fatty acid and hydroxy-aryl esters of mono-, di- and oligosaccharides and carbohydrate polyols, under various reaction conditions [8–11].

Enzymes immobilized on solid supports are commonly used in most studies, since immobilization is the most important strategy leading to improved stability, facile separation, and reusability of the enzymes, including lipases, also allowing the industrial-scale production of high added-value products in pharmaceutical, chemical and food industry [12–14]. The selection of a suitable reaction media is highly important, as the enzymatic synthesis of SFAEs shows limitations in terms of enzyme inactivation in hydrophilic organic solvents in which carbohydrates and their polyols are soluble, low reactivity associated with immiscibility of the raw materials, difficulties in controlling the degree of substitution, thus constraining the synthesis of the foreseen products and their properties [15,16]. Organic solvents or ionic liquids were usually involved for the synthesis of SFAEs, as low water activity of the reaction media is required to favor the esterification reaction instead of the hydrolysis reaction, also providing the optimal amount of water needed by the enzyme [17–19]. Organic solvents were thoroughly investigated as reaction media for SFAEs synthesis. Binary mixtures such as *tert*-butanol/pyridine and *tert*-butanol/DMSO were used for the synthesis of xylitol esters with C4–C14 saturated fatty acids catalyzed by CalB [20] and for the N435-mediated esterification of arabinose [15] and fructose-oligosaccharides with C8–C18 fatty acids [16,21], respectively. Hexane [22] and acetone [23,24] were reaction medium for the synthesis of xylitol stearate [22] and for esterification of the carbohydrate polyols erythritol, arabitol, ribitol, xylitol and D-sorbitol with C8, C10, C12 and C14 acids, and immobilized CalB lipase as catalyst [23, 24]. However, organic solvents compatible for carbohydrate modifications can be toxic, a major source of waste, and can inactivate the biocatalyst. On the other hand, ionic liquids are considered expensive, show ecotoxicity and low biodegradability. Therefore, alternative approaches were needed to overcome these problems related to the selection of the appropriate reaction media [25].

Deep eutectic solvents (DES) recently emerged as a possible solution, being nontoxic, biodegradable, and biocompatible. They are a mixture of two or more compounds, hydrogen bond donors and hydrogen bond acceptors, that are solid at ambient temperature but present a melting point depression and become liquid when combined at a particular molar ratio. They are easy to prepare, by mixing and heating until a homogenous phase is obtained. Typical DESs comprise a quaternary ammonium salt as (HBA) and alcohols, amides, carboxylic acids, carbohydrates, or polyols as (HBD). If the components are natural occurring primary metabolites, the resulting mixture is considered a natural deep eutectic solvent (NADES) [26,27]. The preparation costs are usually low, they present wide polarity range, low toxicity, low volatility, thermal and chemical stability, and biodegradability. Initially, DESs were

developed for application in biorefineries to solubilize polysaccharides like cellulose and other biopolymers including lignin. In the past years, more attention was given to their use as reaction media in synthetic chemistry, including biocatalyzed processes [25,28,29].

Several research works investigated the potential of NADESs for mediating bioprocesses catalyzed by lipases in the topic of glycolipid synthesis. The influence of NADES composition and properties on the active structure and catalytic efficiency of the enzyme could be essential. Cao et al. [30] showed that the secondary structure of CalB was relatively preserved in alcohol-based DESs, whereas it was denatured in carboxylic acid-based DESs. Inactivation of CalB was higher in hydrophilic DESs, while the effect of hydrophobic DESs was lower. The amount of carboxyl groups in hydrophobic DESs and of hydroxyl group in hydrophilic DESs, as well as the length of carbon chains had positive effects on the catalytic efficiency of CalB, in the model reaction of benzyl alcohol acetylation [30]. The positive influence of polyhydroxy alcohols as the HBDs component of the NADES was also demonstrated in a thermal stability study, using the same substrates. NADESs with HBDs containing multiple hydroxyl groups were reported to maintain the active structure of the enzyme by substituting the hydration layer of the enzyme surface, thus protecting the enzyme at higher temperature. Among NADESs with ChCl as HBA and different polyols (glycerol, xylitol, and D-sorbitol) as HBDs, the highest thermal stability of CalB lipase was exhibited using xylitol [31]. The higher catalytic efficiency of CalB lipase in hydrophobic DESs compared to hydrophilic ones was confirmed by Hollenbach et al. [32] in the synthesis of glucose monodecanoate, as the yields obtained in a hydrophobic DES consisting of (-)-menthol and decanoic acid were higher than previously reported for the hydrophilic DESs ChCl:urea and ChCl:glucose [33]. Hoyos et al. used a cosolvent system consisting of ChCl:glucose NADES with DMSO and *tert*-butanol for production of glycolipids (19–25% conversion) by transesterification of glucose and vinyl laurate [34].

The use of renewable feedstocks is an important issue in formulation of reactive NADESs for enzymatic synthesis of fatty acid esters of sugars or sugar polyols. Siebenhaller et al. reported the synthesis of glucose-6-octanoate, xylose-5-octanoate, glucose dipalmitate, glucose dioleate, and glucose dilinoleate, mediated by immobilized *C. antarctica* B lipase, using a reactive NADES obtained from beechwood cellulose fiber hydrolysate and ChCl [35,36]. The same research group stated that water concentration was essential for the lipase catalyzed synthesis of arabinose laurate esters in ChCl:arabinose, the yields increasing at 4% water content in NADES. [21].

The structural diversity of the carbohydrate feedstocks and the regioselectivity of the lipases allowed the desired modification of the physico-chemical properties of the amphiphilic sugar derivatives. The utilization of sugar alcohols, particularly D-sorbitol and xylitol, as raw materials was an important approach in this context.

The utilization of NADESs for such reactions came into focus recently and only a few reports were published in this topic. Kim et al. described the N435 lipase mediated acylation of D-sorbitol with capric, lauric, and myristic acids in eutectic mixtures made with NaOH, DMSO and 2-methyl-2-butanol (considered adjuvants) as alternatives for the organic medium (equimolar mixture of 2-methyl-2-butanol and acetonitrile). The highest conversions (based on the consumed fatty acid) for the reaction of D-sorbitol with lauric acid were 89% in organic medium and 97% in eutectic mixtures, at 60°C and 24 h reaction time, with molecular sieves added to remove the water formed in the reaction. Monoesters were the main reaction product and di-esters were obtained with a concentration of about 1/3–1/6 of the monoester. However, these eutectic mixtures cannot be considered as real DESs and the possible influence of NaOH and of the molecular sieves, which could also have catalytic effect, was not checked [37].

Delavault et al. completed an extensive study for the biocatalytic synthesis of carbohydrate polyol fatty acid esters. Screening of 16 commercial lipases and optimization of several reaction parameters like as enzyme loading, reaction time, temperature, water content and

substrate concentration were assessed to increase the yield of D-sorbitol-6-O-laurate by transesterification of D-sorbitol with vinyl laurate in NADES. 28% conversion was achieved at 50°C, 0.5 M vinyl laurate, 50 g/L N435, after 48 h, in NADES formed of choline chloride (ChCl), D-sorbitol, and 5 wt% water [38]. The same research group reported the use of microwave dielectric heating for the preparation of NADES, as well as for the one-pot extraction of long chain fatty acids from oleaginous yeast biomass and N435 mediated synthesis of fatty acid esters of xylitol and D-sorbitol, significantly reducing the processing time compared to conventional heating [39].

Several studies concerning the synthesis of sugar and sugar polyol esters were accomplished using molecular sieves to remove the water formed during the reaction. The high yields reported in some of these works might be considered questionable if reference experiments with only molecular sieves without enzyme were not performed, as being demonstrated that molecular sieves can catalyze themselves the acylation of sugars [40].

The surfactant properties of fatty acid diesters of sugar polyols were not thoroughly investigated because the diesters were usually the secondary products of the enzymatic process. Only in the work of Arcos et al. the HLB values of D-sorbitol 1,6-diester were calculated as being in the same range to those for the corresponding monoacyl glycerols [23].

Consequently, more research work is still required concerning the utilization of R-NADESs for synthesis of SFAEs, to find the best conditions which could maximize the product yields, optimize the downstream processing, minimize the process costs and environmental impact. Exploiting the intrinsic properties of NADES, in this study we explored a new approach for the enzymatic synthesis of fatty acid diesters of carbohydrate polyols, using R-NADES both as solvent and reagent. Following the preparation and characterization of several carbohydrate polyols-based R-NADES, the influence of R-NADES composition and properties on the esterification activity and operational stability of immobilized lipase was thoroughly investigated. An extensive study, including experimental design, enabled a rigorous selection of the operational conditions for production of carbohydrate polyol-based surfactants with significantly higher yields than previously reported and at significantly higher substrate concentration, which increases the process economics. Arabitol dilaurate was synthesized for the first time as a single reaction product of a biocatalytic process, with high yield and purity. Another important aspect of this work is the innovative utilization of a simulated annealing protocol for prediction of the effect of NADES on the structural stability of the enzyme, and to support the experimental results concerning the formation of diesters in NADES.

2. Materials and methods

2.1. Materials

ChCl, $\geq 98\%$, D-arabitol, $\geq 99\%$, D-sorbitol, $\geq 98\%$, Lipase acrylic resin from *Candida antarctica* B, recombinant, expressed in *Aspergillus niger* (LAR) were purchased from Sigma Aldrich. Dimethylsulfoxide (DMSO), methanol, potassium hydroxide (KOH), hexane, tetrahydrofuran (THF) were from Merck. Xylitol, 99%, lauric acid (LA), 98% were from Alfa Aesar. 1-propanol and ethanol were from Chimopar (Bucharest, Romania). Phenolphthalein was purchased from Fluka.

2.2. Preparation and characterization of R-NADES

Three reactive NADESs were prepared in a parallel reactor (STEM Omni Reaction Stations - Electrothermal) by mixing ChCl as HBA and each of the carbohydrate polyols xylitol, D-arabitol, and D-sorbitol as HBD, at (1:1) molar ratio, at 100 °C, 800 rpm, until homogenous fluid mixtures were obtained. The mixtures were then cooled at room temperature and further characterized to determine thermal and rheological

properties. The prepared R-NADES were stored in a dry environment, at room temperature.

Thermogravimetric analyzes (TG) were carried out on a TG 209 F1 Libra thermogravimetric analyzer (Netzsch, Germany), in nitrogen atmosphere, in the temperature range 30–700 °C, with a heating rate of 10 K/min.

Differential scanning calorimetry (DSC) measurements were performed using the DSC 204 F1 Phoenix differential scanning calorimeter (Netzsch, Germany), under nitrogen atmosphere, at temperature ranging from -80 °C to 150 °C, with a heating rate of 10 K/min. Recorded data were processed with the Netzsch Proteus-Thermal Analyzes software version 6.1.0. (NETZSCH-Geraetebau GmbH, Germany).

Dynamic viscosities (η) of the NADES were measured by rotational viscometer (DV2TRV, Ametek Brookfield), using a stainless-steel spindle type SC4-21, for temperatures ranging between 40–80 °C and are expressed in cP. The relation between centipoise and the SI unit for viscosity (Pa·s) is: 1 cP = 1 mPa·s.

2.3. Determination of lipase esterification activity

The esterification activity of lipase was determined for synthesis of propyl laurate as previously described by Corici et al. [41], in a solvent-free system (as reference) and in R-NADES, based on the initial reaction rate methodology. Lipase activity was expressed in activity units, U, where one unit of enzyme is the amount of propyl laurate (μmol) produced per minute by 1 g of enzyme ($\mu\text{mol min}^{-1} \text{g}_{\text{enzyme}}^{-1}$). All measurements were carried out in triplicate, and the mean value of the activity and the standard deviation were calculated.

2.3.1. Determination of lipase esterification activity in solvent free synthesis of propyl laurate

The reaction mixture consisted of 2 mmol of LA and 2 mmol of 1-propanol and 10 mg of lipase. Reactions were started by addition of the biocatalyst, and were carried out at 55°C, in 2 ml capped vials, stirred at 350 rpm in a thermomixer (Thermomixer Confort, Eppendorf, Germany). The progress of the reaction was monitored by titration of the residual acid content with ethanolic KOH 0.1 M solution with phenolphthalein as endpoint indicator. The amount (μmol) of propyl laurate produced at different times during the linear phase of the reaction, was calculated from the determined converted LA, according to the (1:1) stoichiometry of the reaction.

2.3.2. Determination of lipase esterification activity in R-NADES

The reaction mixture consisted of 0.4 mmol of LA, 0.4 mmol of 1-propanol and 0.8 g R-NADES. Reactions were initiated by addition of 50 units of lipase (determined in the solvent free esterification of 1-propanol with LA, as described at 2.3.1), were carried out at 70° C, in 2 ml capped vials, and stirred at 1000 rpm. The progress of the reaction and the amount of the propyl laurate produced were determined as described at 2.3.1.

2.4. Determination of lipase stability in NADES

Mixtures of 0.8 g NADES (ChCl:polyol (1:1)) and 50 units of lipase were incubated at 70°C for 0, 24, 48 and 72 h. After incubation, 0.4 mmol of LA and 0.4 mmol of 1-propanol were added to the mixture and the reactions were carried out at 70° C, at 1000 rpm. The extent of esterification was monitored by determining the residual acid content by titration with ethanolic KOH 0.1 M solution with phenolphthalein as end-point indicator. All measurements were carried out in triplicate. Stability of lipase was expressed as relative activity (%) and was calculated by ratio of the catalytic activity following incubation in R-NADES for a determined duration (E_t) and the activity without incubation (E_0) (both expressed as U/g enzyme).

2.5. Lipase catalyzed esterification of sugar alcohols with LA in carbohydrate polyols-based R-NADES

The general method used for the small-scale synthesis of lauryl esters of carbohydrate polyols is as follows: In a 2 ml Eppendorf tube, LA was mixed with ChCl:polyol (1:1) R-NADES at different molar ratios of LA:polyol. The reaction was initiated by the addition of immobilized lipase at a defined enzyme load (U/g polyol). The reaction mixture was incubated at different temperatures, at 600 rpm, in a Thermomixer, for different times. At the end of the reaction the enzyme was separated after complete dissolution of the reaction mixture in 3 ml of DMSO at 70 °C. Three control reactions were carried out at similar reaction conditions, but removing one component at a time, as follows: (i) without enzyme, (ii) without NADES and (iii) without LA. The reaction mixture and controls were analyzed by HPLC. The concentration of LA in the samples was determined from the LA calibration curve built up by HPLC analysis, carried out as described in Section 2.8.1. LA conversion was determined based on the moles of LA from the control without enzyme and from the sample. No conversion of lauric acid was observed for the control reactions. All reactions, including controls, were performed in duplicate.

2.6. Experimental design and statistical analysis

For optimization of the synthesis of lauryl esters of D-arabitol in ChCl:D-arabitol (1:1) R-NADES, response surface methodology (RSM) with Box Behnken design was applied. The Design Expert 12.0 software (StateEase, USA) was used for regression and graphical interpretation of the results. The first step of optimization was to determine the reaction parameters and their levels, that are significant for reaction's performance.

Chosen optimization variables were A: temperature, B: enzyme load and C: molar ratio LA: D-arabitol. Minimal (−1), central point (0) and the maximal (+1) levels of variables are listed in Table 1. The variation range for each parameter was set based on preliminary experimental data. The selected temperature range is between 45 °C, which is above the melting point of the R-NADES and of lauric acid, and 80 °C, the onset of enzyme inactivation. The maximal level for enzyme loading was four times the loading used in preliminary reactions conducted to evaluate the reactivity of the lipase for D-arabitol esterification (data not shown). The molar ratio LA:D-arabitol was defined based on preliminary studies (data not given). Fifteen experiments were designed to be performed in a random way at different combinations of the variables, in duplicate (Table S1). Reactions were carried out according to the method described in subsection 2.5.

The response variables are fitted by a quadratic model, selected based on Adjusted R². Visualization of the response surface was done with the design software.

2.7. Synthesis of lauryl esters of D-arabitol, xylitol and D-sorbitol in corresponding R-NADES at preparative scale

The reaction mixture formed of 0.16 g (0.8 mmoles) LA, 0.235 g

Table 1
Variables and their levels used in the design for optimization of the synthesis of lauryl esters of D-arabitol.

Variables	Levels		
	-1	0	+1
Temperature (°C)	45	62.5	80
Enzyme load (U/g arabitol)*	150	375	600
LA/Arabitol (mol/mol)	1	1.5	2

* For calculation of enzyme load, the esterification activity of LAR as determined for synthesis of n-propyl laurate in the ChCl:D-arabitol (1:1) R-NADES is used.

ChCl:D-arabitol (1:1), containing 0.8 mmoles D-arabitol and 0.1 g lipase (800 U/g D-arabitol) was incubated in Thermomixer, at 70°C, 600 rpm, for 24 h. On a fritted glass funnel, the reaction mixture was washed with hexanol to remove the unreacted LA and afterwards with THF to extract the reaction product. These were confirmed by the HPLC analysis of the collected washing phases. For further purification, the product collected after the evaporation of THF was washed with equal volumes of water, methanol, and hexane. 0.16 g of white solid was obtained after drying. The structural confirmation of the reaction product was performed based on mass, infrared and NMR spectroscopy. 1,5-dilauryl-D-arabitol (1,5-DLDA) was identified as the main reaction product.

Preparative syntheses of 1,5-dilauryl xylitol and 1,6-dilauryl-D-sorbitol were carried out using the same experimental protocol.

2.8. Analysis, characterization, and structural analysis of reaction products

2.8.1. Chromatographic analysis

Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used to follow the progress of the reaction and the purity of the isolated products. TLC was made on alumina silica gel plates (Merck, Kenilworth, NJ, USA, DC-Autofolien Kiesegel 60 F254) of 20 × 20 cm and 0.2 mm thick, using a mixture of ethyl acetate: hexane (1:1) as mobile phase. Visualization of spots was completed by immersing the plate in an ethanolic solution of phosphomolybdic acid of a 5% concentration, followed by heating. The values of the retention factor R_f for the polyol, LA, and dilauryl esters were: R_f LA = 0.85, R_f DLX = 0.49, R_f DLDA = 0.62, and R_f DLDS = 0.23, respectively.

HPLC used an Agilent System HPLC 1260 INFINITY II chromatograph equipped with a Kinetex 5 µm Polar C18 100 Å 250 × 4,6 mm column, a quaternary G7111B pump, a column thermostat (Agilent Technology Germany), and a RID WR G7162A detector. A methanol/water 90:10 mixture was used for elution of compounds, at a flow rate of 0.2 ml/min, at 50 °C, for 50 min. OpenLab CDS Workstation Software was used for visualization of the chromatograms. LA conversions (mol %) were calculated based on the calibration curve of LA.

2.8.2. Structural analysis

The identification and structural characterization of isolated reaction products was based on mass spectrometry (ESI-MS), infrared spectroscopy (FTIR) and one-dimensional and two-dimensional nuclear magnetic resonance spectroscopy (1D NMR and 2D NMR). Melting temperature was determined by DSC.

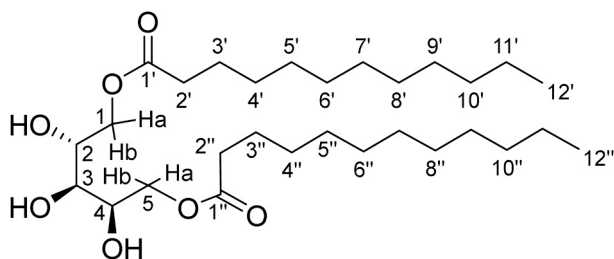
ESI-MS analysis. ESI-MS spectra of products were recorded using an Esquire 4000 (Bruker) Mass Spectrometer interfaced to an Electro Spray Ionization (ESI) by using a positive ion mode. The sample was solubilized in MeOH solution and 0.1% v/v of formic acid.

NMR spectroscopy: High-resolution ¹H and ¹³C NMR spectra were recorded on a Bruker Avance III spectrometer operating at 500 MHz, interfaced with a workstation running a Windows operating system and equipped with a TopSpin 3.5 software package. Py-d5 was used as a solvent, Chemical shifts (¹H) (ppm) were referenced to the solvent signals and to TMS. ¹³C NMR signal multiplicities were based on attached proton test (APT) spectra and assigned based on ¹H-¹³C correlation experiments (Heteronuclear Multiple Quantum Correlation spectroscopy, HMQC, and Heteronuclear Multiple Bond Correlation spectroscopy, HMBC). 1 H signals were assigned by using ¹H-¹H correlation experiments (Correlation Spectroscopy, COSY).

FTIR spectra were acquired in attenuated total reflectance mode, (ATR) on a Bruker Vertex 70 spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with Platinum ATR, Bruker Diamond Tip A225/Q.I. 64 of co-added scans were collected in the range of 4000–400 cm^{−1} with a resolution of 4 cm^{−1}.

Below, the spectral characteristics of the isolated purified reaction products:

1,5-Dilauryl-D-arabitol



Structure 1. Chemical structure of 1,5-Dilauryl-D-arabitol

Mw (g/mol): 516.794; *Melting point* (°C): 105.8; *Purity* (%): > 99.9.
FTIR (cm⁻¹): 3500–3200 (ν(O-H)), 2954, 2916, 2848 (ν(C-H), alkane chain), 1728 (ν(C=O) ester), 1180 (ν(C-O) ester); 1115, 1086, 1061 (ν(C-O) secondary alcohol).

¹H NMR: δ_H (Py-d5, 500 MHz, ppm): 7.10 (s.l., 1 H, OH); 6.75 (s.l., 1 H, OH); 6.61 (s.l., 1 H, OH); 5.51 (dd, 1 H, J₁=11.2 Hz, J₂=2.2 Hz, CH₂a); 5.01–4.99 (m, 1 H, CH); 4.90–4.74 (m, 4 H, CH₂a, CH₂b, CH₂b, CH); 4.27 (d, 1 H, J=8.5 Hz, CH), 2.35–2.31 (m, 4 H, 2'-CH₂, 2''-CH₂), 1.65–1.59 (m, 4 H, 3'-CH₂, 3''-CH₂); 1.26–1.20 (m, 32 H, 4'÷11'-CH₂, 4''÷11''-CH₂); 0.88–0.85 (m, 6 H, 12'-CH₃, 12''-CH₃).

¹³C NMR: δ_C (Py-d5, 125 MHz, ppm): 173.9 (C=O); 173.7 (C=O); 72.1 (CH); 70.1 (CH); 68.7 (CH); 68.1 (CH₂); 67.0 (CH₂); 34.5 (2'-CH₂); 34.4 (2''-CH₂); 32.1 (10'-CH₂, 10''-CH₂); 29.8 (4'-CH₂, 4''-CH₂); 29.7 (5'-CH₂, 5''-CH₂); 29.6 (6'-CH₂, 7'-CH₂, 6''-CH₂, 7''-CH₂); 29.4 (8'-CH₂, 8''-CH₂); 25.3 (9'-CH₂, 9''-CH₂); 25.2 (3'-CH₂, 3''-CH₂); 22.9 (11'-CH₂, 11''-CH₂); 14.3 (12'-CH₃, 12''-CH₃).

1,5-Dilauryl-xylitol

Mw (g/mol): 516.794, *Melting point* (°C): 69.3; *Purity* (%): > 99.

FTIR (cm⁻¹): 3500–3200 (ν(O-H)), 2956, 2918, 2850 (ν(C-H) alkane chain), 1728 (ν(C=O) ester), 1180 (ν(C-O) ester), 1104, 1083, 1062 (ν(C-O), secondary alcohol).

¹H NMR: δ_H (Py-d5, 500 MHz, ppm): 6.90 (s.l., 1 H, OH); 6.60 (s.l., 1 H, OH); 6.08 (s.l., 1 H, OH); 5.06–4.30 (m, 7 H, 1-CH₂, 5-CH₂, 2-CH, 3-CH, 4-CH); 2.49–2.34 (m, 4 H, 2'-CH₂, 2''-CH₂); 1.72–1.62 (m, 4 H, 3'-CH₂, 3''-CH₂); 1.24–1.23 (m, 32 H, 4'-CH₂÷11'-CH₂, 4''-CH₂÷11''-CH₂); 0.89–0.85 (m, 6 H, 12'-CH₃, 12''-CH₃).

¹³C NMR: δ_C (Py-d5, 125 MHz, ppm): 173.4 (C=O); 173.2 (C=O); 72.0 (3-CH); 70.7 (CH); 69.5 (CH); 66.6 (O-CH₂); 66.3 (O-CH₂); 34.2 (2'-CH₂, 2''-CH₂); 31.8 (10'-CH₂, 10''-CH₂); 29.6–29.1 (m, 4'-CH₂÷9'-CH₂, 4''-CH₂÷9''-CH₂); 25.0 (3'-CH₂, 3''-CH₂); 22.6 (11'-CH₂, 11''-CH₂); 14.0 (12'-CH₃, 12''-CH₃).

1,6-Dilauryl-D-sorbitol

Mw (g/mol): 546.814, *Melting point* (°C): 75.7; *Purity* (%): > 95.

FTIR (cm⁻¹): 3500–3200 (ν(O-H) broad), 2956, 2918, 2850 (ν(C-H) alkane chain), 1737 (ν(C=O) ester), 1180 (ν(C-O) ester), 1100, 1085, 1038, 1026 (ν(C-O) secondary alcohols (strong) and primary alcohol (weak)).

¹H NMR: δ_H (Py-d5, 500 MHz, ppm): 7.06 (s.l., 1 H, OH); 6.67 (s.l., 3 H, 3xOH); 5.10–4.35 (m, 8 H, 1-CH₂, 6-CH₂, 2-CH, 3-CH, 4-CH, 5-CH); 2.52–2.30 (m, 4 H, 2'-CH₂, 2''-CH₂); 1.61 (s.l., 4 H, 3'-CH₂, 3''-CH₂); 1.21 (s.l., 32 H, 4'-CH₂÷11'-CH₂, 4''-CH₂÷11''-CH₂); 0.87 (s.l., 6 H, 12'-CH₃, 12''-CH₃).

¹³C NMR: δ_C (Py-d5, 125 MHz, ppm): 173.7 (C=O); 173.5 (C=O); 73.4 (3-CH); 72.3 (CH); 70.4 (CH); 70.3 (CH); 67.5 (O-CH₂); 66.7 (O-CH₂); 34.2 (2'-CH₂); 34.1 (2''-CH₂); 31.8 (10'-CH₂, 10''-CH₂); 29.6 (m,

6'-CH₂÷8'-CH₂, 6''-CH₂÷8''-CH₂); 29.4 (9'-CH₂, 9''-CH₂); 29.3 (5'-CH₂, 5''-CH₂); 29.1 (4'-CH₂, 4''-CH₂); 25.0 (3'-CH₂, 3''-CH₂); 22.6 (11'-CH₂, 11''-CH₂); 14.0 (12'-CH₃, 12''-CH₃).

2.9. Computational simulations

2.9.1. Modelling of lauric acid, NADES and CalB lipase

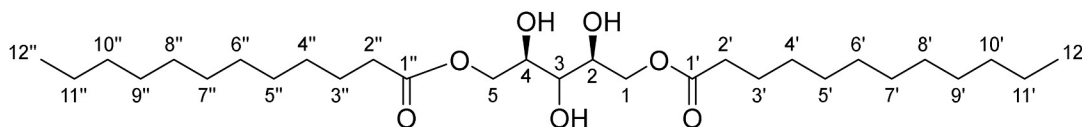
Reactive NADESs with carbohydrate polyols (D-arabitol, D-sorbitol, xylitol) mixtures were constructed using the program Packmol [42]. All CalB + R-NADES systems were constructed by placing the protein at the center of the box and then filling the box with the R-NADES mixture according to experimental molar ratios, i.e., with molar ratios ChCl: polyol:lauric acid (LA) (1:1:1). The constructed cubic box mixtures included 1000 ChCl pairs, 1000 polyol, 1000 LA, respectively with CalB and a choline ion to reach electroneutrality. The structure of Lipase B from *Candida antarctica* (CalB) was available in the Protein Data Bank (PDB ID: 1TCA) [43]. The structures of the R-NADES components and the lauric acid were obtained from the CHARMM Small Molecule Library from CHARMM-GUI [44].

2.9.2. Molecular dynamics

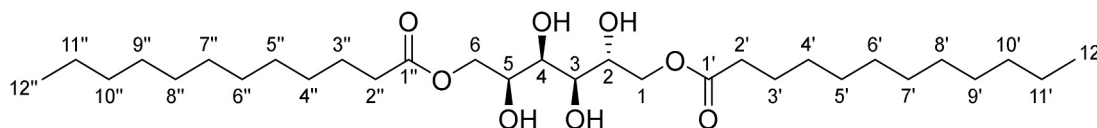
Prior to performing MD simulations, a minimization of the system was performed in two steps: first the protein was restrained, and the solvent energy minimized for 10000 steps and second, an unrestrained minimization was performed for another 10000 steps. Following minimization, the solvent system was relaxed for 1 ns at 343.15 K and at constant pressure (1 bar) while the protein structure was restrained, checking that no further changes were observed in the density of the systems. To accelerate the mixing of the NADES components, we subsequently followed a simulated annealing scheme similar to [45]. Thus, the temperature was linearly increased to 500 K during 100 ns and remained at 500 K for a duration of 200 ns. We performed a sampling of configurations every 10 ns during the last 100 ns, obtaining 10 different poses for each system. All poses were subsequently cooled down to 343.15 K following an exponential cooling schedule, as it is the most basic procedure for cooling systems with rugged energy landscapes [46]. The cooling process was performed during 100 ns by using the exponential function $T = 156.85 * \exp(-0.05 * t) + 343.15$, in which the parameters of the function were chosen manually to guarantee an initial fast rate cooling during the first 20 ns (achieving 400 K), following a much slower cooling until the end of the simulation. During all the simulated annealing process the structure of the protein was restrained. Then, we employed each configuration at the end of the 10 cooling simulations to run 10 MD replicas of 100 ns length at 343.15 K of each system with the protein structure unrestrained.

All MD simulations were performed with the software package GROMACS v.2022 [47]. Charmm36 [48] and CGenFF 4.6 [49,50] force fields were employed for the parameterization of the system. Verlet integrator and LINCS [51] constraints were employed with a time step of 1 fs. Temperature was controlled utilizing a modified Berendsen thermostat [52], while an isotropic pressure of 1 bar was kept using Parrinello-Rahman barostat [53], with a time constant of 2 ps and an isothermal compressibility of $4.5 \cdot 10^{-5} \text{ bar}^{-1}$. Long-range electrostatic interactions were described by the smooth Particle-Mesh Ewald REF (PME) method by using a PME order of 4. Lennard-Jones and electrostatic interactions were calculated with a cut-off radius of 1.2 nm.

The structural analysis of the MD trajectories was conducted using the VMD software package [54] and GROMACS [47]. The calculation of



Structure 2. Chemical structure of 1,5-Dilauryl-xylitol



Structure 3. Chemical structure of 1,6-Dilauryl-D-sorbitol

the cavity volumes was performed with Nanoshaper [55].

2.9.3. Molecular docking of ligands on CalB

The x-ray structure of CalB obtained from Protein Data Bank (PDB ID: 1TCA) while substrate molecules were generated as previously described by [56] with ChemDraw software and minimized using MOPAC with the AM1 semi-empirical method prior docking. The lipase was prepared by removing all the water molecules, heteroatoms, any co-crystallized solvent and substrates and the polar hydrogens and partial charges were added to the structure using ADT 1.5.6. When AutoDock was used, for each experiment 500 dockings were calculated with docking box (60 × 60 × 60) Å, Grid Point Spacing 0.250 Å centered on the binding cavity. Auto-Dock version 4.2 was used for performing the docking calculations with Lamarckian genetic algorithm. Genetic Algorithm population size was set at 1500 and Maximum number of evaluations was set at 25000000. 500 docking generations, each one composed of 250 docking poses were generated. The same system was also docked with AutodockVINA [57] by employing the same docking box with exhaustiveness 500 and energy range 50.

Alternatively, carbohydrate polyol ligand SMILES strings were obtained from PubChem and their 3D structures were generated in pdbqt format with MMFF94 Force Field charges using Open Babel [58] with essentially the same results.

Specifically, for the diesterification of arabitol and lauric acid, we used AutoDock Vina with exhaustiveness 500 and energy range 5 to provide a plausible diesterification reaction pathway through the following steps:

- 1) docking of LA on CalB;
- 2) docking of D-arabitol on CalB with SER 105 esterified with LA;
- 3) docking of MLDA on CalB;
- 4) docking of LA on CalB with MDLA bound;
- 5) docking of MLDA on CalB with SER 105 esterified with LA;
- 6) docking of DLDA on CalB.

Esterification of SER 105 was performed manually.

All docking poses were energy minimized and relaxed by 1 ns MD simulation.

3. Results and discussions

3.1. Preparation and characterization of carbohydrate polyols-based NADES

Three binary hydrophilic R-NADES based on ChCl as HBA and carbohydrate polyols (xylitol, D-arabitol and D-sorbitol) as HBDs, at (1:1) molar ratio, were prepared and characterized to determine the melting temperature (T_m), the thermal stability and the viscosity-temperature profile (Table 2).

Thermogravimetric analysis revealed high thermal stability up to 278 °C for all carbohydrate polyol-based R-NADES (Fig. S1). The melting temperatures (Fig. S2), ranging between 38 °C and 49 °C, are significantly lower than that of the constituents of the mixture, namely 302 °C for ChCl, 92–96 °C for xylitol and D-sorbitol and 102 °C for D-arabitol. Surprisingly, the melting temperature of the ChCl:D-arabitol (1:1) R-NADES is lower than that of the NADES based on xylitol, which might be due to the difference in the chirality of the two pentahydroxypentols. We could argue that the chiral D-arabitol (2R,4R-pentane-

Table 2

Composition and properties of R-NADES.

NADES composition	Molar ratio	Decomposition temperature (°C)	T_m (°C)	Viscosity at 70 °C (cP)	Appearance
ChCl:D-arabitol	1:1	276.6	38.6	300	fluid at $T > 40$ °C, colorless
ChCl:xylitol	1:1	277.5	41.0	235	fluid at $T > 40$ °C, colorless
ChCl:D-sorbitol	1:1	277.9	48.4	655	fluid at $T > 50$ °C, colorless

1,2,3,4,5-pentol) can form different interactions with ChCl as compared to the achiral xylitol (2R,4S-pentane-1,2,3,4,5-pentol). Indeed, earlier studies report that the melting point of deep eutectic solvents is influenced by the structure and strength of the interactions between the hydrogen bond donor and hydrogen bond acceptor [23]. Low melting temperatures for NADES with ChCl as HBA and xylitol and D-sorbitol as HBDs were also reported by other research groups [59]. To our best knowledge, R-NADES containing D-arabitol as HBD are not described in literature.

The viscosity of the three R-NADES was measured between 40 and 80 °C (Fig. 1). For maximum accuracy, the readings were made at a torque range from 10% to 100%, that made viscosity measurements possible at different temperature intervals. Viscosities of R-NADES decreased with increasing temperature following an Arrhenius-like behavior, as at higher temperature the internal resistance of molecules decreases and cause the molecules to flow more easily. The viscosity profiles show that the obtained polyol-based R-NADES are stable fluids with adequate viscosities between 40 and 80°C, the optimum temperature range for an enzymatic reaction.

Results show a strong relation between the viscosity and the composition of the deep eutectic solvent, primarily linked to the chain length and the number of hydroxyl groups of the HBD polyol. The viscosity of D-sorbitol-based NADES is considerable higher than that of the pentol-based R-NADESs, over the entire temperature range. This is in agreement with the literature data [59,60] that show that the hydrogen bond network driving to a lower mobility of free species within the mixture, and electrostatic or van der Waals interactions contribute to the

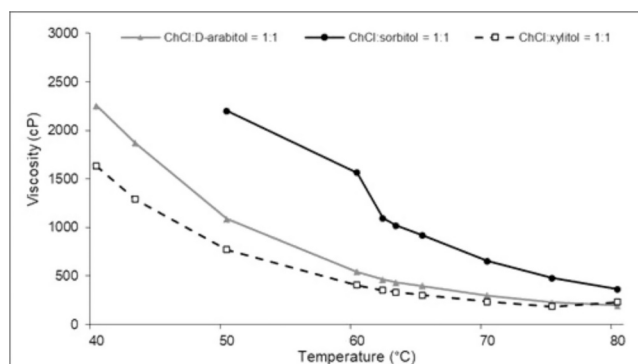


Fig. 1. Viscosity curves (cP) of the carbohydrate polyol-based NADES, assessed in the 40–80 °C temperature range.

high viscosity of deep eutectic solvents.

3.2. Experimental and theoretical assessment of the effects of NADES composition and properties on the esterification activity and stability of the immobilized lipase

3.2.1. Experimental assessment of the esterification activity of the CalB lipase in polyol-based R-NADES

The esterification activity of industrial lipase B from *Candida antarctica* immobilized on acrylic resin (LAR, formerly Novozym 435) was evaluated in the esterification of 1-propanol with LA, in carbohydrate polyol-based R-NADES, compared to the solvent free system as reference. The lipase showed significant esterification activity in all the tested R-NADES but lower than in solvent free system (Fig. 2A). Carbohydrate based R-NADES are quite viscous even at the reaction temperature of 70 °C, thus the diffusion and collision of reactant molecules could be slowed down compared to that in solvent free system. Moreover, interactions between R-NADES and enzyme must be considered, that in some cases can be activating by stabilizing the tertiary structure of the enzyme but in other cases can be inhibiting [32].

For the stability study, the lipase was preincubated at 70 °C in each R-NADES for 0, 24, 48 and 72 h. Then, the esterification activity was measured using the same model reaction of propyl laurate synthesis. Such a study was necessary considering the requirement of conducting the reaction at higher temperatures, to decrease the viscosity of the reaction mixture.

Excellent stability of this lipase in all three tested R-NADES was observed (Fig. 2B).

The highest stability of the immobilized CalB lipase at 72 h incubation time at 70 °C was exhibited in ChCl:D-arabitol (1:1) R-NADES (97%), followed by ChCl:xylitol (1:1) (89%) and ChCl:D-sorbitol (1:1) (82%). This strongly suggests that the interaction between the polyol component of the NADES and the amino acid residues at the surface of the enzyme plays an essential role in the stabilization of the biocatalyst. Although measured at a higher temperature than in other reported studies, our results are in good agreement with the conclusions of the thermal stability study of Cao et al. [30] who stated that HBDs containing multiple hydroxyl groups preserve the active structure of the enzyme by substituting the hydration layer of the enzyme surface, thus protecting the enzyme at higher temperature.

3.2.2. Theoretical evaluation of the effect of NADES on the structural stability of CalB lipase

The stability of the CalB in NADES was studied by molecular dynamics (MD) simulations. The residues of the catalytic site were defined as those known to be interacting with the substrates in the acyclic and alcohol/aminic subsites and the stereospecific pocket (see Fig. 3a) [61]. MD simulations of CalB in water solvent were also included for the sake of comparison. Results of the MD trajectories indicate that the conformation of the active site is highly stable in all three NADES over the trajectory. The root mean square deviation (RMSD) values of the active site residues using as template the crystal structure pdbID:1tca remain stable at 1 Å along the trajectory when the protein is solvated in any of the NADES (see Fig. 3b). These results contrast with the high RMSD values of 4 Å achieved at the end of the simulations when using water solvent.

Lipases have a funnel-like binding site, which contributes to the selectivity of the catalytic reaction [62]. Thus, the morphology of the entrance of the cavity was studied more in detail by evaluating the minimum distance between residues Ile189 and Leu279 and between Leu144 and Ala282 (see Fig. 3a). As a reference, we evaluated both distances in the reference crystal structure (pdbID: 1tca) being 3.2 Å for Ile189-Leu279 distance and 8.1 Å for Leu144-Ala282 distance. The MD simulations show that the evolution of both distances (see Fig. 3c and S3) is significantly stable, oscillating around the reference values when CalB is solvated in all three NADES. On the contrary, a water environment leads to the increase of these distances along the trajectories, indicating the modification of the cavity entrance, as already showed in [62]. Moreover, the average volume of the catalytic cavity was calculated considering the most representative conformations of the trajectories (see Section 2.9.2 in Material and Methods for a more detailed explanation). Average volume values of 226, 334 and 268 Å³ were obtained for NADES containing arabitol, xylitol and sorbitol, respectively. Considering that the volume of the cavity in the reference crystal structure (pdbID: 1tca) is 332 Å³, they seem to be slightly smaller for arabitol and sorbitol.

Additional MD simulations of CalB in all three NADES and lauric acid (LA), with LA:polyol molar ratio 1:1, were performed to evaluate the influence of the LA + NADES mixture in the structural stability of the CalB active site. During the annealing phase (see Section 2.9.2 in Materials and Methods), the system spontaneously evolved towards a biphasic phase in which the lipase is located at the lipid-NADES interface with the active site oriented toward the hydrophobic phase (see Fig. 3c and the video in the Suppl. Material). This behavior agrees with

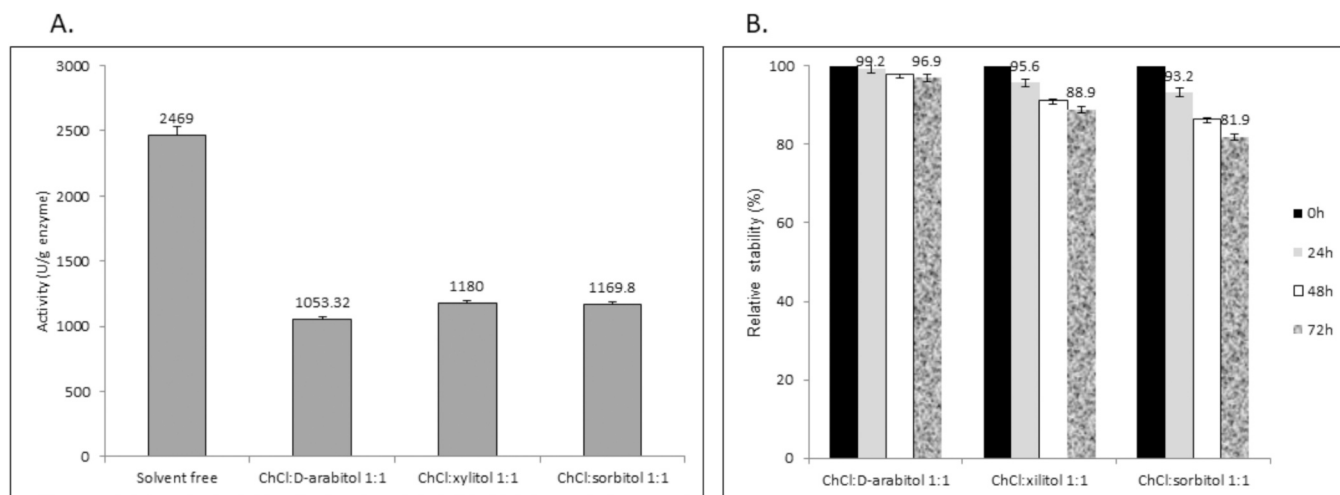


Fig. 2. A) Esterification activity (U/g enzyme) of LAR determined in the acylation of 1-propanol with lauric acid, in solvent free system, at 55°C, and in polyol-based R-NADES at 70°C. B) Stability of immobilized *Candida antarctica* B lipase (LAR) in polyol-based R-NADES (ChCl:carbohydrate polyol (1:1)), assayed for the esterification of 1-propanol with LA at 70 °C and (1:1) molar ratio of reagents.

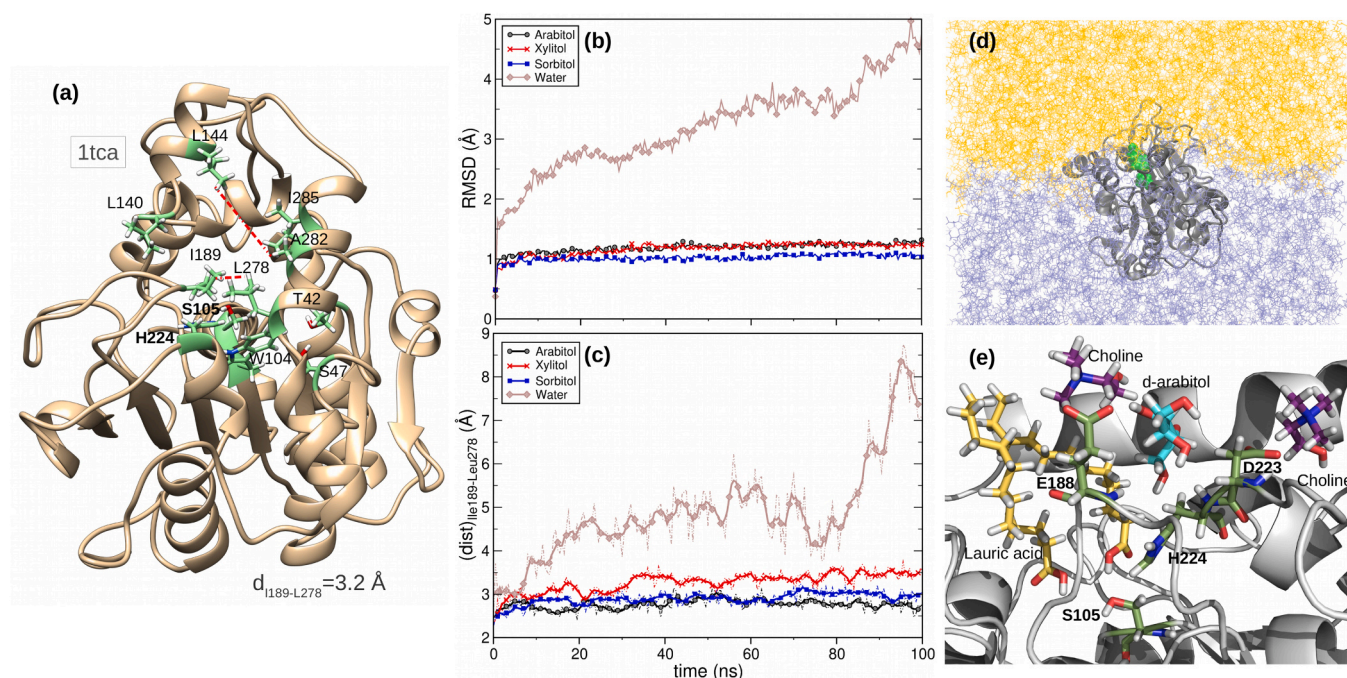


Fig. 3. Computational analysis of the structural stability of the CalB active site. (a) Graphical representation of CalB lipase (pdbID: 1tca). Active site residues are colored in light green, while red dashed lines indicate the minimum residue-residue distances. Evolution of (b) root mean squared deviation (RMSD) of the CalB active site and (c) the Ile189-Leu278 minimum distances for CalB in NADES (ChCl:polyol 1:1) and in water solvent along the MD trajectories. Values are averaged over 10 trajectories for each system. (d) Graphical representation of the lipid-NADES interface after the annealing stage. Lauric acid molecules are yellow, NADES is light blue, while the catalytic residues Ser105 and His224 of CalB (dark grey, cartoon) are represented as green surface. (e) Graphical representation of NADES components, lauric acid molecules and the lipase active site residues.

the experimental observation that CalB lipases show an enhanced catalytic rate at hydrophobic interfaces when bulky substrates are involved [63]. A similar structural analysis was performed in the LA+NADES systems (see Figs. S4-S6), showing that the conformation of the active site was also stable at the interface of the lipid-NADES biphasic system. Interestingly, the evaluation of the residue-residue distances showed slightly higher distances with respect to the NADES mono-phase solvent (see Figs. S4 and S5). Also, higher average volume values of the cavity were observed, as they achieve 307 ± 3 , 334 ± 3 , and 357 ± 3 Å³ for arabitol, xylitol and sorbitol, respectively, which are more similar than the one obtained with the reference structure. This behavior agrees with the hypothesis of a more open active site when the lipase is located at the interface [64].

Supplementary material related to this article can be found online at [doi:10.1016/j.cattod.2023.114373](https://doi.org/10.1016/j.cattod.2023.114373).

To understand the mechanism of catalyzed esterification when the lipase is located at the lipid-NADES interface, the interactions between substrates and the active site residues were analyzed in all trajectories. The most representative scenario is depicted in Fig. 3e. Two lauric acid molecules enter the catalytic site (80% of probability) occupying the acyclic subsite. The location of the carboxylic groups is one of them inside the active site interacting with Ser105 (88%), while the other can be found either outside (65%), or inside (23%). No polyol molecule was found inside the cavity in the simulations, but at least one molecule was located at the entrance of the cavity interacting with the His224 backbone (64%). Two polyols at the cavity entrance region were found with much smaller probability (17%), while three or more polyols were never found in the simulations. Interestingly, two choline molecules are found in the same entrance interacting with Glu188 and Asp223 (80%). This configuration enables a region of charged groups inside the lipid phase that may stabilize the location of a polyol molecule close to the alcohol/aminic subsite, as well as preserving the active structure of the enzyme.

3.3. Catalytic efficiency of LAR for synthesizing lauryl esters of carbohydrate polyols in R-NADES

The synthesis of lauryl esters of the selected carbohydrate polyols, catalyzed by immobilized LAR lipase, was evaluated in preliminary experiments in ChCl:polyol reactive NADES. Screening small scale reactions were carried out with 300 U enzyme/g polyol, at 70°C, 67 h reaction time, at a 1:1 molar ratio between LA and the polyol component of the R-NADES of 1. Surprisingly, addition of the lauric acid to the reactive NADES resulted in the formation of a two-phase system, due to the separation of the hydrophobic lauric acid layer from the hydrophilic NADES core containing the solid enzyme. Extensive stirring was required to homogenize the reaction mixture and avoid mass transfer limitations. The product formation was demonstrated by chromatography (HPLC and TLC). The highest lauric acid conversion (23 mol%) was obtained for the ChCl:arabitol (1:1) NADES. The esterification of xylitol and sorbitol attained comparable levels of LA conversion, i.e., 16.5 mol% and 18 mol% respectively, but lower than that obtained for the esterification of D-arabitol. We suggest that this difference in LA conversion might be induced by the differentiation in the stereo-conformation of the polyols that influence both the enzyme-substrate interactions and the strength of the intermolecular interactions of the constituents of NADES.

Based on these results, the esterification of D-arabitol with lauric acid in R-NADES ChCl:D-arabitol (1:1) was selected as model reaction for optimization of reaction parameters to maximize the conversion and product yield.

3.4. Optimization of the esterification reaction in R-NADES by experimental design

The first step of process optimization focused on the identification and selection of significant variables and their levels. Critical parameters identified are (a) temperature, (b) viscosity of the R-NADES, that defines

the viscosity of the whole reaction mixture as well, (c) reaction time, (d) molar ratio LA/D-arabitol and (e) enzyme load. Since the temperature is highly correlated with the viscosity of the medium and with enzyme activity and stability, we selected temperatures in the 45–80 °C range. Enzyme load and molar ratio are important variables and based on the results obtained in the small-scale experiments discussed in subchapter 3.3, we have considered increasing the upper level of this variable two-fold. To select a proper level of the reaction time, since next to temperature, long incubation time significantly increases enzyme inactivation, a time-course experiment was carried out to evaluate the impact of time on conversion (Fig. 4). Up to 24 h the LA conversion increased significantly to reach about 60 mol%. However, only 6.7% and 12% increase of LA conversion were obtained after 48 h and 72 h, respectively. Therefore, the reaction time selected for optimization studies was 24 h.

To assess the effects of reaction parameters on the generation of products, a factorial experimental design was developed. The selected Box Behnken optimization model allows to vary the independent variables on 3 levels (–1, 0, 1). The three variables considered were the temperature (45, 62.5 and 80 °C), the loading of enzyme (150, 375 and 600 U/g D-arabitol) and the LA:D-arabitol molar ratio (1, 1.5 and 2). Two blocks each consisting of 12 reactions at different values of the variables and three reactions at the central point (e.g., mid values for each variable) were performed according to the method described in subchapter 2.5. The reactions were stopped at 24 h. The reaction mixtures were analyzed by HPLC, and the LA conversion was determined. For the control reactions no conversion of LA or other peaks in the chromatograms other than those corresponding to the raw materials were found. The values of the independent variables used, and the values of the response obtained (observed LA conversion) are summarized in Table S1.

With all subsets' selection, a quadratic model with 7 regression coefficients was selected with the highest adjusted R^2 and the better fit statistics. (Table 3).

The low model p-factor (<0.0001) the F-value of 22.15 denotes that the model is significant, and that there is a very low chance, i.e., 0.01% chance that this large F-value could happen due to noise. The p-value of linear term influencing factor (A, B), interaction factors (AB, BC), and squared factors (A^2 , B^2 , C^2) is less than 0.05 at 95% confidence level, therefore these factors have a substantial contribution to conversion. Factors A, B, A^2 and B^2 , with the largest F-values, are the most significant. The large Adequate Precision (16.703, Table 3) confirms that the signal to noise ratio is satisfactory, and the model can be used to traverse the design space. Analysis of the residuals' predictions plot shows a good linear correlation of the experimental data compared to the predicted data. The Adjusted R^2 is high (0.8783, Table 3) and close to the model Multiple R^2 (0.9212, Table 3) and suggests that there is a good

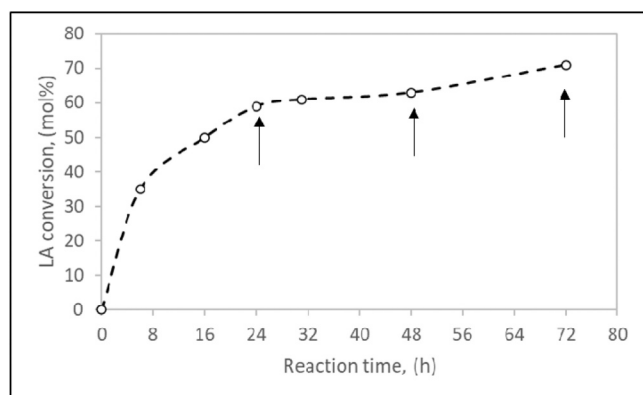


Fig. 4. The LA conversion for the esterification of D-arabitol with LA, in CHCl_3 : D-arabitol (1:1), at 70°C, catalyzed by LAR (400 U/g D-arabitol), at an LA/D-arabitol molar ratio of 1, at different reaction time.

Table 3

Fit statistics and estimated regression coefficients and adjusted R^2 obtained for the quadratic BBD model.

Parameter	Estimated regression coefficients	F value	p-value	
Model		22.15	< 0.0001	significant
b_0 : Intercept	-109.09484			
A: Temperature	4.33565	51.05	< 0.0001	significant
B: Enzyme load	0.02266	89.25	< 0.0001	significant
C: LA/D-arabitol molar ratio	-50.90375	0.0376	0.8482	
AB: Temperature*Enzyme load	0.00107	4.58	0.0456	significant
AC: Temperature*LA/D-arabitol	-0.03400	0.0225	0.8823	
BC: Enzyme load*LA/D-arabitol	-0.06416	13.26	0.0057	significant
A^2 : (Temperature) ²	-0.03293	23.90	0.0001	significant
B^2 : (Enzyme load) ²	0.00087	4.54	0.0464	significant
C^2 : (LA/D-arabitol molar ratio) ²	25.51583	9.56	0.00060	significant
Multiple R^2	0.9212			
Adjusted R^2	0.8783 (>0.8)			
Adequate precision	16.703			

agreement between the experimental and predicted values of conversion. Estimated regression coefficients of significant variables were fitted in the quadratic model equation below, where Y is the predicted response and A, B, and C are the model variables (or factors). This equation in terms of actual values of the factors can be used to predict the response (i.e., LA conversion) for given levels of each factor.

$$Y = -109.095 + 4.335A + 0.023B - 50.9C + 0.001AB - 0.034BC - 0.064AC - 0.032A^2 + 0.0008B^2 + 25.52C^2$$

Interactions between variables were visualized by constructing response surface (Fig. 5A) and contour plots (Figs. 5B and 5C), which explain the interaction between the response and the experimental data as function of the levels of two variables with the third variable at its central value.

The highest LA conversion (60.6 mol%) was reached at high enzyme loads (600 U/gram D-arabitol), at high temperatures (above 66 °C) and for a molar ratio LA/D-arabitol of 1. The LA conversion was hardly influenced by increasing the enzyme load. However, the highest possible enzyme load for maximal conversion was not achieved, as illustrated by the increasing trend for enzyme load in Fig. 5D. Since the LA conversion is augmented at the borderlines of the design space for variable B, i.e., enzyme load, the experimental design should be extended towards higher enzyme amounts.

The information acquired from the analysis of the surface and contour plots suggest that higher conversions may be possible to be obtained at higher enzyme load. Therefore, to validate the model and this assumption, using the regression equation generated by the model given in equation 2, we have calculated predicted values of the LA conversions for several levels of the enzyme load, two included in the model (i.e., 400 U/g D-arabitol and 600 U/g arabitol), and one higher (i.e., 800 U/g D-arabitol), at two temperatures 60 °C and 70 °C, at a LA:D-arabitol molar ratio of 1, and 24 h. The additional experiments followed the same procedure used for the design of experiments, with additional conversion measurements at 48 h and 72 h (Table 4).

As shown in Table 4, there is a very good correlation between the predicted values for LA conversion and the experimentally determined values for each experiment. Carrying out the reaction at higher enzyme loading amount (800 U/g D-arabitol) at the specified conditions (70 °C, molar ratio LA:D-arabitol of 1), an LA conversion of 80 mol% was obtained already at 24 h reaction time. Increasing the reaction time to 48 h and 72 h, respectively, increased the LA conversion when reactions were

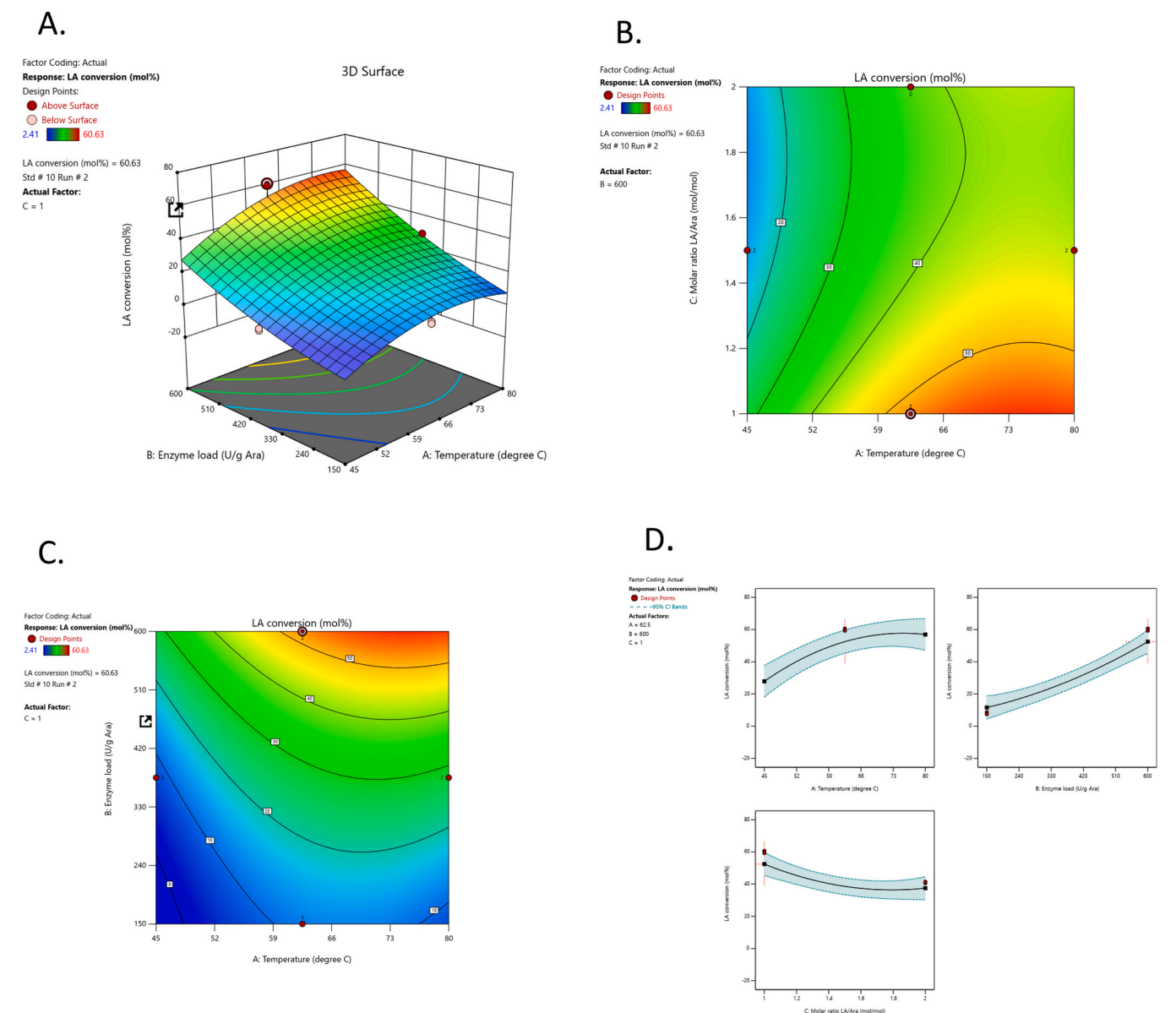


Fig. 5. Graphic visualization from Response Surface Methodology (RSM). A) Surface plot for LA conversion, for molar ratio LA/D-arabitol = 1:1. B) Contour plot temperature vs. LA/D-arabitol molar ratio at enzyme load = 600 U/g; C) Contour plot for enzyme load vs temperature, at LA/D-arabitol molar ratio = 1; D) Interaction plot between LA conversion and factors.

Table 4

Experimental validation of the optimization model.

Entry	T (°C)	Enzyme load (U/g D-arabitol)	LA conversion (mol%)			
			Predicted	Experimentally measured		
				24 h	48 h	72 h
1	60	400	52.30	54 ± 0.97	65 ± 0.63	73 ± 0.16
2	70	600	59.31	65 ± 1.28	74 ± 1.56	80 ± 0.82
3	70	800	75.36	78 ± 1.73	82 ± 1.67	85 ± 1.56

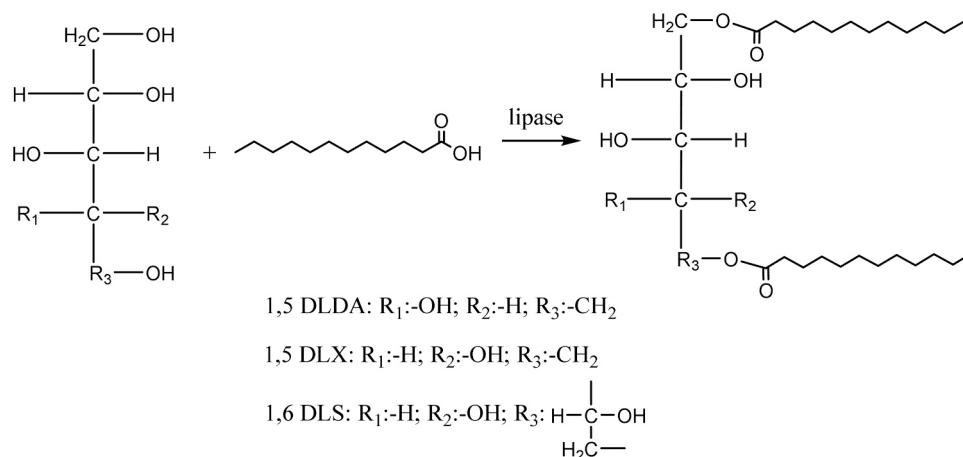
carried out at the lower enzyme loadings. Nevertheless, this effect is minimal at higher enzyme concentration, confirming the assumptions made that the enzyme load and not the reaction time is the critical parameter of the process.

3.5. Preparative synthesis of the carbohydrate polyol laurate diesters and their characterization

Following the validation of the optimal reaction conditions obtained based on the factorial experimental program, preparative synthesis of lauryl esters of D-arabitol, xylitol and D-sorbitol, mediated by the commercial immobilized CalB lipase (LAR) was at conditions predicted for maximizing LA conversion, given in Table 4, Entry 3. All reactions (Scheme 1) and product isolation steps were carried out as described in subchapter 2.7.

Results are summarized in Table 5. The structural characterization of the reaction products was performed based on ESI-MS, FTIR, ^1H NMR, ^{13}C NMR, 2D ^1H - ^{13}C HSQC NMR and 2D ^1H - ^{13}C HMBC NMR.

Reactions proceeded with a medium to high conversion of lauric acid of 80 mol%, 56% and 62% for the esterification of D-arabitol, xylitol and D-sorbitol. Surprisingly, LA conversion increased with the same factor for all three substrates (i.e., 3.5) as compared to the results obtained in the preliminary screening experiments, for a 2.7-fold increase of enzyme



Scheme 1. Reaction scheme of the enzymatic synthesis of esters of D-arabitol, xylitol and D-sorbitol.

Table 5

Yields of enzymatic synthesis of esters of D-arabitol, xylitol and D-sorbitol and their properties.

Substrate NADES	Product	LA ^{a)} conversion (mol%)	Product yield (mol%)	Polyol conversion (mol%)	T _m (°C)	HLB
ChCl:D-arabitol, 1:1	1,5-DLDA	80	95	38.7	105.8	5.8
ChCl:xylitol, 1:1	1,5-DLX	56	90	25.0	69.3	5.8
ChCl:D-sorbitol, 1:1	1,6-DLS	62	86	26.5	75.7	6.6

^{a)} Reactions were performed at 70 °C, with 0.25 g R-NADES, and 800 U/g polyol enzyme catalyst, molar ratio LA/polyol = 1, for 24 h.

load and 2.8-fold decrease of reaction time. This confirms the assumption that enzyme-substrate and enzyme-NADES interactions are the main factors controlling the reaction. The isolated products were obtained as white amorphous solids. The structure of the products as well as their purity was elucidated by a combination of spectroscopic, calorimetric, and chromatographic techniques.

1,5-DLDA has a melting temperature of 105.8 °C, which is very close to that of the D-arabitol, (i.e., 102 °C), but a significant drop in the T_m is observed for 1,5-DLX and 1,6-DLS, from 92° to 96–69.3 °C and

75.7 °C, respectively (Fig. S7). DSC shows only one sharp, symmetric peak for the D-arabitol ester, indicative of a single product, very pure (Fig. S7A). A discrete tailing of the peak measured for the xylitol product suggests the presence of a minute trace impurity (Fig. S7B). Nevertheless, DSC shows that the isolated 1,5-DLDS contains traces of lauric acid (T_m: 43.8 °C) and of a second compound that can be assigned to sorbitol lauryl monoester with an estimated T_m at 66 °C (Fig. S7D). However, the concentration of the monoester impurity is very low, and it could not be detected by TLC.

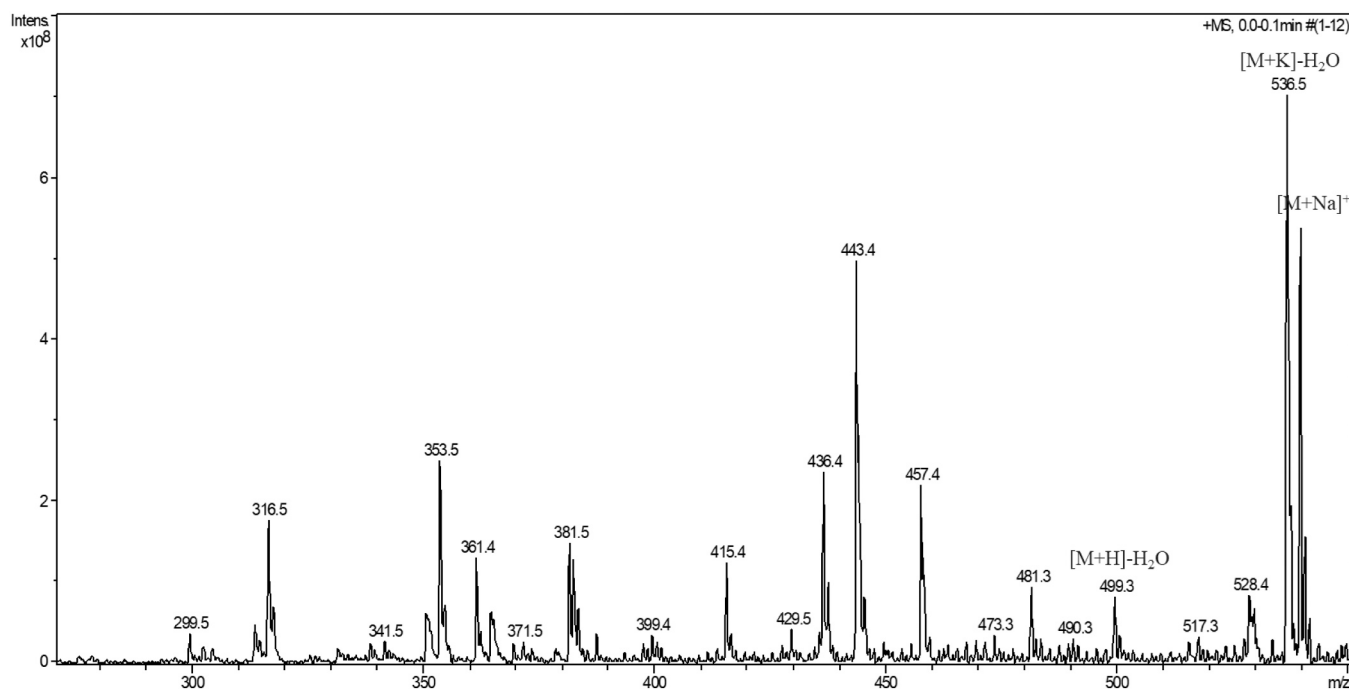


Fig. 6. The ESI-MS spectra of 1,5-dilauryl-D-arabitol ester.

FTIR spectra of the isolated purified products (Fig. S8) confirm the results from DSC. Only the three characteristic stretching vibrations of the -C-O secondary alcohol groups in the FTIR spectra of 1,5-DLDA and 1,5-DLX, which clearly demonstrates the high purity of these two products (Fig. S8A and S8B). A broad band with six overlapping peaks in the stretching vibration of -C-O primary and secondary alcohol spectral region (1120–1000 cm^{-1}) and a broadening of the -C=O ester band at 1737 cm^{-1} show the presence of the sorbitol monolauryl ester and free

lauric acid as trace impurities (Fig. S8C).

The ESI-MS spectrum of the purified D-arabitol ester, the presence of m/z : 539 $[\text{M}+\text{Na}]^+$, 537 $[(\text{M}+\text{K})-\text{H}_2\text{O}]$, 517.3 $[\text{M}+\text{H}]^+$, 499.3 $[(\text{M}+\text{H})-\text{H}_2\text{O}]$ demonstrated the formation of the diester (Fig. 6). This is consistent to the expected molecular mass for 1,5-dilauryl-D-arabitol diester (1,5-DLDA), of 516.8 g/mol.

The NMR spectra demonstrated that the reaction occurred with high regioselectivity, exclusively at the primary hydroxyl groups of the

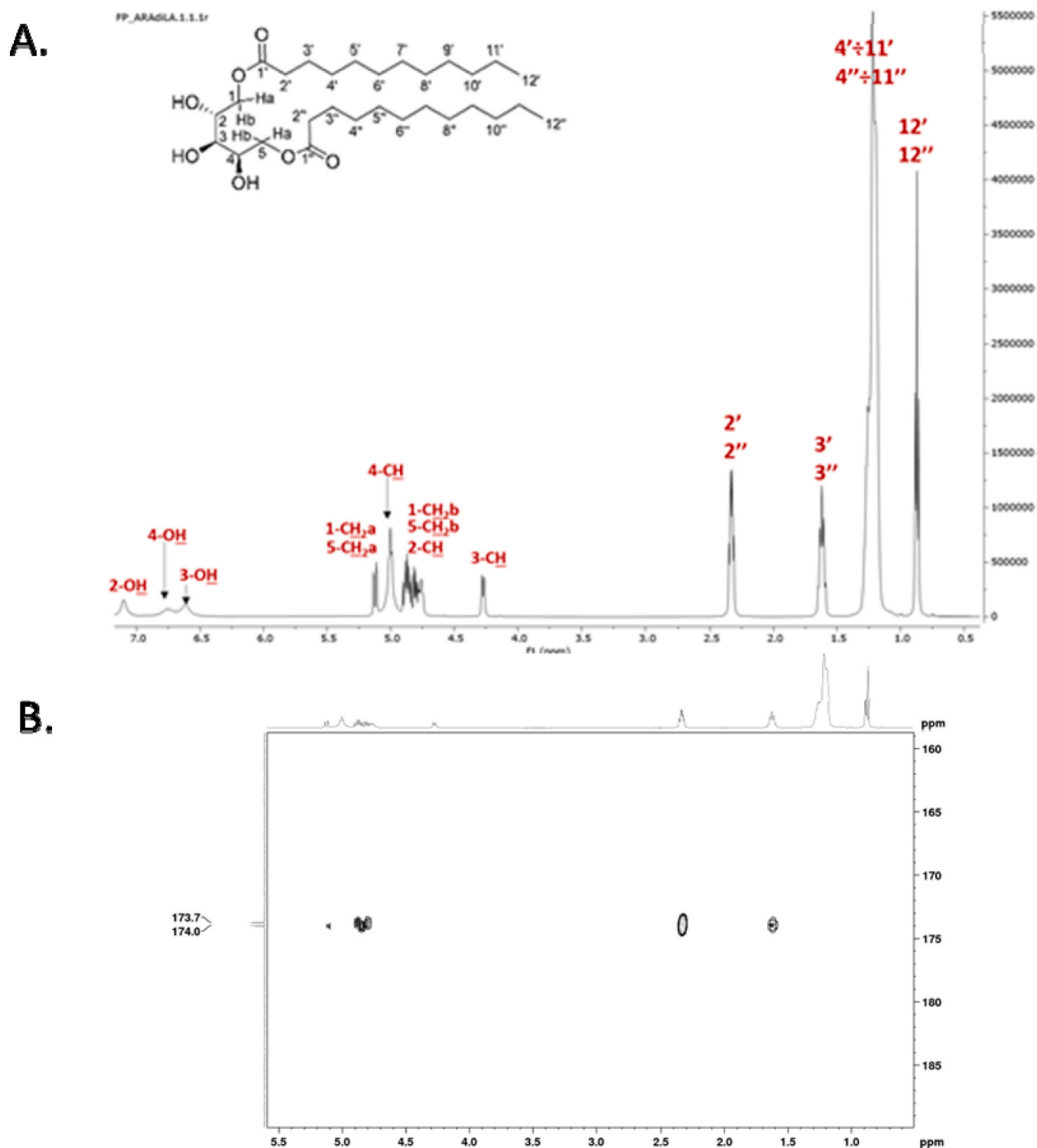


Fig. 7. A) ^1H NMR spectrum of 1,5-DLDA. B) Zoom of the 2D ^1H - ^{13}C HMBC spectrum, the long-distance coupling between the two carbonyl carbons (173.9 ppm and 173.7 ppm) and the four methylene protons at 5.51 ppm and 5.01–4.78 ppm, and with the methylene protons of the aliphatic acid chain.

polyols, namely OH-1 and OH-5 groups for D-arabitol and xylitol, and OH-1 and OH-6 for sorbitol. The ratio of the protons of the hydrocarbon chain of LA to the protons of the hydrocarbon chain of polyol in the ^1H NMR spectrum is 46 H:7 H for D-arabitol (Fig. 7A) and xylitol and 46 H:8 H for D-sorbitol, which corresponds to diesters of D-arabitol, xylitol and D-sorbitol, respectively. In the ^{13}C NMR spectrum of the D-arabitol ester, two signals are observed at 173.9 ppm and 173.7 ppm, which correspond to the two carbonyl carbon atoms in the diester. Although we would expect the two carbonyl carbons to be equivalent, they have different chemical shifts due to the presence of the two chiral carbons (2-C and 4-C) that have different configurations (2 R, 4 R). The presence of the two chirality centers at the 2-C and 4-C carbon atoms makes the protons from the methylene groups 1-CH₂ and 5-CH₂ diastereotopic.

The ^{13}C NMR spectra of 1,5-DLX and 1,6-DLDS show that the diesters are impurified in a small proportion with the monoester. Three signals are observed for carbonyl carbon and the number of peaks for the carbon atoms in the polyol chains for xylitol and D-sorbitol is double. Indeed, this is in good agreement with the findings from FTIR and DSC analyses discussed above. The DEPT-135 ^{13}C spectrum of 1,5-DLDA is shown in Fig. S9.

The coupling in the 2D ^1H - ^{13}C HSQCED spectrum (the direct couplings of the methylene and methine carbon atoms respectively have different phases) between the two methylene carbon atoms 1-CH₂ and 5-CH₂ from 68.1 ppm and 67.0 ppm and the protons at 5.51 ppm and 5.01–4.78 ppm, respectively, demonstrate that these protons are the 1-CH₂ and 5-CH₂ diastereotopic protons of the D-arabitol moiety.

In the 2D ^1H - ^{13}C HMBC spectrum, the long-distance coupling over three bonds between the two carbonyl carbons (173.9 ppm and 173.7 ppm) and the four methylene protons at 5.51 ppm and 5.01–4.78 ppm, respectively, is observed. We also observe the distance coupling over two and respectively three bonds between the two carbonyl carbon atoms and the methylene protons 2'-CH₂, 2''-CH₂ (2.35–2.31 ppm) and respectively 3'-CH₂, 3''-CH₂ (1.65–1.59 ppm). These long-distance couplings demonstrate that esterification with LA has occurred at both ends of the D-arabitol. According to the above, it can be concluded that the diester of D-arabitol with LA was obtained at the hydroxy groups in positions 1 and 5.

1,5-dilauryl-D-arabitol (1,5-DLDA), 1,5-dilauryl-xylitol and 1,6-dilauryl-D-sorbitol were identified as the main reaction products, showing an almost quantitative conversion of the monoester into diester and a high conversion of the substrate polyols. The fast and almost complete conversion of the monoester was also confirmed by HPLC that showed that even after a few hours of reaction, at half of the maximum conversion obtained under the optimized conditions, the diester was the main reaction product.

The high selectivity towards the diester can be related to the high preference of CalB for more hydrophobic substrates, thus the monoester could be a better substrate and lead to quantitative formation of the diester. If CalB operates preferentially at lipase-NADES interfaces, as MD simulations predicted, the monoester high solubility in the lipid phase in which the active site is orientated, and the very low concentration of polyols in the same phase, may support the high selectivity towards the diester formation.

3.6. Molecular docking study of enzymatic synthesis of sugar alcohol diesters in NADES containing carbohydrate polyols as hydrogen bond donors

A first molecular docking study was performed by using the diesters as substrates and Autodock Vina based on Monte Carlo search and rapid gradient-optimization conformational search coupled with a simple scoring function for free energy estimation [65]. As previously demonstrated [56] for substrates larger than dimers, AutodockVina was proper for docking studies probably because such larger substrates are characterized by higher flexibility and a larger number of torsions which can

be more efficiently accounted for by a Monte Carlo based protocol. Among the first 10 generated poses ranked by energy the productive poses were selected by identifying those where the substrate assumes a Near Attack Conformation (NAC) compatible with the productive attack of the catalytic serine (Ser105 of CalB) to the carbon atom of the ester groups of the ligands.

In Table 6 the values of the d as required and θ values compatible with NAC and the correspondent docking energy were included. Even if the values of the docking energies are not very low the protocol led to NAC compatible θ and d within 0.2/0.3 Å and the results are in good agreement with the experimental data was obtained.

3.7. A plausible reaction pathway for diesterification

Since the sole product of the reaction for arabitol/lauric acid mixtures was 1,5-DLDA we investigated by a combination of docking, energy minimization and short molecular dynamics runs a possible reaction pathway with the following steps:

- 1) lauric acid esterification with SER 105
- 2) transesterification with arabitol
- 3) second lauric acid esterification with SER 105 with the binding cavity occupied by the arabitol-mono-laurate
- 4) transesterification with arabitol-mono-laurate to produce the final diester product.

With standard choices of parameters all relevant bound configurations could be obtained by autodock vina, without any biasing condition. The only restraining bias was applied afterwards to properly orient the carbonyl oxygen in two of the docking poses, without modifications to the overall position of the molecules. In general, the docking study shows that all intermediate configurations along the reaction pathway do not produce any strain in the cavity of the protein, on the contrary they are obtained among the best configurations produced by the docking algorithm. These considerations are illustrated in Fig. 8, together with the hypothesized reaction pathway.

4. Conclusions

In this study three different carbohydrate polyol-based NADES, with physical and fluid properties suited for biocatalysis, were successfully obtained, and characterized. Immobilized CalB showed considerable catalytic activity and high thermal stability in NADES and was able to efficiently convert polyols into their lauryl esters with high yield. Computational simulations helped to rationalize the stability of the CalB active site in NADES, as well as the selectivity towards the catalysis of lauryl diesters. Through docking poses the plausibility of a diesterification reaction pathway could be demonstrated. The results are unprecedented in literature reports and open new pathways to further explore the potential of NADES as reaction medium for the enzymatic synthesis of long alkyl chain esters of oligosaccharides. The new approach that we propose in this paper has the huge advantage of high substrate concentration of at least 30 mol%, which is considerably higher than the common levels of 0.1–0.5 M in organic solvent systems. Integration of experimental work with computation and molecular modelling brings a higher level of understanding of interactions and

Table 6

AutoDock Vina data relative to the docking free energies computed only for the poses corresponding to the lowest energy values distances and angles were calculated taking as a reference the C atom of the -COOR ester group.

Substrate	E * abs [kcal/mol]	d [Å]	θ [°]
LA2AR	-1.2	3.5	93.5
LA2XL	-1.4	2.2	61.0
LA2SRB	-0.4	3.4	56.5

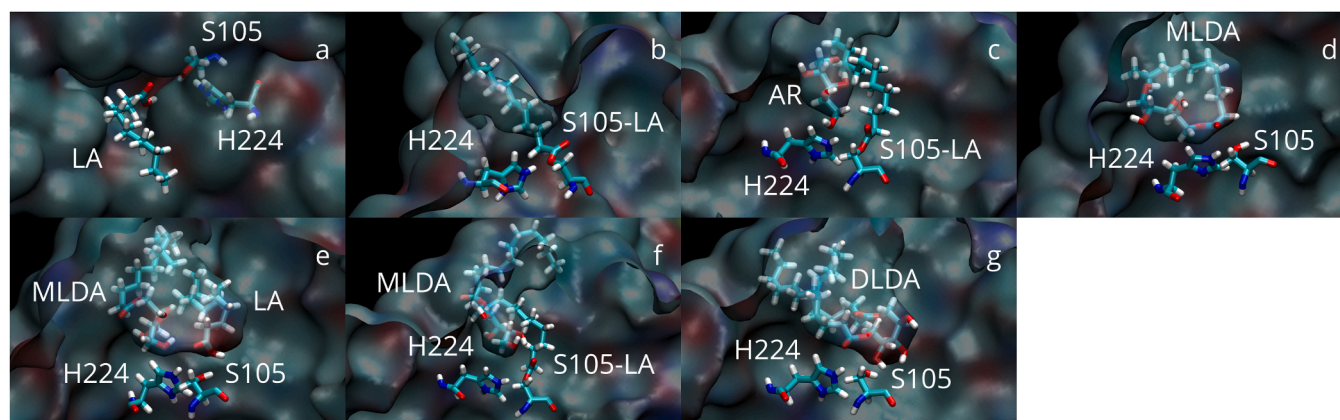


Fig. 8. A plausible diesterification reaction pathway illustrated through docking poses. In all figures the protein is shown by its molecular surface, residues SER 105 (esterified or not) and HIS 224 are shown in licorice and are readily identified by the presence of one and three nitrogen atoms, respectively, displayed in blue. Esterification of SER 105 was performed manually defining the corresponding topology in charmm36 forcefield and obtained by energy minimization starting from unreacted compounds. a) lauric acid in the cavity with the protonated carboxylic group in proper arrangement close to SER 105 hydroxyl group; b) the product of esterification of SER 105; c) arabitinol in the cavity with one end hydroxyl group close to the ester group of esterified SER 105; d) the monoester product of transesterification of arabitinol with SER 105; e) a second lauric acid in the cavity with the carboxyl group close to the hydroxyl group of SER 105; f) the product of esterification of SER 105 in the presence of the monoester in the cavity; g) the diester as the product of transesterification of the monoester with esterified SER 105.

mechanism of reaction in such complex systems. Moreover, the exclusive use of nontoxic, reusable biomaterials significantly decreases the environmental burden. In our experience, the utilization of R-NADES can be extended above lipase esterification reaction to other biocatalytic processes and could be implemented as well to chemo catalytic conversions.

The diesters of carbohydrate polyols may have valuable functional properties which would render them of interest for food, cosmetic, pharmaceutical, and detergent industries. The surfactant properties depend on the chemical structure, the degree of substitution of the hydroxyl groups, the chain length and saturation of fatty acid. The surfactant properties of several fatty acid monoesters of carbohydrate polyols, including D-sorbitol and xylitol, synthesized in lipase-catalyzed reactions were studied by Piao et al. [24], showing HLB values above 8 which makes them more suitable for the preparation of oil-in-water emulsions. To the best of our knowledge, pure fatty acid diesters of D-arabitol were not yet studied in this respect. Hydrophilic-lipophilic balance (HLB) of 1,5-DLDA calculated with Griffin equation is 5.8, that makes it more suitable for the preparation of water-in-oil emulsions. Our further studies will involve the assessment of surfactant properties of this compound, for possible utilization in cosmetic formulations.

CRediT author contribution statement

Alina Ramona Buzatu: Investigation, Validation, Writing – original draft, Writing – review and & editing; **Miguel Angel Soler:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision; **Sara Fortuna:** Investigation, Writing – original draft, Writing – review & editing; **Ozge Ozkilinc:** Investigation, Writing – original draft; **Diana Maria Dreavă:** Investigation; **Ioan Bîtcă:** Methodology, Investigation; **Valentin Badea:** Formal analysis, Investigation; **Paolo Giannozzi:** Funding acquisition, Writing – review & editing; **Federico Fogolari:** Methodology, Investigation, Writing – review & editing, Supervision, Funding acquisition; **Lucia Gardossi:** Methodology, Writing – review & editing; **Francisc Peter:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Resources, Funding acquisition; **Anamaria Todea:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition; **Carmen Gabriela Boeriu:** Conceptualization, Methodology, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Supervision, Administration, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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