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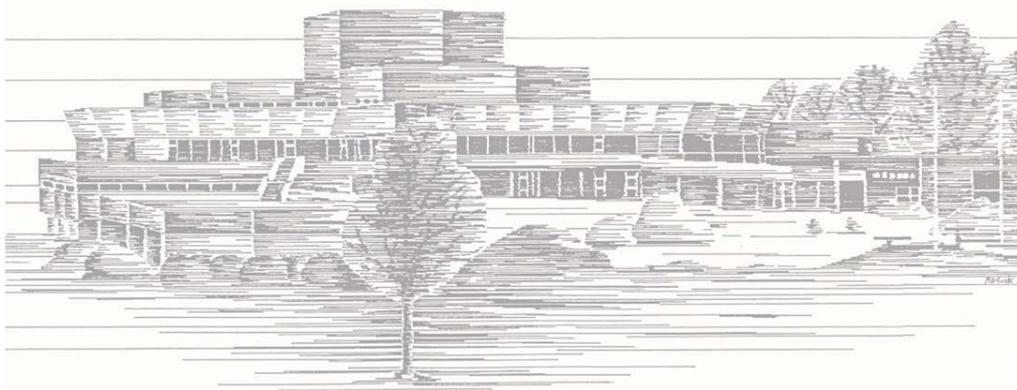
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Manuscript published as chapter of:

Book of Proceedings of STEP_sCON 2018

STEP_sCON 2018 – International Scientific Conference
on Sustainability and Innovation

7 December 2018, Leverkusen, Germany



**Technology
Arts Sciences
TH Köln**

***Pseudozyma antarctica* lipase B catalysis in deep eutectic solvents**

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Abstract

Pseudozyma antarctica Lipase B catalyzed esterification and transesterification in deep eutectic solvents (DES) was investigated in reaction systems with alcohols of different polarity. Coconut oil and crude biodiesel were deacidified successfully with non-immobilized CALBL and final acid values of 1.2 for biodiesel and 0.5 for coconut oil were obtained, while no esterification with ethanol was observed without DES. Water depletion of the lipid phase in the presence of water adsorbing DES causes this difference. Analysis of water contents revealed a 10 fold lower water content of the lipid phase in the presence of a second DES phase than in trials without utilization of DES. In contrast reactions of hydrophilic polyols are suppressed in the presence of DES. While the esterification of fructose and the transesterification with glycerol worked well in the polar solvent 2-methyl-2-butanol, almost no fructose esterification and a decreased transesterification with glycerol were observed in the presence of DES. Analysis of logP values of the substrates explains the substrate dependent differences in reactivity. The polar alcohols are probably bound strongly in the hydrogen-bonding network of the DES phase and are thus not available for lipase catalyzed reactions.

Abbreviations: DES: deep eutectic solvents; ChCl:U: urea based DES; ChCl:G: glycerol based DES; ChCl:F: fructose based DES; CALBL: *Pseudozyma antarctica* lipase B, 2M2B: 2-methyl-2-butanol

1. Introduction

Lipase B from *Pseudozyma antarctica* is a versatile enzyme with an exceptional temperature and solvent stability. In reverse hydrolysis and transesterification reactions the synthesis of all kinds of esters is possible; biodiesel and polyesters are examples of accessible products in this respect [1-4]. The immobilized enzyme was used successfully for the synthesis of polyol esters like monoglycerides and sugar esters [5-7], which are commercially available as emulsifiers in food and cosmetical products. Besides reverse hydrolysis and transesterification reaction, non-natural reactions like perhydrolysis and amide bond

formation are catalyzed by the enzyme [8],[9].

The production of cosmetic esters at low temperature with immobilized *P. antarctica* lipase B under continuous water removal with vacuum is done on industrial scale. It was shown that the enzymatic process is advantageous over the chemical one in regard to product quality and number of process steps [10,11]. Additionally, according to an overall LCA assessment, the enzymatic process is more eco-friendly than the chemical one [12]. Besides production of green emollients for the cosmetic industry, vacuum-based synthesis with immobilized *P. antarctica* lipase B is applied for the production of omega-3 fatty acid and conjugated linoleic acid based triglycerides were commercialized [13,14].

Deep eutectic solvents are a new class of green solvents obtained by complexation of quaternary ammonium salts with hydrogen bond donors [15]. DES, being hydrophilic and non-volatile, share some similarity with ionic liquids in this respect. From initial studies by Gorke *et al.* with immobilized *P. antarctica* lipase B [16], several lipase transformations in DES have been performed successfully [17-20]. Enzymatic synthesis of biodiesel was employed in DES with soybean oil as substrate and up to 88 % of ester was obtained within 24 h with commercially available Novozym 435 as biocatalyst [21,22]. A study of different DES revealed that the best biodiesel production was obtained in mixtures with choline acetate [23]. The half-life time of the immobilized lipase could be enhanced considerably in the presence of water and by reducing the amount of denaturing urea in the DES [24]. It was shown that free lipase B from *P. antarctica* is highly active in hydrophilic solvents including DES at low water contents [25]. The synthesis of cosmetic esters proceeded to near completion, because water adsorbing properties of DES shifted the equilibrium towards ester formation. The same effect was originally observed in esterification reactions in the presence of glycerol [26]. Using the water adsorbing effect of DES, biodiesel in high yields and a low content of residual fatty acids could be synthesized successfully with free *P. antarctica* lipase by our group [27].

The aim of this work was the evaluation of lipase B from *Pseudozyma antarctica* in DES for the synthesis of polyol esters and for esterification with short chain alcohols. Esterification with short chain alcohols was evaluated in deacidification reactions with biodiesel and coconut oil. Preesterification of crude acidic coconut oil may be used as preparatory step for fatty alcohol production via subsequent alkaline transesterification and hydrogenation. Deacidification of biodiesel could be interesting for enzymatic processes starting from acid rich raw materials like deodorizer distillates and tall oil fatty acids. Transesterification of olive oil with glycerol to yield partial glycerides and the synthesis of fructose esters were chosen as example compounds for hydrophilic polyol transformations.

2. Materials and methods

2.1 Materials

All chemicals were of synthesis grade. BSTFA + 1% TMCS and reference standards for GC

calibration were from Sigma Aldrich (Steinheim, Germany). Liquid and immobilized lipase B preparations from *Pseudozyma antarctica* (Lipozyme CALBL and Novozym 435) are products from Novozymes A/S and Accurel MP 1000 is a macroporous polypropylene from Membrana. Refined rapeseed oil, olive oil and coconut oil were obtained from a local supermarket. All other chemicals were brought from Carl Roth GmbH (Karlsruhe, Germany) or VWR International (Darmstadt, Germany).

2,2 Preparation of DES, immobilized lipase and lipid substrates

Choline chloride and urea (ChCl:U) or choline chloride and glycerol (ChCl:G) in a molar composition of 1:2 were weighed directly into bottles, sealed immediately and mixed by vigorous shaking. Liquid DES were obtained within a few hours by incubation on an orbital shaker (Infors HT) at 250 rpm and 60°C. ChCl:F was produced in the same way by mixing choline chloride and fructose in a molar composition of 1:3.

Lipozyme CALBL was immobilized onto Accurel MP 1000 by adsorption as described in [28]. 10 g of Accurel MP 1000 were soaked for 30 min in ethanol. Ethanol was removed and 40 ml of water and 20 ml of Lipozyme CALBL were added and incubated on a rotary shaker overnight at room temperature. The immobilized enzyme was filtered, dried on a sheet of paper and stored at 8 °C.

Biodiesel was produced chemically from refined rapeseed oil. 500 g of oil and 100 g of dried ethanol were heated in stirred reactor to 80 °C and the reaction was started by addition of 8 g sodium ethylate in ethanol (21 wt%). The mixture was stirred under reflux at 95 °C for 2 hours and the glycerol phase was separated after cooling. The lipid phase was acidified with diluted phosphoric acid to a pH of < 5 and the hydrophilic phase was separated. The lipid phase was washed twice with 200 ml of water and then dried with a rotary evaporator. Acid value of the biodiesel was adjusted by adding oleic acid.

20 g of coconut oil were mixed with 3 ml of water and 100 µl of Lipozyme CALBL and incubated for 24 h on a rotary shaker at 45 °C. The water phase was separated and the lipid phase was heated to 80 °C for 1 h to deactivate the enzyme. After analyzing the acid value the partially hydrolyzed coconut oil was mixed with refined coconut oil to adjust the acid value.

2,3 Biocatalytic reactions

In a typical deacidification experiment 20 g of lipid (acidified biodiesel or acidified coconut oil) were mixed with 20 g of DES (CHCl:U or CHCl:G), 1 g ethanol and 1 g of water and the reaction was started by addition of 100 µl of Lipozyme CALBL. The mixtures were incubated in sealed bottles at 30 °C and 250 rpm on an Infors Multitron rotary shaker. Variations in composition are outlined in the results section in detail. Comparative examples were done with 0.1 g of Novozym 435, 2 g of ethanol and no water addition. Samples were taken at different time intervals for further analysis.

For fructose ester syntheses 4.2 g of lauric acid, 4 g of fructose and 20 g ChCl:F were mixed

in sealed flasks, 0.5 ml liquid Lipozyme CALBL and 0.5 ml water or 0.5 g immobilized CALBL without additional water were added and the mixtures were incubated at 250 rpm on an Infors Multitron rotary shaker. In comparative trials 30 g of 2-methyl-2-butanol and 1 g immobilized CALBL were used. Reactions with immobilized enzymes were run at 60 °C while reactions with liquid CALBL were incubated at 50 °C. In trials with water removal 30 g of oleic acid and 20 g fructose were mixed in a stirred reactor with 150 g 2-methyl-2-butanol and 5 g immobilized CALBL was added. A column packed with 20 g molecular sieve 3A and a cooler was mounted on top of the reactor. The temperature was set to 65 °C and vacuum was applied until a reflux was observed.

10 g of olive oil, 5 g of glycerol and 0.5 g of water were mixed with 10 g of 2-methyl-2-butanol in sealed bottles for monoglyceride synthesis by glycerolysis. After addition of 0.2 g of Novozym 435 the mixtures were incubated at 45 °C and 250 rpm on an Infors Multitron rotary shaker. 10 g of 2-methyl-2-butanol and 20 g of ChCl:U were used in experiments with a mixed solvent system. Samples were withdrawn from the reaction mixtures for further analysis at different time intervals.

2.4 Analytical methods and calculations

In routine analysis 10 µL of the lipid phase were dissolved in 940 µl of heptane in a GC-vial and 50 µl silylation agent (BSTFA + 1% TMCS) were added. The samples were sealed and incubated in an oven at 80°C for one hour. Analysis was done with a Shimadzu GC 2010 Plus using a MTX Biodiesel TG column (RESTEK, length 14m, Ø 0.53 mm, film thickness 0.16 µm) connected to a FID detector with helium as carrier gas and a temperature gradient from 75°C to 410 °C. A split ratio of 5 with an injection volume of 1.5 µl was applied.

Calculation of fatty acid content from acid values was done with molecular masses of 282 g/mol for oleic acid, 200 g/mol for lauric acid and 211 g/mol for mixed coconut fatty acids. An acid value of 10 corresponds to 5 % oleic acid, 3.6 % lauric acid or 3.8 % mixed coconut fatty acids. Analysis of lipids was done according to the DGF standard method C-V 2 “Acid value and free fatty acid content (Acidity)” [29] with 0.2 – 2 g of lipid sample dissolved in ethanol. Acid values of samples containing organic solvents were normalized to the lipid content in the organic phase. The acid value was determined by titration with 0.1 M KOH solution against phenolphthalein using a Metrohm Dosimat E535 and calculated with the following equation:

$$AV = \frac{\text{ml KOH consumed} \cdot [\text{KOH}] \cdot M_{\text{KOH}}}{\text{g sample}}$$

The water amount of the lipid phase was determined according to the method of Fischer with an automatic titrator (Metrohm 870 KF Titrinoplus). LogP values were calculated with the program MedChem Designer from Simulations Plus Inc. using the company’s internal calculation model (S+logP) or the logP values according to Moriguchi (MlogP). LogP values of DES could not be given precisely, as DES are multi-component mixtures.

3. Results and discussion

3.1 Deacidification of biodiesel and coconut oil

Acid values of biodiesel and coconut oil were adjusted to 11.5 and 10.9 respectively, which correspond to fatty acid contents of 5.8 % for biodiesel and 4.1 % for acidified coconut oil. Comparative deacidification trials were done in the presence of DES and without DES (Fig. 1). An acid value reduction was observed in the presence of DES in all experiments, while acid values increased in solvent free experiments.

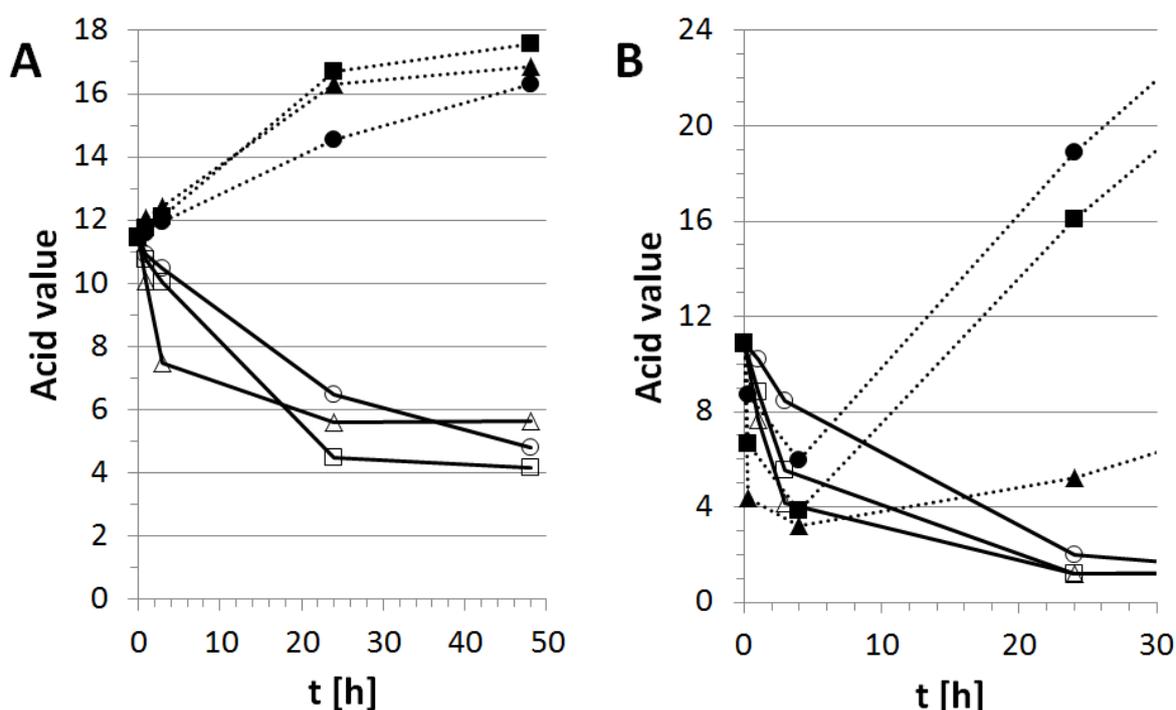


Fig. 1: Time dependent deacidification of biodiesel (A) and coconut oil (B) in dependence of enzyme concentration and presence of DES; open symbols, straight lines = with DES; closed symbols, dotted lines = without DES; A) ●, ○ = 20 μ l CALB L; ■, □ = 100 μ l CALB L; ▲, △ = 1000 μ l CALB L, B) ●, ○ = 20 μ l CALB L; ■, □ = 50 μ l CALB L; ▲, △ = 100 μ l CALB L.

The acid value of biodiesel under standard reaction conditions with 1000 μ l of ethanol and 1000 μ l of water was decreased to 4 (Fig. 1A), which corresponds to a residual acid content of 2 %. A clear dependence of reaction velocity was observed at different enzyme concentrations. The highest velocities were obtained with 1000 μ l of liquid CALBL; however, a final acid value of > 5 remains in the mixture, probably caused by the water content of the liquid enzyme preparation. To elucidate the influence of water in more detail, experiments with different ethanol to water ratios were performed (Table 1). Low water conditions lead to lower acid values; however negatively influenced the enzyme activity. Increasing the concentration of ethanol lead to enzyme deactivation; thus low concentrations of water and low concentrations of short chain alcohol have to be selected for optimum reactivity. Best results with an acid value of 1.2 were obtained with 300 μ l of water, though lower concentrations of water may result in lower acid values after prolonged reaction times.

Dosage of ethanol at low water concentration could be a means to further reduce the acid content to meet a value of 0.5 corresponding to 0.25 % fatty acid, which is the maximum allowed concentration according to the EN 14214 European Biodiesel norm [30].

Trials with acidified coconut oil revealed some differences to biodiesel deacidification. While the influence of enzyme concentration on reaction velocity and the decrease of acid values in the presence of DES are comparable, overall reaction velocities and equilibrium compositions differ significantly (Fig. 1B). Deacidification of coconut oil is faster than esterification in biodiesel at the same enzyme concentration. In the presence of DES final acid values of slightly higher than 1 corresponding to acid contents of 0.4 were reached under standard reaction conditions and the final acid values were less dependent on enzyme concentration. Complex behavior, with deacidification at the beginning and a steep rise in acid value at longer reaction times, was observed in trials without DES. GC analyses revealed, that enzymatic transesterification took place simultaneously. With the formation of partial glycerides the mixture becomes more hydrophilic and obtains some emulsifying properties. Better solubility of water in the partial glycerides containing coconut oil may be the reason for the increase in hydrolysis observed after 24 h of reaction time. Additionally ethanol concentration is decreased by transesterification leading to a higher surplus of water and thus a change in the hydrolysis / esterification equilibrium of the system. In trials with DES a slight increase in acid values is only observed at reaction times exceeding 120 h. A less pronounced effect of ethanol to water ratio was observed with coconut oil in comparison to experiments with biodiesel (Table 1). Lowest acid values of 0.5 – 0.6, corresponding to 0.2 % of free fatty acids, were obtained with 0 – 100 μl of water added to the reaction mixture.

Table 1: Acid values after deacidification of biodiesel (left) and coconut oil (right) in dependence of ethanol / water composition (* = equilibrium was not reached after 120 h)

Substrate	Composition [μl] ethanol/water	Acid value	Substrate	Composition [μl] ethanol/water	Acid value
Biodiesel	1000 / 0	6.7*	Coconut oil	1000 / 0	0.5
Biodiesel	1000 / 100	4.1*	Coconut oil	1000 / 100	0.6
Biodiesel	1000 / 300	1.2	Coconut oil	1000 / 300	1.0
Biodiesel	1000 / 500	2.6	Coconut oil	1000 / 500	0.8
Biodiesel	1000 / 1000	4.1	Coconut oil	1000 / 1000	1.2

3.2 Polyol esters by synthesis and glycerolysis

The synthesis of fructose esters starting from fructose and lauric acid was compared in reaction systems with DES and 2-methyl-2-butanol (2M2B) as solvents. In 2M2B lauric acid

and fructose are solubilized and form a system composed of one liquid phase plus residual non-dissolved fructose as solid second phase. The reaction proceeded smoothly towards ester formation in the presence of immobilized CALBL with a significant improvement in esterification velocity and shift of reaction equilibrium towards esterification in the presence of water absorbing molecular sieves (Fig. 2A). CALBL esterifies preferably the primary hydroxyl groups of fructose and thus mono- and diesters are formed as outlined in Fig. 2B on the basis of the cyclic fructofuranose structure. Almost equimolar amounts of fructose mono- and dilaurate were detected by GC analysis accordingly. In DES a different phase behavior was observed. Fructose is dissolved completely in the DES phase and a liquid two-phase system consisting of a hydrophobic lauric acid and a hydrophilic sugar – DES phase is generated. The two-phase system was not suited well for the synthesis of fructose esters neither with immobilized nor with non-immobilized lipase. Less than 20 % lauric acid was esterified in all cases, though fructose was completely dissolved in the reaction system.

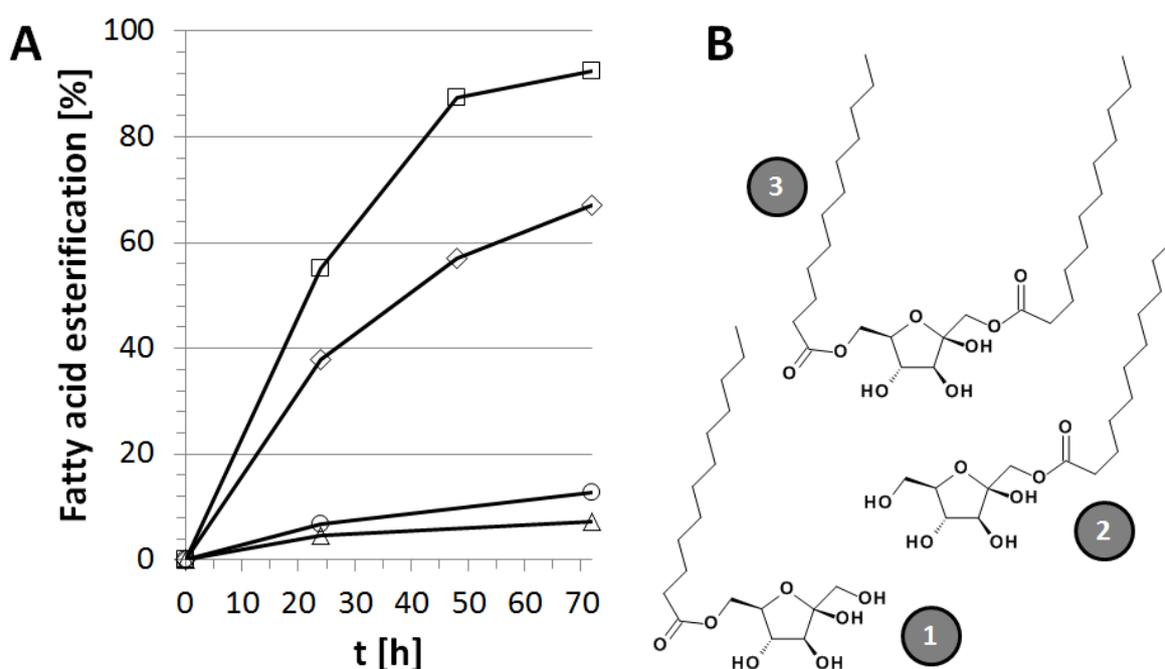


Fig. 2: A) Time dependent esterification in fructose ester synthesis: \triangle = free Lipozyme CALB L dissolved in DES, \circ = immobilized Lipozyme CALB L in DES, \diamond = immobilized Lipozyme CALB L in 2-methyl-2-butanol without water removal, \square = immobilized Lipozyme CALB L in 2-methyl-2-butanol with molecular sieve based water removal; **B)** Fructose monolaurate (1, 2) and fructose dilaurate (3) obtained by enzymatic esterification of the primary hydroxyl groups of fructofuranose.

The immobilized CALBL catalyzed transesterification of olive oil with a surplus of glycerol was evaluated in the presence of 2M2B and in a mixed 2M2B / DES solvent system. In 2M2B a single-phase system composed of polar solvent, hydrophobic olive oil and hydrophilic glycerol is formed. Triglycerides were transesterified to > 98 % and an equilibrium mixture with 60 % monoglycerides and 10 % diglycerides was yielded (Fig. 3A). The addition of 2 % of water resulted in the formation of around 25 % of fatty acids through hydrolysis, thus water

is competing with glycerol in the single phase system. By addition of DES a two-phase system formed, because 2M2B and DES are only partially miscible and phase behavior as well as equilibrium yields changed significantly. Transesterification only proceeded to 90 % of triglyceride consumption and a partial glyceride mixture with 35 % of diglycerides and 20 % of monoglycerides was obtained (Fig. 3B). Transfer of glycerol into the hydrophilic DES phase and thus depletion of glycerol concentration in the 2M2B phase may explain the different equilibrium composition obtained. The formation of fatty acid as byproduct is even stronger than in the pure 2M2B system, though water is adsorbed strongly by the DES phase [25].

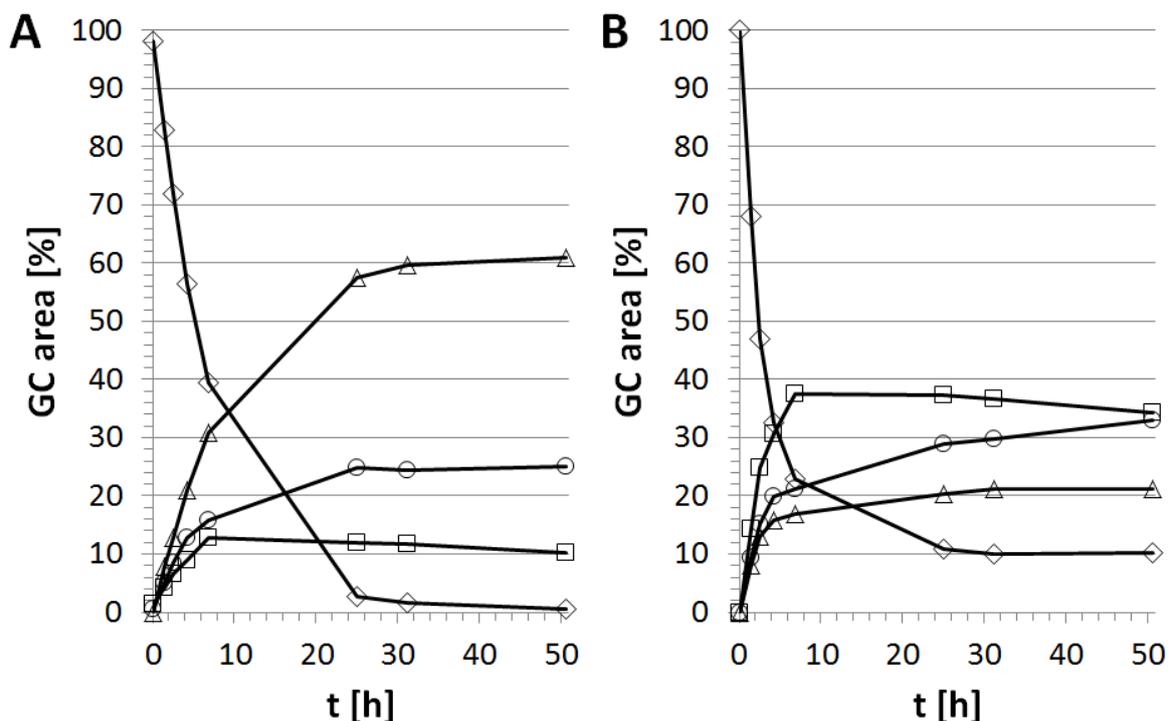


Fig. 3: Time dependent glycerolysis of olive oil in 2-methyl-2-butanol (A) and a mixed 2-methyl-2-butanol / DES solvent system (B); ○ = fatty acids, ◇ = triglycerides, □ = diglycerides, △ = monoglycerides.

3.3 Comparative analysis of lipase B catalysis in DES

Addition of hydrophilic DES solvents to hydrophobic lipid substrates results in the formation of two-phase systems, which can shift the equilibrium towards ester synthesis and prevent the formation of fatty acid byproducts in transesterification reactions through water adsorption [27][25],[27]. This effect was employed successfully for the deacidification of crude biodiesel and coconut oil with ethanol as polar alcohol (Fig. 4). Analysis of water contents in biodiesel deacidification support the water adsorbing effect with 0.4 mg/ml of water in the presence of DES and 4 mg/ml in the lipid phase without DES. Similar water concentrations of 0.3 mg/ml in the lipid phase with DES and 3.4 mg/ml without DES were obtained with immobilized CALBL.

Trials with the hydrophilic polyols fructose and glycerol revealed that the solubility of the polyols is high in DES; however, the water adsorbing effect of DES does not positively influence esterification yield and does not suppress fatty acid formation in transesterification reactions. Hence, DES induced equilibrium shifts are substrate dependent and the positive effects found for alcohols ranging from long chain fatty alcohols to polar methanol cannot be transferred to all lipase substrates.

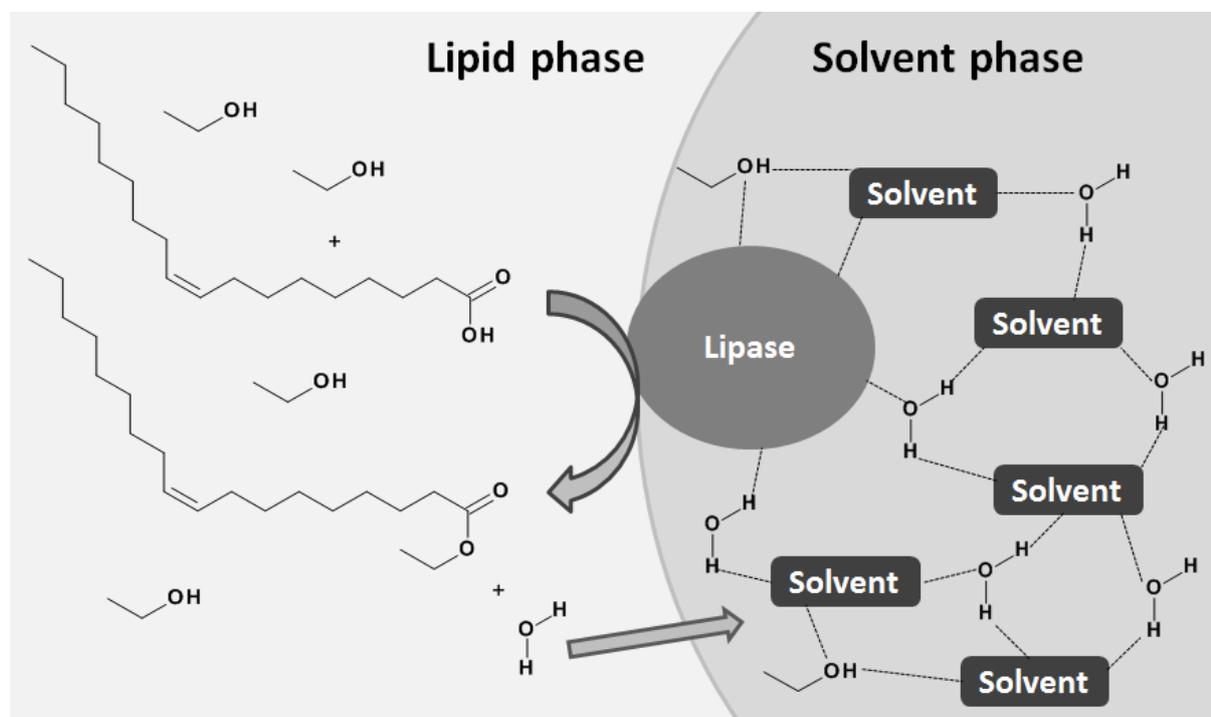


Fig. 4: Scheme of esterification of oleic acid with ethanol as model for biodiesel deacidification. Water is more strongly adsorbed to the hydrophilic phase than the polar alcohol ethanol and the equilibrium is shifted towards ethyl oleate synthesis.

Table 2: LogP values for solvents and substrates used for ester synthesis and transesterification

Substance	logP (S+logP)	logP (MlogP)	Substance	logP (S+logP)	logP (MlogP)
Lauric acid	4.9	2.8	Glycerol	- 1.9	- 1.4
Oleic acid	7.2	4.3	Fructose	- 2.5	- 2.5
Decanol	4.1	2.9	ChCl:U	< - 2	< - 2
Ethanol	- 0.3	- 0.2	ChCl:G	< - 2	< - 2
Methanol	- 0.7	- 0.8	ChCl:F	< - 2	< - 2
Water	- 1.1	- 2.1	2-methyl-2-butanol	0.9	1.2

Analysis of logP values of substrates and solvents (Table 2) can explain the varying effects of DES in dependence of alcohol substrates. While the polar alcohols methanol and ethanol are more hydrophobic than water, logP values of glycerol and fructose are comparable to or even lower than that of water. Therefore partitioning of substrates and water into the lipid and DES phases will be different; for substrates less polar than water a relative increase in substrate to water ratio in the lipid phase is expected, while for substrates similar in logP no significant changes of substrate to water ratio in the lipid phase are predicted. An increase of substrate to water ratio in the lipid phase favors esterification, which was observed for all substrates less polar than water. In contrast no shift towards esterification is expected for hydrophilic polyols and additionally hydrophilic substrate concentration will be low in the lipid phase in the presence of DES, which results in a decreased ester synthesis in comparison to the 2M2B solvent system, which dissolves significant amounts of hydrophilic polyols. The solubility effect is not counterbalanced by the better overall solubility of fructose or glycerol in DES.

Conclusions

DES are suitable solvents for lipase catalyzed esterification and transesterification reactions, which influence esterification yield and fatty acid byproduct formation in dependence of substrate polarity. While no positive effects were observed for hydrophilic polyols with logP values similar to water, an increase in esterification yield was obtained for all polar and non-polar alcohols with logP values higher than water. Thus we could show that the two-phase reaction system is well suited for esterification and transesterification reactions with substrates more hydrophobic than water.

References

- [1] Y. Shimada, Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase, *J. Am. Oil Chem. Soc.* 76, (1999) 789–793.
- [2] T. Tan, J. Lu, K. Nie, L. Deng, F. Wang, Biodiesel production with immobilized lipase: a review, *Biotechnol Adv.* 28 (2010) 628–634.
- [3] B. Chen, J. Hu, E.M. Miller, W. Xie, M. Cai, R.A. Gross, *Candida antarctica* Lipase B Chemically Immobilized on Epoxy-Activated Micro- and Nanobeads: Catalysts for Polyester Synthesis, *Biomacromolecules* 9(2008) 463–471.
- [4] C. Ortiz, M.L. Ferreira, O. Barbosa, J.C.S. dos Santos, R.C. Rodrigues, Á. Berenguer-Murcia, L.E. Briand, R. Fernandez-Lafuente, Novozym 435: the “perfect” lipase immobilized biocatalyst?, *Catal. Sci. Technol.*, 9 (2019) 2380–2420.
- [5] U.T. Bornscheuer, Lipase-catalyzed syntheses of monoacylglycerols, *Enz. Microb. Technol.* 17 (1995) 578-586.

- [6] R.T. Otto, H. Scheib, U.T. Bornscheuer, J. Pleiss, C. Syldatk, R.D. Schmid, Substrate specificity of lipase B from *Candida antarctica* in the synthesis of arylaliphatic glycolipids, *J. Mol. Catal. B: Enz.* 8 (2000) 201–211.
- [7] T. Kobayashi, Lipase-catalyzed syntheses of sugar esters in non-aqueous media, *Biotechnol. Lett.* 33 (2011) 1911–1919.
- [8] C. Carboni-Oerlemans, P. Dominguez de Maria, B. Tuin, G. Bargeman, A. van der Meer, R. van Gemert, Hydrolase-catalysed synthesis of peroxy-carboxylic acids: Biocatalytic promiscuity for practical applications, *J. Biotechnol.* 126 (2006) 140–151.
- [9] V. Gotor-Fernández, E. Busto, V. Gotor, *Candida antarctica* Lipase B: An Ideal Biocatalyst for the Preparation of Nitrogenated Organic Compounds, *Adv. Synth. & Catal.* 348 (2006) 797–812.
- [10] G. Hills, Industrial use of lipases to produce fatty acid esters. *Eur. J. Lipid Sci. Technol.* 105 (2003) 601–607.
- [11] M.B. Ansorge-Schumacher, O. Thum, Immobilized lipases in the cosmetics industry. *Chem. Soc. Rev.* 42 (2013) 6475–6490.
- [12] O. Thum, K.M. Oxenbøll, Biocatalysis – A Sustainable Method for the Production of Emollient Esters, *SOFW Journal* 134 (2008) 44–47..
- [13] U. Schörken, P. Kempers, Lipid biotechnology: Industrially relevant production processes, *Eur. J. Lipid Sci. Technol.* 111 (2009) 627–645.
- [14] S. Busch, P. Horlacher, S. Both, A. Westfechtel, U. Schörken, Green synthesis routes towards triglycerides of conjugated linoleic acid, *Eur. J. Lipid Sci. Technol.* 113 (2011) 92–99.
- [15] A.P. Abbott, G. Capper, D.L. Davies, R.K. Rasheed, V. Tambyrajah, Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* 1, (2003) 70–71.
- [16] J.T. Gorke, F. Sreenc, R.J. Kazlauskas, Deep Eutectic Solvents for *Candida Antarctica*, *ACS Symposium Serie*, 1038 (2010) 169–180.
- [17] E. Durand, J. Lecomte, B. Baréa, G.E. Piombo, E. Dubreucq, P. Villeneuve, Evaluation of deep eutectic solvents as new media for *Candida antarctica* B lipase catalyzed reactions, *Process Biochem.* 47 (2012) 2081–2089.
- [18] E. Durand, J. Lecomte, P. Villeneuve, Deep eutectic solvents: Synthesis, application, and focus on lipase-catalyzed reactions, *Eur. J. Lipid Sci. Technol.* 115 (2013) 379–385.
- [19] P. Xu, G. Zheng, M.Zong, N. Li, W. Lou, Recent progress on deep eutectic solvents in biocatalysis. *Bioresour.. Bioprocess.* 4 (2017) 34.
- [20] M. Pätzold, S. Siebenhaller, S. Kara, A. Liese, C. Syldatk, D. Holtmann, Deep Eutectic Solvents as Efficient Solvents in Biocatalysis. *Trends Biotechnol.* (2019) 10.1016/j.tibtech.2019.03.007.

- [21] H. Zhao, G.A. Baker, S. Holmes, New eutectic ionic liquids for lipase activation and enzymatic preparation of biodiesel, *Org. Biomol. Chem.* 9 (2011). 1908–1916.
- [22] H. Zhao, C. Zhang, T.D. Crittle, “holine-based deep eutectic solvents for enzymatic preparation of biodiesel from soybean oil, *J. Mol. Catal. B: Enz.* 85–86 (2013) 243–247.
- [23] Z.L. Huang, B.P. Wu, Q. Wen, T.X. Yang, Z. Yang, Deep eutectic solvents can be viable enzyme activators and stabilizers, *J. Chem. Technol. Biotechnol.* 89 (2014) 1975–1981.
- [24] E. Durand, J. Lecomte, B. Baréa, P. Villeneuve, Towards a better understanding of how to improve lipase catalyzed reactions using deep eutectic solvents based on choline chloride, *Eur. J. Lipid Sci. Technol.* 116 (2014) 16–23.
- [25] B. Kleiner, U. Schörken, Native lipase dissolved in hydrophilic green solvents: A versatile 2-phase reaction system for high yield ester synthesis, *Eur. J. Lipid Sci. Technol.* 117 (2014) 167–177.
- [26] Y. Watanabe, T. Nagao, Y. Nishida, Y. Takagi, Y. Shimada, Enzymatic production of fatty acid methyl esters by hydrolysis of acid oil followed by esterification, *J. Am. Oil Chem. Soc.* 84 (2007) 1015–1021.
- [27] B. Kleiner, P. Fleischer, U. Schörken, Biocatalytic synthesis of biodiesel utilizing deep eutectic solvents: A two-step-one-pot approach with free lipases suitable for acidic and used oil processing, *Process Biochem.*, 51 (2016) 1808-1816.
- [28] M. Persson, I. Mladenoska, E. Wehtje, P. Adlercreutz, Preparation of lipases for use in organic solvents, *Enz. Microb. Technol.* 31 (2002) 833-841.
- [29] Deutsche Einheitsmethoden zur Untersuchung von Fetten, Fettprodukten, Tensiden und verwandten Stoffen (DGF standard methods), 2nd Edition. Wissenschaftliche Verlagsgesellschaft Stuttgart; (2015) ISBN 978-3-8047-3024-3.
- [30] EN 14214:2012: Liquid petroleum products - Fatty acid methyl esters (FAME) for use in diesel engines and heating applications - Requirements and test met