Deacetylation of mannosylerythritol lipids in hydrophobic natural deep eutectic solvents

RESEARCH ARTICLE

Deacetylation of Mannosylerythritol Lipids in Hydrophobic Natural Deep Eutectic Solvents

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Abstract: Mannosylerythritol lipids (MELs) are a promising group of biosurfactants due to their high fermentation yield, self-assembly and biological activity. During fermentation by *Pseudozyma aphidis*, a mixture of MELs with different levels of acylation is formed, of which the fully deacetylated form is the most valuable. In order to reduce the environmental impact of deacetylation, an enzymatic process using natural deep eutectic solvents (NADES) has been developed. We tested the deacetylation of a purified MELs mixture with immobilized *Candida antarctica* lipase B enzyme and 2-ethylhexanol as co-substrate in 140 h reactions with different NADES. We identified hydrophobic NADES systems with similar yields and kinetics as in pure 2-ethylhexanol solvent. Our results indicate that deacetylation of MELs mixtures in NADES as a solvent is possible with yields comparable to pure co-substrate and that hydrophobic NADES without carboxylic acid compounds facilitate the reaction to the greatest extent.

Introduction

Natural deep eutectic solvents (NADES) have emerged in recent years as a green alternative to common organic solvents due to their benign properties[1]. NADES are mixtures of two or more naturally derived compounds (like primary metabolites or resources from waste streams, such as quaternary ammonium salts, amines, sugars, organic and amino acids)[2], which form strong secondary interactions in the form of an intermolecular hydrogen bonding network[3]. This leads to the stabilization of the mixtures in the liquid state (with the minimum freezing temperature at the eutectic composition), which facilitates their use as solvents. NADES have low toxicity, they are non-volatile, non-flammable, biodegradable and often biocompatible[4,5]. In addition, NADES are considered designer solvents, which means that their properties can be tailored to the specific application by changing the components and their ratio in the mixture[6,7]. A drawback of NADES is their often high viscosity (due to the strong secondary interactions in the system), which can limit mass transfer in applications[8].

Due to their beneficial properties and versatility, the application of NADES is considered in many fields. The possible applications have been reviewed by Zhang et al.[9]. In practice, NADES are currently used in electrochemistry[10] and separation processes[11]. However, their use in biochemistry is also being considered. The first enzymatic reaction in a eutectic mixture was described by Gill and Vulfson[12] (while the first enzymatic reaction explicitly in DES was reported by Gorke et al.[13]), and since then many experimental and simulation studies have been carried out on the subject[14–17]. Recently, Xu et al. wrote a review on the experimental research in this field[18]. Two main concerns in carrying out enzymatic reactions in NADES are the enzyme-solvent interactions (changes in enzyme structure or complete denaturation)[19] and the viscosity of the solvent[20]. Since many of the NADES used have components that can form strong interactions with the enzyme residues (e.g., urea or carboxylic acids), their use can alter the behavior of the catalyst. However, experimental studies observed similar or in some cases even higher activities of the enzyme than in common organic solvents[13,21,22]. In addition, simulations also confirmed the stability of the enzyme structure in NADES[19,23–25]. The other possible issue is the significantly higher viscosities of many NADES than common solvents[8,20]. Nevertheless, experimental research has not reported any mass transfer limitations in NADES[13,21,22].

One interesting application for NADES in an enzyme reaction of the deacetylation of mannosylerythritol lipids (MELs), a process that has already been researched in our group using organic solvents. NADES would be a way to do this in a more environmentally friendly way, with a product that is free from traces of organic solvents.

Mannosylerythritol lipids are a promising class of biosurfactants[26]. They represent a green and circular alternative to chemical surfactants as they are produced from renewable resources by microorganisms[27]. MELs are composed of a 4O-β-D-mannopyranosyl-erythritol hydrophilic head and one or more fatty acid chains as hydrophobic tails. They are produced by fungi of the genus *Ustilago* or yeasts of the genus *Pseudozyma* during the fermentation of vegetable oils with high yields exceeding 100 g/L[28]. The MEL class consists of many similar molecules and it is divided by the degree of acetylation, the amount of fatty acid chains and sometimes by their chirality[29]. The term MEL is used here to refer to the commonly produced di- and fully deacetylated MEL. These MELs are named MEL-A, MEL-B, MEL-C or MELD according to their degree of acetylation at C6' and C4' (see Figure 1).

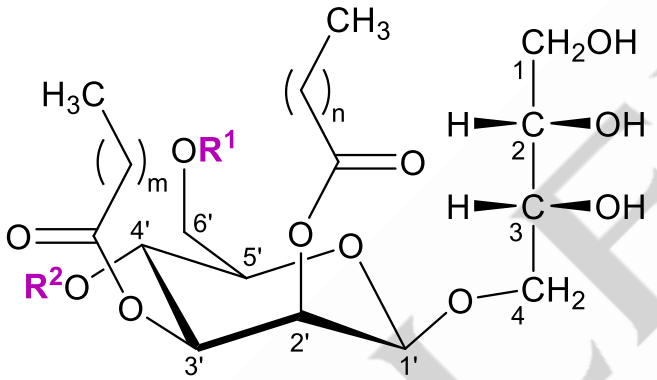


Figure 1. General structure of mannosylerythritol lipids (MELs). MEL-A: $R^1 = R^2 = Ac$; MEL-B: $R^1 = Ac$, $R^2 = H$; MEL-C: $R^1 = H$, $R^2 = Ac$; MEL-D: $R^1 = R^2 = H$.

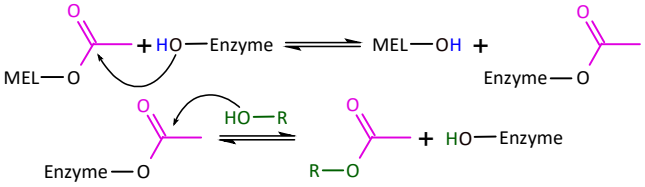
MELs are a subject of interest due to their interfacial properties, highly biodegradable structure and non-toxicity[30]. In addition, MELs exhibit effects relevant to biomedical applications, such as skin care properties, repair of damaged hair and anti-inflammatory properties[28,31,32]. Because of these properties, MELs have potential applications in cosmetics, food, pharmaceuticals and environmental protection. For most applications, the fully deacetylated MELD is the most suitable because of its higher solubility in water, its excellent interfacial activity even at lower concentrations and, most importantly, because it cannot be further deacetylated[33]. The other MELs have the potential to lose an acetyl group and release acetic acid into the product. This should be avoided, not only because of the change in interfacial properties and polarity, but also because of the strong odor of acetic acid.

However, the proportion of MEL-D produced by the yeast or fungus is very low in the MELs mixture. This limitation can be overcome either by selection or engineering of fungal strains with higher MEL-D production[34–36] or by selective deacetylation in a subsequent step. For this reason, a method was developed by Fukuoka et al. to produce MEL-D from the other MELs via a deacetylation reaction[33]. This method uses immobilized *Candida antarctica* lipase B (iCALB) enzyme as a biocatalyst and ethanol as a co-substrate for the alcoholysis reaction. Fukuoka et al. were able to convert more than 99% of MEL-B to MELD after seven days and saw a partial conversion of MEL-A to MEL-C[33]. Wijnants et al. optimized this enzymatic reaction in 2019 by testing different organic co-substrates (C2-C8 alcohols) and their water content[37]. In their subsequent work they also screened other enzymes for deacetylation and iCALB proved to be the optimal candidate[38]. They discovered that water has a negative effect on the reaction and that the chirality of the erythritol has a large effect. They showed that the alcohol substrate has an effect on the reaction rate of certain deacetylations. The conversion of MEL-A to MEL-C is fastest in 2-ethylhexanol, while the conversion of MEL-C to MEL-D is fastest in isoamyl alcohol. The overall yield of MEL-D from a mixture of the four types of MELs was highest in 2-ethylhexanol.

Since no research has been reported on the deacetylation of MELs in NADES, we investigated whether NADES could be an alternative to conventional organic solvents in the deacetylation process. We also compared how the reaction rates change with the different solvents. As a benchmark, we compared the results of 2-ethylhexanol (co-substrate) and toluene (commonly used organic solvent in lipase catalysis) as solvents with NADES. As NADES, we considered choline chloride-ethylene glycol as a commonly studied hydrophilic NADES and three hydrophobic NADES (thymol-octanoic acid, thymol-menthol, and menthol-coumarin). Since MELs are surfactants, we investigated solvents of widely varying polarity to see which works best.

Results and Discussion

The goal of the studied reaction is to deacetylate MEL-A, MEL-B and MEL-C to form MEL-D. The simplified reaction scheme is shown in Scheme 1 with iCALB as the enzyme and 2-ethylhexanol as the alcohol co-substrate. The samples were analyzed by HPLC and the concentration of each type of MEL was calculated for the 140 hours of monitored reaction time. The data were fitted with first order kinetics and the initial reaction rate was calculated as the slope of a linear regression of the data points in the first five hours of the reaction. The data of the analysis are detailed in the Supporting Information.

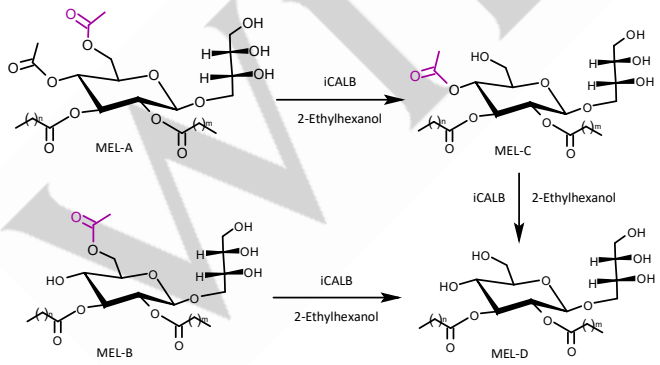


Scheme 1. Reaction mechanism of MELs deacetylation.

The performances of the different solvents were compared by the initial conversion rate of different MEL compounds and the final

yield of the main product, MEL-D. First, we plotted the relative amount of the different compounds in the mixture as the function of time. Goossens et al.[38] determined the reaction pathway of the enzymatic deacetylation of MELs. The acetyl group on the C6 carbon is more accessible, thus MEL-A reacts first into MEL-C, followed by a slower conversion step further into MEL-D. MEL-B, on the other hand, converts directly into MEL-D in a faster reaction. They did not observe the direct conversion of MEL-A into MEL-B.

However, the works of Goossens et al. and Fukuoka et al. did not discuss the differences in conversion rate of the different steps of MEL deacetylation[33,38]. Based on the review by Chen et al. on lipase selectivity, we assume that the different conversion rate of the C4' and C6' acetyl groups is the combined effect of steric hindrance, structural flexibility of the enzyme, and hydrogen bonds present in the active site[39]. The active site of the CALB enzyme contains three amino residues, a serine (SER105), a histidine (HIS224) and an asparagine (ASP187) residue[40]. In alcoholysis, the serine acts as a nucleophile and attacks the carbonyl carbon of the ester. The histidine promotes this attack by accepting a proton from the serine. The essential hydrogen bonds for catalysis of the tetrahedral intermediate of the substrate and the enzyme residues are the two N-H bonds between the alcohol oxygen and the histidine residue and between the serine residue and the histidine residue, plus two N-H bonds between the oxyanion oxygen of the tetrahedral intermediate and the binding sites of the enzyme residues[41]. The acetyl group on the C6' carbon has more flexibility to form these hydrogen bonds because it is not directly attached to the sugar ring (see Scheme 2). In addition, in the case of the C4' group, the proximity of the lipid chains at the second and third carbon atoms of the chain provides a steric hindrance to the reaction. Regional structural flexibility can also influence the reactivity of certain sites, which is modified by the solvent used[39]. There is evidence that in solvents with high hydrophobicity and low dielectric constant, lipases exhibit higher structural rigidity and therefore lower activity for less fitting substrates[42].



Scheme 2. Reaction pathways of the enzymatic deacetylation of a MEL mixture with 2-ethylhexanol.

In our calculations we assumed a similar reaction pathway in NADES as Goossens et al. described[38]. Our results imply the same pathway as Goossens et al. described[38], as the conversion of the different MEL compounds follow a similar trend in each solvent system (See Figures 2 to 5).

Deacetylation in Reference Solvents

First, we tested the conversion of MELs in 2-ethylhexanol. Since this solvent is at the same time the co-substrate, present in large excess, we expect the fastest conversion and highest yield of any solvent tested. This assumption turns out to be correct, as the depletion of MEL-A and MEL-B is the fastest (see Figure 2), while the final yield of MEL-D was the highest (52.7 % relative concentration in all MELs) among all tested systems (see Table 1). The reaction of MEL-B in 2-ethylhexanol is relatively fast and no MEL-B is observed in the mixture after 40 hours. The conversion of MEL-A to MEL-C is also rapid, with only 10 % remaining in the system after 75 hours. However, the conversion of MEL-C to MEL-D is very slow, leading to an increase in the relative concentration of MEL-C in the system (from the initial 10% to 42 % at the end of the reaction). In the reaction pathway from MEL-C to MEL-D, about 12 % of MEL is converted during the monitored reaction time, which is about the initial amount of MEL-C in the mixture. (The amount of converted MEL-C was calculated as the change of the amount of MEL-C in the mixture during the reaction minus the amount of reacted MEL-A.) Together with the calculated reaction rates, this means that the conversion of the C-compounds is much slower than the conversion of the B-compound. The reason for this is the earlier discussed higher reactivity of the C6' acetyl group, which allows a much faster conversion of MEL-B to D and MEL-A to C (in agreement with the results of Fukuoka et al.[33]).

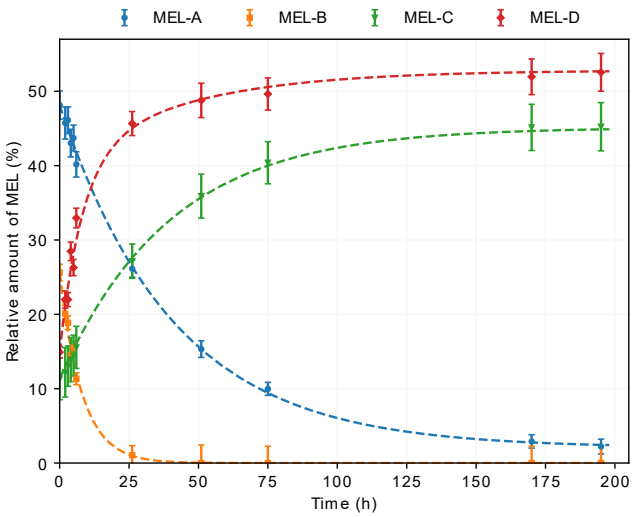


Figure 2. Conversion of each type of MEL in 2-ethylhexanol. The whiskers represent the 95 % confidence interval of the calculated concentration based on the calibration (see Experimental section, HPLC quantification for details).

Toluene was chosen as the second solvent because it is a commonly used volatile organic compound in lipase reactions. It usually has good yields and reaction rates because it has low viscosity and immobilized enzymes retain their activity well in toluene. Accordingly, we observed good conversion rates of MEL-B and a similar, but slightly lower (51.7 % vs. 52.7 %) final yield of MEL-D as with 2ethylhexanol as a solvent (see Figure 3). The conversion of MEL-A to MEL-C was slower, resulting in the

relative concentration of MEL-C remaining constant during the reaction. The reaction rates were all lower than in the pure co-substrate, but this was expected due to the lower concentration of 2-ethylhexanol (see Table 1).

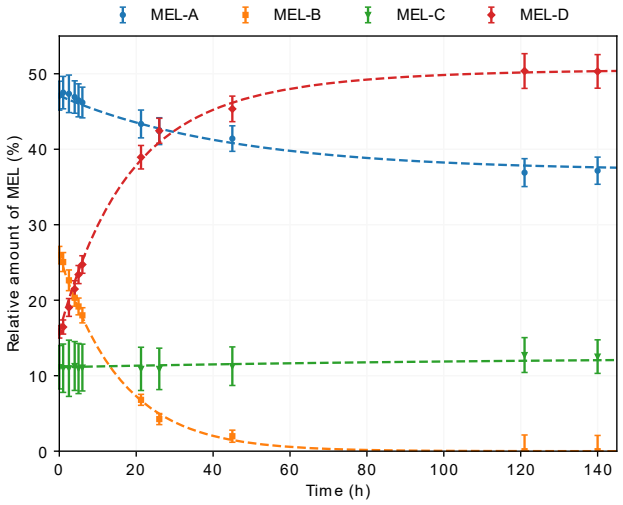


Figure 3. Conversion of each type of MEL in toluene. The whiskers represent the 95 % confidence interval of the calculated concentration based on the calibration (see Experimental section, HPLC quantification for details).

Deacetylation in NADES

The first NADES system we tested was choline chloride-ethylene glycol in eutectic composition (1:2 molar ratio). This system has been used successfully in lipase reactions and provides increased thermal stability to the enzyme[21,25]. However, due to the high viscosity of the system (49 mPa·s at 25 °C), we expected a slower reaction due to possible mass transfer limitations. However, there was no visible transformation in the system and sampling was stopped after 50 hours. Therefore, we assume that the reaction did not take place because the substrates and the media formed a heterogeneous system due to the different polarities. The possible denaturation of the enzyme was discarded as earlier studies show the activity of the same enzyme in these NADES[21]. This led us to focus on more apolar NADES, which may be better solvents for the substrates.

The first apolar NADES system was the eutectic composition (3:2 molar ratio) of menthol and octanoic acid. This system has a lower viscosity than choline chloride-ethylene glycol (15 mPa·s at 25 °C), which also makes it a better candidate for the reaction. However, again no deacetylation of the four MEL compounds was observed over time. In addition, the absolute amount of MELs decreased during the reaction, while the peak associated with the triacetylated MELs increased (see Figure S4). This occurred because the presence of carboxylic acid in the NADES induced the reverse reaction, i.e., esterification of the mannosyl hydroxyl group, resulting in triacetylated MELs (see Figure S5). The acylation of MELs in the presence of organic acids has already been described by Recke et al.[43], but we expected that the strong secondary interactions between the NADES compounds would prevent such side reactions. This example highlights the importance of considering similar potential side reactions in NADES media.

Since the carboxylic acid compound induced side reactions, we abandoned acidic compounds. The next system was the 1:1 molar mixture of thymol and menthol. This system has a relatively high viscosity of 53 mPa·s at 25 °C, which is higher than the choline chloride-ethylene glycol system (49 mPa·s) at the same temperature. Nevertheless, we observed similar yields and reaction rates in this system as in toluene (see Figure 4). While the final yield was somewhat lower than in toluene (49.6 % vs. 51.7 %), we observed a slightly faster conversion of MEL-A to MEL-C. However, the conversion rate of MEL-C to MEL-D was lower than in toluene or in 2-ethylhexanol. Consequently, the relative concentration of MEL-C slightly increased during the reaction. This may be due to the higher viscosity of the system. The conversion of MEL-B to MEL-D was similar to that of toluene as solvent and the system reached full depletion approximately after 80 hours.

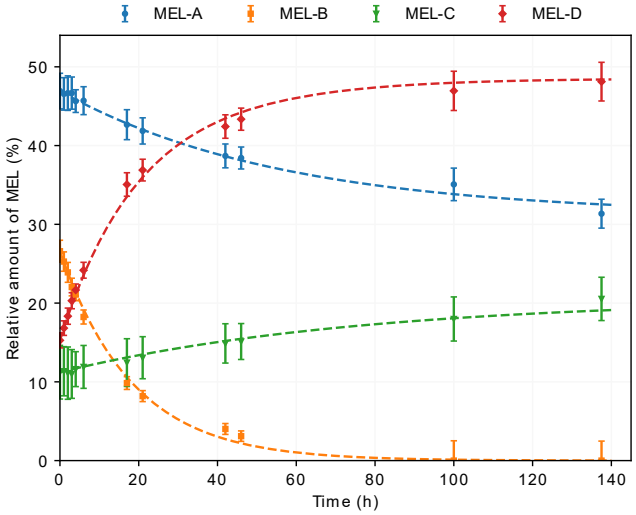


Figure 4. Conversion of each type of MEL in thymol-menthol NADES. The whiskers represent the 95 % confidence interval of the calculated concentration based on the calibration (see Experimental section, HPLC quantification for details).

The last NADES system tested was the 1:1 molar ratio of thymol-coumarin. This system has a lower viscosity than the thymol-menthol (29 mPa·s vs. 53 mPa·s at 25 °C), so a higher initial reaction rate was expected. The results (see Figure 5) partially confirmed our expectations, as MEL-B was depleted faster. Compared to the thymol-menthol system, faster reaction rates were also measured for the conversion of MEL-A to MEL-C and MEL-C to MELD (see Table 1). However, the final yields did not change significantly compared to either toluene or thymol-menthol NADES. MEL-D reached a relative concentration of 49.9 %, while the amount of MEL-C increased slightly to 19.5 %. Overall, of the eutectic systems tested, this last NADES had the highest conversion yield of MEL-D and also the highest reaction rates. It also had higher initial reaction rates than toluene. Despite the higher rates, the thymol-coumarin system did not achieve a higher yield of MEL-D than in toluene.

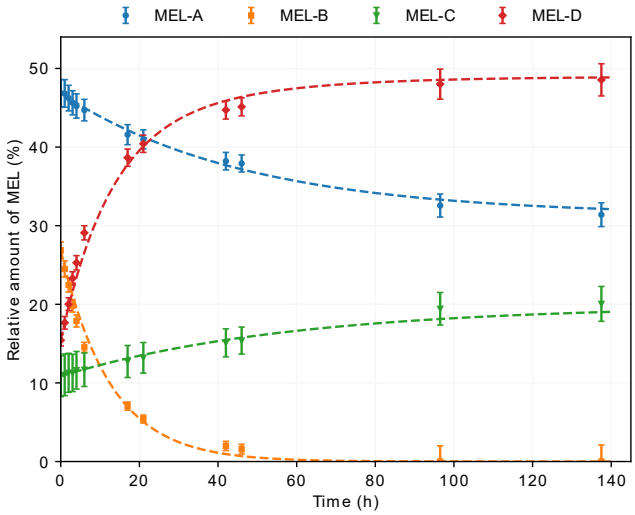


Figure 5. Conversion of each type of MEL in thymol–coumarin NADES. The whiskers represent the 95 % confidence interval of the calculated concentration based on the calibration (see Experimental section, HPLC quantification for details).

Comparison of Reaction Rates, Final Yield and Utilization

In Table 1 we compare the final yields and initial reaction rates of the solvents that facilitated the target reaction. The kinetics and final yield of MEL-D in apolar NADES are comparable to the results obtained with pure 2-ethylhexanol and in toluene, where the thymol-coumarin system even surpasses toluene with respect to the initial conversion rate of MEL-D. To this end, feasible NADES alternatives for MEL deacetylation have been identified.

However, the effect of NADES on reaction kinetics and yield needs to be further investigated. Our hypothesis was that the high viscosity of the NADES would play an important role in the reaction rates. However, the measured reaction rates show no correlation with viscosity. The two- and threefold changes in viscosity (cf. 49, 53 and 29 mPa·s at 25 °C viscosity of choline chloride-ethylene glycol, thymol-menthol and thymol-coumarin systems, respectively) lead to only marginal changes in the initial reaction rates (see Table 1). Considering the amount of substrate in the mixture (55 wt% of MELs and co-substrate), the effect of MELs and 2-ethylhexanol on viscosity should also be considered and the whole system should be treated as a ternary mixture. However, this would include the change in composition over time due to the reaction and formation of products (change in MEL composition and 2-ethylxyl acetate), which would complicate the analysis. Therefore, only the viscosity of pure binary mixtures was considered in this study.

The differences in the partition coefficients (logP, see Table 1) highlight the role of solubility. In the strongly polar choline chloride-ethylene glycol NADES no reaction took place. The apolar systems, both organic solvents and NADES, performed much better. However, as in the case of viscosity, no clear trend was observed. This underlines that a single characteristic property of the NADES is not sufficient to predict the performance of the reaction in the given solvent. In the case of the hydrophilic choline chloride-ethylene glycol NADES, the lack of solubility of the substrates completely blocks the reaction. Comparing the initial reaction rates of toluene and apolar NADES, in the latter the conversion of MEL-A to MEL-C was slightly faster, but the reaction of MEL-C to MEL-D was slightly slower. This may be due to the altered flexibility of the enzyme caused by the change in polarity of the solvent (see Table 1)). The increased flexibility may provide better accessibility to the active site of the enzyme for the C4' acetyl group, which is directly connected to the mannosyl group (see Scheme 2). The selection and investigation of additional NADES systems will require further research, as only a few commonly used NADES have been studied here.

The plateau at the end of the monitored time range also requires further investigation. The conversion to MEL-D appears to stop after 140 hours in every solvent system, but 50 % of the deacetylated MEL compounds are still available. This may indicate denaturation of the enzyme. Although previous studies have reported the general long-term stability of enzymes in other NADES systems even at elevated temperatures[21,25], the eutectic solvents used in this study were not investigated for the thermal stability of the enzyme. The depletion of the co-substrate was not taken under consideration as it was applied at four times the stoichiometric concentration.

Compared to the reaction in pure co-substrate, the yield in NADES systems decreases only slightly. Two reasons for using only stoichiometric amounts of co-substrate are toxicity and economic feasibility. Although 2-ethylhexanol has low toxicity, it oxidizes to 2-hexanoic acid, which is teratogenic[44]. In addition, the price of 2-ethylhexanol is 2495 USD/ton[45], while toluene is 1016 USD/ton[46]. In comparison, the material cost of thymol-menthol system is 1457 USD/ton and in case of thymol-coumarin it is 1473 USD/ton[47–49]. From this aspect, the use of NADES could be the middle ground between economic feasibility and environmental safety.

Compounds of NADES, such as menthol, could act as co-substrates and initiate side reactions. In the literature it is described that the strong secondary interactions in the NADES decrease the reactivity of the compounds, just as they prevent the denaturing effect of urea, for example[6,25]. Nevertheless, we experienced side reaction only in one case, and not with the alcohols, but the co-hydrolysis of the lipid tails by the octanoic acid.

It's worth mentioning that Goossens et al. achieved a higher conversion yield of MEL-D in their deacetylation process[38]. This was partly due to the much higher enzyme concentration used (50 g/L vs. 6 g/L here) and partly due to optimization steps with the reaction parameters. This underlines that although NADES are a promising reaction medium for this deacetylation reaction, it will be necessary to optimize the reaction parameters in addition to finding ideal NADES systems as solvents.

Table 1. Reaction rate and summary of the deacetylation reaction in different solvents.

Concentration at start	Viscosity [mPa·s][b]	LogP[c]	MEL-A [%]	MEL-B [%]	MEL-C [%]	MEL-D [%]
2-ethylhexanol	10.3	2.82	47.98±1.75	25.51±1.03	11.50±2.59	15.01±0.72
Toluene	0.56	2.68	47.10±1.91	25.97±1.15	11.12±2.87	15.81±0.82
Choline chloride-Ethylene glycol	49	-3.77/-1.43	49.53	27.91	11.23	11.33
Menthol-Octanoic acid	15	3.20/2.90	45.23	26.48	11.67	16.63
Thymol-Menthol	53	3.28/3.20	46.99±2.27	26.61±1.40	11.13±3.42	15.27±0.96
Thymol-Coumarin	29	3.28/1.39	46.91±1.78	26.81±1.10	10.90±2.69	15.37±0.76
Concentration 140 hours[e]			MEL-A [%]	MEL-B [%]	MEL-C [%]	MEL-D [%]
2-Ethylhexanol	10.3	2.82	3.04±0.87	0.0±2.24	45.12±3.03	51.84±2.33
Toluene	0.56	2.68	37.16±1.80	0.0±2.08	12.53±2.22	50.31±2.23
Choline chloride-Ethylene glycol	49	-3.77/-1.43	50.13	28.77	10.31	10.79
Menthol-Octanoic acid[d]	15	3.20/2.90	NA	NA	NA	NA
Thymol-Menthol	53	3.28/3.20	31.17±1.77	0.0±2.41	20.63±2.66	48.20±2.38
Thymol-Coumarin	29	3.28/1.39	31.30±1.47	0.0±2.06	20.14±2.15	48.56±1.99
Reaction rate (%/h)[a]			A→C	B→D	C→D	Overall D
2-ethylhexanol	10.3	2.82	-1.383±0.067	-2.2449±0.302	-0.774±0.070	3.058±0.322
Toluene	0.56	2.68	-0.337±0.089	-1.348±0.106	-0.012±0.061	1.639±0.079
Choline chloride-Ethylene glycol	49	-3.77/-1.43	-0.020	-0.061	0.086	-0.005
Menthol-Octanoic acid[d]	15	3.20/2.90	-	-	-	-
Thymol-Menthol	53	3.28/3.20	-0.215±0.122	-1.402±0.069	0.155±0.142	1.525±0.097
Thymol-Coumarin	29	3.28/1.39	-0.423±0.096	-2.115±0.154	0.124±0.055	2.353±0.181

[a] Based on the first 5 hours of the reaction. [b] Measured at 25 °C. [c] Water-octanol partition coefficient values predicted by ACD Labs Perceptra platform. The values for HBA and HBD compounds are marked separately, as the partition coefficient values of NADES are not readily available in literature. [d] Due to the triacetylation sidereaction, comparable concentration at 140 hours or conversion rates could not be determined. [e] In case of choline chloride-ethylene glycol and menthol-octanoic acid measurements were stopped after 50 hours due to the lack of conversion or side reactions.

Due to the high viscosity of NADES and their strong interaction with substrates, separation of the final product is an important consideration. The study of the separation step is beyond the scope of this paper. However, many feasible methods for product recovery are discussed in the literature. For example, the review by Hansen et al. lists the recovery of various products, including biochemical products[50]. Applicable strategies for biosurfactant recovery are solid phase[51] and liquid-liquid extraction[52], supercritical CO2 extraction or anti-solvent[53,54]. We note that these methods are being tested at the laboratory scale and therefore the best method for economically relevant scales remains to be determined. Nevertheless, Isci and Kaltschmitt's recent review of DES recycling reports that recovery above 90% can be achieved[52]. Given the low water solubility of the final thymol-menthol and thymol-coumarin systems and the amphiphilic nature of the MEL compounds, a simple water

extraction after the reaction may be the most straightforward method.

Conclusion

In this study, we compared the deacetylation of MELs mixtures with iCALB and 2-ethylhexanol in different NADES and reference solvents. To evaluate the effect of the solvent on the reaction kinetics and yield, we compared the results of 2-ethylhexanol and toluene as reference organic solvents with hydrophilic and hydrophobic NADES containing a quaternary ammonium salt (choline chloride), diol (ethylene glycol), carboxylic acid (octanoic acid), terpenes (menthol and thymol), and coumarin. Of these systems, the hydrophilic NADES formed by choline chloride and ethylene glycol and the hydrophobic menthol-octanoic acid proved to be inefficient. The former due to the lack of solubility of the substrates and the latter due to the reverse reaction induced by the presence of the carboxylic acid. We found that the deacetylation in thymol-coumarin and menthol-thymol have similar final yields of MEL-D as the two common organic solvents. While the deacetylation rate to MEL-D is the fastest in pure 2-ethylhexanol (which is also the co-substrate), the thymol-coumarin system outperforms the toluene system. The reaction pathway is the same in all systems tested, MEL-A reacts to MEL-C and MEL-C to MEL-D, while MEL-B reacts directly to MEL-D. While the reaction of MEL-B to MEL-D goes well in all systems, the A-C-D pathway is much slower and shows large differences between the 2-ethylhexanol, toluene and NADES systems, which may be related to the different polarities of the solvents. The menthol-octanoic acid system shows a significant side reaction as octanoic acid forms triacylated compounds with MELs. These results demonstrate the feasibility of enzymatic deacetylation of MELs in NADES media. However, further research is needed to understand and optimize the solvent effect on the MEL-A pathway. To this end, molecular dynamics simulations could reveal the interaction energies between solvents, substrates and the enzyme, which could explain the altered reactivity of different MELs in different solvents.

Experimental Section

Materials

Choline chloride (99 %, Thermo scientific, China), ethylene glycol (laboratory reagent grade, ≥ 99%, Fisher chemical, USA), DL-menthol (99 %, Janssens Chimica, Belgium), octanoic acid (99 %, Acros organics, Germany), thymol (laboratory reagent grade, Fisher chemical, India), and coumarin (≥ 99%, Thermo scientific, France) were used for the preparation of the NADES systems. 2-ethylhexanol (99%, Acros organics, Germany) and Immozyme CALB-T2-150XL immobilized lipase enzyme (Chiralvision) were used for the catalytic reaction. Toluene (laboratory reagent grade, ≥ 99%, Fisher chemical, UK) was used as the reference solvent for the deacetylation reactions. For preparatory flash chromatography and analytical HPLC, methanol (HPLC grade, ≥ 99.8%, Fisher chemical, UK), isopropanol (HPLC grade, ≥ 99.8%, Chem-Lab NV, Belgium), acetone (HPLC grade, ≥ 99.8%, Fisher chemical, UK), dichloromethane (HPLC grade, ≥ 99.8%, Fisher chemical, Germany) and formic acid (≥ 98%, Acros organics, Germany). Hydranal composite 5 (Honeywell Fluka) was used to measure water content.

Production of MELs

To obtain a concentrated MEL mixture, we used the product of Goossens et al.[29]. The detailed fermentation and isolation procedure is discussed in their work[29]. In our research, we obtained the concentrated MEL-enriched phase together with some yeast cells and residue water. This mixture was first dissolved in ethyl acetate and dried with anhydrous Na2SO4. The solution was then filtered through a Whatman paper filter. The ethyl acetate solvent was removed by rotary evaporation at 60 °C and 300 mbar. This left a brown viscous liquid containing, apart from MELs, vegetable oil and free fatty acids from the fermentation. To remove the latter two, the mixture was dissolved in a mixture of n-hexane:methanol:water 1:6:3 (v:v:v). The aqueous bottom phase was collected and washed twice with n-hexane. The water and methanol were evaporated again using a rotary evaporator, again resulting in a brown viscous liquid.

This concentrated crude MEL mixture still contained small amounts of residual free fatty acids, residual oil and triacylated MELs. To purify the samples from these residues, the mixture was separated by flash chromatography. We used a flash chromatography system (BUCHI Pure C-815 Flash with an ELSD detector) with a 25 g silica column (Chromabond® Flash RS 25 SiOH, 40 - 63 µm). The eluents used were dichloromethane, isopropanol and methanol. The separation method is described in the Supporting Information (see Figure S1). To obtain pure MEL samples, the flash chromatography appropriate samples of the above method were combined. For the pure samples of MEL-A and MEL-B, an additional purification step was performed on the combined MEL-A-MEL-B samples as described in the Supporting Information (see Figure S2).

HPLC quantification

An HPLC method was developed to quantitatively determine the concentration of each type of MEL. 20 µL of sample diluted with dichloromethane to 1000 ppm (all MELs combined) was injected into a Nova Pak® silica column (Waters, 60 Å, 4 µm, 3.9 mm x 150 mm) protected by a µPorasilTM guard column (Waters, 10 µm, 3.9 mm x 20 mm). The eluents used were dichloromethane and isopropanol, both spiked with 0.16% formic acid. The detailed HPLC method is described in the Supporting Information (see Figure S3). An Agilent 1260 infinity II HPLC system was coupled to an Agilent 1260 infinity II ELSD with nebulizer. The ELSD temperature was set to 30 °C, the evaporator chamber temperature was set to 30 °C, and the carrier gas flow rate was set to 1.3 standard liters per minute (SLM). An example of the chromatogram obtained is shown in Figure 6.

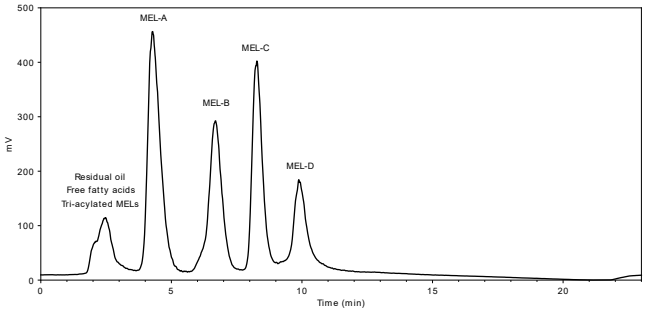


Figure 6. Example of a HPLC chromatogram from a MEL separation.

To determine the concentration and retention of each type of MEL in a mixture, the HPLC system was calibrated with ELSD using a standard of each type of MEL as described in the previous section. The concentration of MELs was calibrated in the range of 50-400 ppm.

The uncertainty (95% confidence interval) of the calibration curves was greater than the uncertainty of the concentration calculated from the deviation between the triplicate results, so the uncertainty of the calibration is used as the uncertainty of the calculated concentration. Detailed calculations are provided in the Supporting Information.

Preparation of NADES

The components of the NADES were weighed to obtain the required molar ratio and combined in an Erlenmeyer flask. The Erlenmeyer flask was sealed with a glass stopper to limit the amount of water from the air that would dissolve in the NADES. The sealed flask was heated to 80 °C in a glycerol bath and held at that temperature with stirring until a clear liquid was obtained. After cooling to room temperature, the water content of the NADES was measured by Karl Fischer titration (Mettler Toledo V30 Volumetric KF Titrator) to ensure that it was less than 1 %.

Deacetylation Reaction

The following reaction setup and conditions were used for the deacetylation reaction. Reactions were performed in glass vials placed in a glycerol bath and kept at a constant temperature of 60 °C throughout the reaction. The reaction mixture contained 5 g of solvent (reference solvent or NADES), 300 mg of 2-ethylhexanol and 300 mg of crude MEL mixture. The vials were placed in the glycerol bath and stirred for five minutes to allow the reaction mixture to reach reaction temperature. The reaction was initiated by adding 30 mg of crushed enzyme to the mixture. The immobilized enzyme was crushed with a mortar and pestle to increase the accessible surface area of the beads prior to the reaction. The crushed enzyme was sieved and the particles collected between 75 µm and 355 µm were used for the reaction. The reaction was sampled by removing 100 µL from the reaction mixture with an autopipette and diluting the sample with 4 ml of dichloromethane. To immediately stop the reaction upon dilution, cold dichloromethane (-17 °C) was used; and the diluted samples were filtered with a 45 µm PTFE syringe filter to remove the immobilized enzyme from the mixture.

Supporting Information

Part of the data that support the findings of this study are openly available in Figshare at https://doi.org/10.6084/m9.figshare.24745593. Part of the data that supports the findings of this study are available in the supplementary material of this article.

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Keywords: Biosurfactants • Candida antarctica lipase B • Deep eutectic solvents • Lipases • Mannosylerythritol lipids

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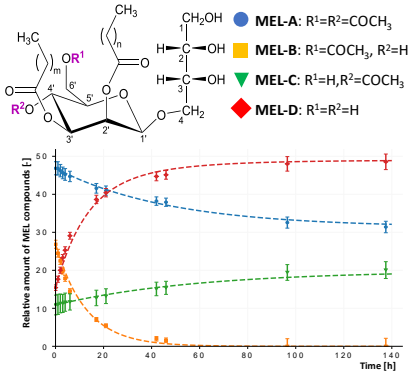

Enzymatic deacetylation of mannosylerythritol lipid (MEL) biosurfactant mixtures in naturally derived deep eutectic solvents has comparable yields and kinetics to conventional organic solvents. Here, the example of thymol-coumarin 1:1 mixture is shown where the diacetylated (MEL-A) and monoacetylated (MEL-B and MEL-C) are converted to fully deacetylated compound (MEL-D) with 50 % yield after 140 hours.

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