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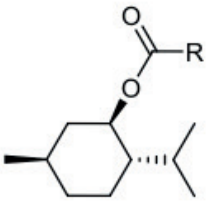
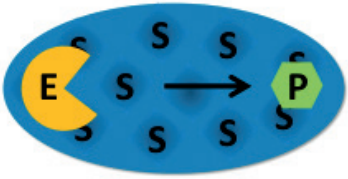
Evaluation of Deep Eutectic Solvents as Alternative Reaction Media in Biocatalysis





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Evaluation of Deep Eutectic Solvents as Alternative Reaction Media in Biocatalysis

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You live and learn. At any rate, you live.
— Douglas Adams

Publications

M. Hümmer, S. Kara, A. Liese, I. Huth, J. Schrader, D. Holtmann:
Synthesis of (–)-menthol fatty acid esters in and from (–)-menthol and fatty acids — novel concept for lipase catalyzed esterification based on eutectic solvents
Molecular Catalysis (2018), vol. 458, pp. 67-72
DOI: https://doi.org/10.1016/j.mcat.2018.08.003

M. Pätzold, A. Weimer, A. Liese, D. Holtmann:
Optimization of solvent-free enzymatic esterification in eutectic substrate reaction mixture
Biotechnology Reports (2019), vol. 22, e00333
DOI: https://doi.org/10.1016/j.btre.2019.e00333

M. Pätzold, B. O. Burek, A. Liese, J. Z. Bloh, D. Holtmann:
Product recovery of an enzymatically synthesized (–)-menthol ester in a deep eutectic solvents
Bioprocess and Biosystems Engineering (2019), vol. 42, pp. 1385–1389
DOI: https://doi.org/10.1007/s00449-019-02125-6

M. Pätzold, S. Siebenhaller, S. Kara, A. Liese, C. Syldatk, D. Holtmann:
Deep eutectic solvents as efficient solvents in biocatalysis
Trends in Biotechnology (2019), vol. 37, pp. 943-959
DOI: https://doi.org/10.1016/j.tibtech.2019.03.007

M. Pätzold, D. Holtmann:
Eutektische Lösungsmittel in der Biokatalyse
Biospektrum (2019), vol. 25, pp. 458-460
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M.-L. R. W. Hansen, M. A. Petersen, J. Risbo, M. Hümmer, A. Clausen:
Implications of modifying membrane fatty acid composition on membrane oxidation, integrity, and storage viability of freeze-dried probiotic, *Lactobacillus acidophilus* LA-5
Biotechnology Progress (2015), vol. 31, pp. 799-807
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J. Taucher, S. Baer, P. Schwerna, D. Hofmann, M. Hümmer, R. Buchholz, A. Becker:
Cell disruption and pressurized liquid extraction of carotenoids from microalgae
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S. Milker, M. Pätzold, J. Z. Bloh, D. Holtmann:
Comparison of deep eutectic solvents and solvent-free reaction conditions for aldol production
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DOI: https://doi.org/10.1016/j.mcat.2019.01.012

Conference talks

M. Hümmer, D. Holtmann, J. Schrader, S. Kara, A. Liese:
One-for-two — Use of menthol-based deep eutectic solvents for lipase-catalyzed esterifications
5th International Conference on Biocatalysis in Non-Conventional Media
9 – 11 May 2017, Rostock, Germany

M. Hümmer, K. Schoppel, J. Schrader, S. Kara, A. Liese, D. Holtmann:
Deep eutectic solvents in der Biokatalyse — 2-in-1 Reaktionsmedien für die Veresterung von Menthol
6. Stiftungstag, DECHEMA Research Institute
6 December 2017, Frankfurt a.M., Germany

M. Hümmer, J. Schrader, D. Holtmann, S. Kara, A. Liese:
Two-in-one reaction media — Menthol-based deep eutectic solvents for lipase-catalyzed esterifications
Himmelfahrtstagung
7 – 9 May 2018, Magdeburg, Germany

M. Hümmer, J. Schrader, D. Holtmann, S. Kara, A. Liese:
(–)-Menthol-based DES as substrate and solvent for the lipase-catalysed esterification of (–)-menthol
ACHEMA Congress
11 – 15 June 2018, Frankfurt a.M., Germany

Poster presentations

M. Hümmer, J. Schrader, D. Holtmann:
Evaluation of deep eutectic solvents as reaction media for biotransformation
4. Stiftungstag, DECHEMA Research Institute
9 December 2019, Frankfurt a.M., Germany

M. Hümmer, D. Holtmann:
Deep eutectic solvents as novel reaction media for biotransformations
4th Workshop of the FSP "Integrierte Biotechnologie und Prozesstechnik"
17 February 2016, Hamburg, Germany

M. Hümmer, D. Holtmann:
Rheological characterization of deep eutectic solvents as alternative reaction media for biocatalysis
8th International Congress on Biocatalysis
28 August – 1 September 2016, Hamburg, Germany

M. Hümmer, J. Schrader, A. Liese, D. Holtmann:
Deep eutectic solvents as novel reaction media for biotransformations
ProcessNet-Jahrestagung und 32. DECHEMA-Jahrestagung der Biotechnologen
12 – 15 September 2016, Aachen, Germany

M. Hümmer, D. Holtmann:
Rheological characterization of deep eutectic solvents as alternative reaction media for biocatalysis
8th Green Solvents Conference
16 – 19 October 2016, Kiel, Germany

M. Hümmer, J. Schrader, A. Liese, S. Kara, D. Holtmann:
Deep eutectic solvents as novel reaction media for biotransformations
5. Stiftungstag, DECHEMA Research Institute
7 December 2016, Frankfurt a.M., Germany

M. Hümmer, K. Schoppel, D. Holtmann, J. Schrader, S. Kara, A. Liese:
(–)-Menthol:lauric acid deep eutectic solvent — substrate and solvent for the biotransformation of menthol in a rotating bed reactor
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M. Hümmer, J. Schrader, A. Liese, S. Kara, D. Holtmann:
(–)-Menthol-based deep eutectic solvents — substrate and solvent for the biotransformation of (–)-menthol
6. Stiftungstag, DECHEMA Research Institute
6 December 2017, Frankfurt a.M., Germany

M. Hümmer, J. Schrader, A. Liese, S. Kara, D. Holtmann:
(–)-Menthol-based deep eutectic solvents — substrate and solvent for the biotransformation of (–)-menthol
7. Stiftungstag, DECHEMA Research Institute
5 December 2018, Frankfurt a.M., Germany

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Abstract

Enzyme catalysis is often associated with aqueous reaction media, but water is rather an inadequate solvent for hydrophobic substrates. In this study 'deep eutectic solvents' (DESs) were evaluated as novel reaction media for biocataylsis. For instance, a DES composed of both substrates, i.e. a DES made of (–)-Menthol:lauric acid, was used as combined solvent and substrate for the lipase catalysed synthesis of (–)-menthyl laurate, so that no additional solvent was necessary. The optimisation of the reaction resulted in a batch productivity of 443 $g_{ester}\,L^{-1}\,d^{-1}$ at 95 % conversion (24 h). Excess (–)-menthol was separated in a vacuum distillation step and was reused for a second enzymatic DES reaction.

Die Enzymkatalyse wird häufig mit wässrigen Reaktionsmedien assoziiert, aber Wasser ist ein eher unzureichendes Lösungsmittel für hydrophobe Substrate. In dieser Arbeit wurden tief eutektische Lösungsmittel (DES) als neuartige Reaktionsmedien für die Biokatalyse evaluiert. Für die Lipase katalysierte (–)-Menthyllaurat Synthese wurde beispielsweise ein DES, das aus beiden Substraten besteht, d.h. ein DES aus (–)-Menthol:Laurinsäure, als Solvens und Substrat verwendet, so dass kein Zusatzlösungsmittel nötig war. Die Reaktionsoptimierung führte zu einer Produktivität von 443 $g_{ester}\,L^{-1}\,d^{-1}$ bei 95 % Umsatz (24 h). Überschüssiges (–)-Menthol wurde destillativ abgetrennt und in einer zweiten DES Enzymreaktion wiederverwendet.

Zusammenfassung

Enzymreaktionen werden häufig mit Wasser basierten Reaktionsmedien verbunden, wobei die Biotransformation hydrophober Substrate aber aufgrund von geringen Substratkonzentrationen häufig nicht effizient in Wasser durchführbar ist. Während 'unkonventionelle' Reaktionsmedien, wie z.B. organische Lösungsmittel oder ionische Flüssigkeiten, schon länger für Biotransformationen eingesetzt werden, sind sogenannte tief eutektische Lösungsmittel (DES, engl. 'deep eutectic solvents') relativ neue Reaktionsmedien in der Biokatalyse.

Im Rahmen der vorliegenden Arbeit wurden DES als alternative Lösungsmittel evaluiert. Neben der physikochemischen Charakterisierung einiger DES, lag der Fokus auf der Untersuchung Lipase katalysierter Reaktionen in DES. Für die Veresterung des Duft- und Aromastoffs (–)-Menthol mit verschiedenen Fettsäuren konnten die DES direkt aus den Substraten hergestellt werden. Eine *Candida rugosa* Lipase war geeignet, um (–)-Menthylfettsäureester in (–)-Menthol:Caprylsäure, (–)-Menthol:Caprinsäure und (–)-Menthol:Laurinsäure DES ohne weiteren

Lösungsmittelzusatz zu synthetisieren. Für das DES Reaktionssystem aus (–)-Menthol:Laurinsäure (3:1 mol/mol) war die Reaktion unter kontrollierter Wasseraktivität (a_w) effizienter als die Veresterung in DES-Wasser Gemischen oder im reinen DES. Bei einem konstanten a_w (0,32) konnte eine Batch-Produktivität für (–)-Menthyllaurat von $287\,\mathrm{g\,L^{-1}\,d^{-1}}$ und ein Fettsäureumsatz von 65 % erreicht werden (24 h). Die Esterbildung im DES (–)-Menthol:Laurinsäure (3:1 mol/mol) konnte mittels statistischer Versuchsplanung hinsichtlich der Reaktionstemperatur (45 °C), der Enzymmenge (60 mg) und des a_w (0,55) optimiert werden, so dass die Batch-Produktivität auf $443\,\mathrm{g\,L^{-1}\,d^{-1}}$ und der Umsatz auf 95 % (24 h) gesteigert werden konnten. Durch den Einsatz des DES als 2-in-1 Reaktionsmedium, d.h. als verflüssigte Substrate und gleichzeitig als Lösungsmittel, konnte die Veresterung mit hohen Substratkonzentrationen durchgeführt werden. Die Esterkonzentration erreichte dabei $1{,}36 \pm 0{,}04\,\mathrm{M}$ (2,25 d) bei einer maximalen Esterbildung von $174\,\mathrm{mM\,h^{-1}}$ innerhalb von 6 h. Die Reduktion des Molverhältnisses des (–)-Menthol:Laurinsäure DES bewirkte eine weitere Steigerung der Esterbildung.

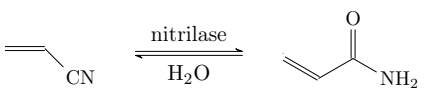
Das (–)-Menthol:Laurinsäure (3:1 mol/mol) DES-Reaktionssystem konnte so optimiert werden, dass die Fettsäure praktisch vollständig umgesetzt wird (3 d, 35 °C, a_w 0,16). Überschüssiges (–)-Menthol aus zwei Reaktionen konnte durch Vakuumdestillation abgetrennt werden. Der Ester (735 mg) wurde mit einer Reinheit von 94 % gewonnen, während das abgetrennte (–)-Menthol (622,5 mg, 4 mmol) für die Herstellung eines neuen DES genutzt wurde. Bei der anschließende Veresterung mit recyceltem (–)-Menthol erreichte der Fettsäureumsatz 98,7 %. Anhand der Reaktions- und Prozessoptimierung dieser Beispielreaktion konnte gezeigt werden, wie DES effizient für die Biokatalyse genutzt werden können. DES sind insbesondere als 2-in-1 Reaktionsmedien vorteilhaft und können als Alternative zu anderen 'unkonventionellen' Reaktionsmedien für die Biokatalyse angesehen werden.

Contents

1 Introduction

1.1 Biocatalysis - using enzymes as catalysts

The use of living organisms to prepare or preserve foods by biotransformation processes dates back to ancient times, long before anything was known about the existence or function of microorganisms and enzymes. The spontaneous fermentation of mashed grains by yeast during beer or bread production can be considered as the oldest bioprocesses, which were established as early as 7000 BC in Sumeria and Babylonia and 4000 BC in Egypt, respectively [1]. During the last century, biocatalysis has evolved in three major waves, starting with the use of living cell components as catalysts to achieve useful chemical conversions at the beginning of the 20th century [2]. The second wave of biocatalysis occurred during the 1980s and 1990s and was determined by the rise of early protein engineering methods to modify enzymes for unusual substrates, enabling the enzymatic production of pharmaceutical intermediates and fine chemicals [2]. It was also in this decade that a breakthrough of biocatalysis was seen in the industrial environment. In 1985, the enzymatic synthesis of acrylamide (see Scheme 1.1) was implemented in the petrochemical industry on a several thousand tons/year scale. This reaction can be regarded as the first successful biocatalytic production of a bulk chemical [3, 4]. In contrast to the chemical process, the enzymatic production reaches almost 100 % conversion and yield, superseding the recovery of unreacted acrylnitrile, and additionally the reaction proceeds at low temperatures, preventing the polymerisation of acrylamide [4].



acrylonitrile acryl amide

Scheme 1.1: The production of acrylamide from acrylonitrile using nitrilase is an example of an early enzymatic process implemented in industry.

The third, present wave of biocatalysis is affected by directed evolution methods to develop robust catalysts withstanding exceptional synthetic reaction conditions

[2]. The integration of advanced tools, such as molecular genetics, metagenomics and bioinformatics, with innovative process developments (e.g. multi-enzyme cascades, enzyme immobilisation or microreactors) is even believed to emerge the fourth wave of biocatalysis [5]. Today, biocatalysis is regarded as a powerful tool to complement the toolbox of synthetic chemistry rather than replacing it [6]. The combination of chemo- and biocatalysts in cascade reactions enables new synthetic routes and retrosynthetic concepts to obtain complex organic compounds such as pharmaceuticals [7].

1.2 Enzymes - naturally occurring biocatalysts

In nature, enzymes are universally occurring biomolecules and catalyse metabolic reactions in all domains of life (eukaryota as well as prokaryota) under physiological reaction conditions. While only a few enzymes are made of RNA, most enzymes are proteins, sharing basically the same structural elements. The basic structure of an enzyme is determined by its amino acid sequence, a random coil polypeptide chain with a defined order of covalently bonded amino acids. Due to the non-covalent hydrogen bond interactions within the polypeptide chain, different three-dimensional structures can be formed, such as α-helices, β-sheets or loops. However, only when these structural elements of a polypeptide are arranged in a certain overall, three-dimensional shape determined by non-covalent interactions (e.g. ionic, van-der-Waals or hydrogen bond interactions), the protein becomes functional. In terms of an enzyme, this means that certain side chains point towards each other to form the active site, which makes the enzyme catalytically active. In general, there is a relationship between enzyme structure and function. If an enzyme looses its characteristic three-dimensional conformation, for instance by thermal unfolding, the enzyme would not be functional anymore and become inactive. The process of folding and unfolding can be reversible, if the energy input only affects non-covalent bonds determining the secondary and tertiary structure, or irreversible, if covalent bonds within the polypeptide chain are disrupted.

As one of their unique properties, enzymes accelerate the rate of chemical reactions by lowering the activation energy needed for the conversion of a substrate to a

product. An enzyme can help to overcome the activation energy of a reaction due to the formation of transient covalent bonds between the amino acid residues of the active site and the substrate, on the one hand. On the other hand, there are multiple weak non-covalent interactions of the amino acid side chains with the substrate contributing to the release of binding energy. The interactions between the enzyme (E) and the substrate (S) can be illustrated by the formation of an enzyme-substrate complex (ES) as intermediate reaction step to release the product (P). The following reaction equation (1.1) represents a general enzymatic reaction including the rate constants for the forward (k_1, k_2) and the back reaction steps (k_{-1}, k_{-2}).

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E} + \mathrm{P} \tag{1.1}$$

Their extraordinary specificity makes enzymes great catalysts and underlines their importance for syntheses of complex chemicals. The specificity of enzymes is closely related to their three-dimensional structure. In the 19$^{\text{th}}$ century enzyme specificity was first described by the key-lock model, which stated that the substrate would fit to its enzyme like a key in a lock [8]. Although this model recognises the unique specificity of enzymes accepting only substrates accurately fitting into the active site, the model cannot explain why enzymes are such good biocatalysts. If the substrate fits tightly in the active site, the ES complex would be stabilised and thus prevent the formation of the product. In contrast to that, structural flexibility of proteins is very important to understand why enzymes can efficiently function as catalysts. Instead of an accurate match of the substrate to the enzyme's catalytic center, the induced-fit model added protein conformational flexibility, like a hand in a glove, to Fischer's key-lock theory [9]. The induced-fit theory proposed that enzyme action requires the precise arrangement of catalytic groups, that the substrate binding induces a favourable change of the enzyme's three-dimensional structure at the active site, and that this structural change will align the catalytic groups appropriately [9]. Thus, the structural flexibility of a protein contributes to the stabilisation of the intermediate ES complex and eventually enables the release of the transformed product. Enzyme specificity can be absolute, if only one reaction is catalysed, or related to certain chemical groups or bonds, on which the enzyme acts (i.e. group or linkage specificity). Additionally, enzymes can be

stereospecific converting only one stereoisomer. Therefore, enzymes usually catalyse reactions with high regio- and stereoselectivity.

These unique features are to date more than relevant for the development of industrial biocatalysis. Whereas in the past the industrial use of enzymes was predominantly found for processed foods, starting with Chr. Hansen's Teknisk-Kemiske Laboratorium in 1874 for the commercial production of rennet[1], biocatalysis has evolved due to the extraordinary specificity of enzymes to cover also complex applications, e.g. in pharmaceutical industry or in the production of fine and bulk chemicals. For instance, the synthesis of the key side chain of atorvastatin (a cholesterol lowering drug) with two chiral centres and a requirement of $>99.5\,\%$ enantiomeric excess (e.e.) and $99\,\%$ diastereomeric excess (d.e.) can be accomplished by a variety of enzymatic steps [10]. The biocatalytic synthesis is advantageous over conventional synthetic approaches in terms of costs, reaction conditions or generation of less byproducts due to the high regio- and stereoselective nature of the enzymes (e.g. alcohol dehydrogenase, nitrilase or lipase can be used for the synthesis among others) [10]. The use of a recombinant *Lactobacillus brevis* alcohol dehydrogenase enables the regioselective reduction of a diketoester to yield the chiral atorvastatin intermediate with an e.e. of more than $99.5\,\%$ within a reaction time of 24 h at $72\,\%$ isolated yield [10]. These examples illustrate how biocatalysis has evolved over the the past century, e.g. from isolating cheese-making enzymes from cow's stomachs to highly specific recombinantly produced enzymes to support the synthesis of complex chiral molecules.

1.3 Enzyme classes

Since there is a huge variety of enzymes being part of the metabolic pathways in all living organisms, enzymes were classified according to the type of reaction they catalyse. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology proposed seven different classes of enzymatic reactions to assign enzymes with a Enzyme Commission (EC) number [11]. The EC number is

[1] Chr. Hansen A/S company website: `https://www.chr-hansen.com/de/about-us/history`, accessed on 09/11/2019

a code of four numbers providing information about the enzyme and the reaction it catalyses (EC -.-.-.-). Whereas the first number indicates the major enzyme class, the following three numbers further categorise and specify the reaction type or enzyme action, respectively (e.g. on which groups or bonds the enzyme acts). Table Table 1.1 summarises the major enzyme classes and gives some general enzyme examples.

Table 1.1: Major enzyme classes according to EC classification with catalysed reaction types and examples of enzymes.

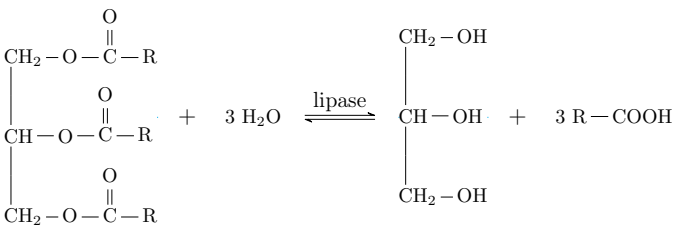
EC class	**Nomenclature**	**Reaction catalysed**	**Enzyme examples**
EC 1.-.-.-	Oxidoreductases	Redox reactions	Dehydrogenases, oxidases
EC 2.-.-.-	Transferases	Transfer of functional groups	Transaminases, kinases
EC 3.-.-.-	Hydrolases	Cleavage of hydrolytic bonds	Lipases, amylases
EC 4.-.-.-	Lyases	Non-hydrolytic addition or elimination of groups	Decarboxylases, aldolases
EC 5.-.-.-	Isomerases	Intramolecular rearrangement, isomerisation	Isomerases, racemases
EC 6.-.-.-	Ligases	Formation of covalent bonds with ATP consumption	Synthases, carboxylases
EC 7.-.-.-	Translocases	Transport of compounds through the cell membrane	Ornithine translocase

In 2000, biocatalysis has been said to gain more ground in industry due to an increasing demand for environmentally safe production processes, and at that time about 100 biotransformations have already been carried out in industry, involving enzymes of almost all EC classes (oxidoreductases to isomerases) [12]. Lipases (EC 3.1.1.3) belong to the class of hydrolases and represent an outstanding class of biocatalysts with regard to their industrial relevance. From an industrial point of

view, lipases feature many attractive properties, such as high catalytic efficiency and stability, high chemo-, regio- and enantioselectivity, activity in organic solvents, and the fact that lipases do not require any cofactors and can be produced in large quantities makes these enzymes extremely attractive for organic syntheses [13, 14]. The plethora of positive lipase characteristics has even raised the question, whether these enzymes are still important biocatalysts, but the answer to this question is yes [13]. Despite their widespread and versatile application, lipases are still regarded as important biocatalysts. While the use of lipases for kinetic resolutions has lost its industrial momentum, the application of lipases is still attractive in different technological fields, and a growing demand for lipases is expected for detergent, food and biodiesel production [13].

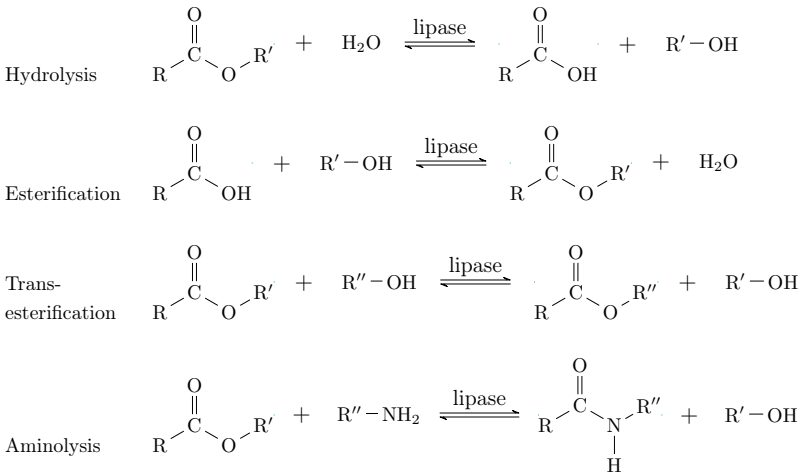
1.4 Lipases for enzymatic ester synthesis

From a physiological perspective, lipases or more specifically triacylglycerol ester hydrolases occur ubiquitously in almost all organisms to hydrolyse oils and fats [15]. The enzymatic cleavage of the ester bonds of a triglyceride produces fatty acids and glycerol (see Scheme 1.2), which is an essential reaction for the digestion of fats.



Scheme 1.2: Catalytic activity of lipases on triglycerides to release glycerol and free fatty acids.

For synthetic applications, lipases are used to catalyse a variety of different reactions (see Scheme 1.3), which can be grouped as hydrolysis, esterification, transesterification or aminolysis reactions [16]. Since it was reported that lipases can be active under non-aqueous conditions in the 1980s [17], lipases have been increasingly used for synthetic organic reactions. In fact, lipases were said to be the most



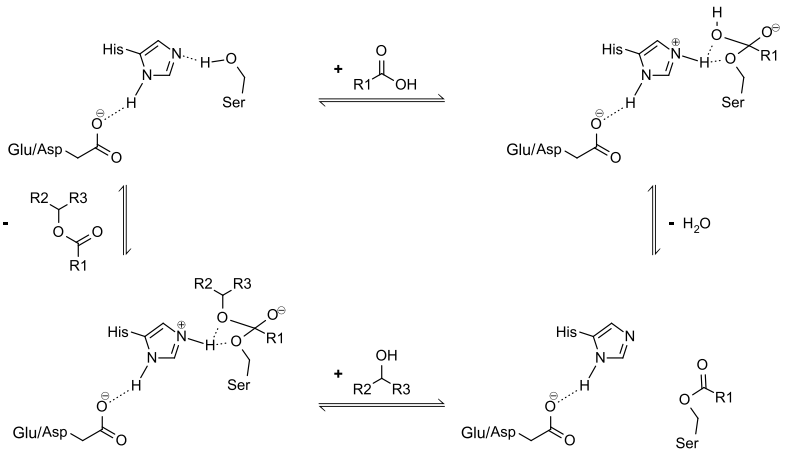
Scheme 1.3: Reaction spectrum catalysed by lipases.

used enzymes in synthetic organic chemistry, as they catalyse the hydrolysis of carboxylic acid esters, but also the ester synthesis under reverse reaction conditions [18]. Among the catalysed reaction types, enzymatic ester synthesis is of great importance due to the broad use of ester products in the food, cosmetic, energy or pharmaceutical industry. For instance, lipase catalysed esterifications are used to produce glycerides from fats or oils, modified vegetable oil, biodiesel (fatty acid methyl esters) or biosurfactants (glycolipids) [16]. Beyond that, lipases are important biocatalysts in the production of flavour and fragrance compounds, where enzymatic esterifications are established to synthesize optically pure intermediates or flavour products [19]. Lipase catalysed esterification is especially attractive to synthesize flavour and fragrance compounds, since valuable ester materials might only occur in small quantities and their isolation from natural sources is therefore difficult and cost-intensive. Different flavour esters (e.g. isoamyl, geranyl, citronellyl, menthyl or cinnamyl esters) have been synthesized by lipases in organic or solvent-free reaction media, and rarely also in other non-conventional reaction media like ionic liquids (ILs) or supercritical fluids [20]. Enzymatically synthesized esters are also important ingredients for personal care products. In cosmetic prod-

ucts, esters are not only used as aroma compounds, but also as functional active ingredients or as specialty esters, delivering certain properties to a product [21]. Therefore, the use of lipase catalysed esterifications to synthesize flavour and cosmetic products is an important application in view of an increasing demand for more sustainable chemical production processes.

Commercially available lipases used for synthetic applications are often derived from microorganisms, and genetic engineering tools have increased recombinant lipase production from bacteria or yeast [22]. All lipases share a common structural architecture, the so-called α/β-hydrolase fold, which describes an architecture of eight β-strands connected by α-helices in the core of these enzymes [15, 23]. Another structural characteristic of lipases is their catalytic triad of serine, histidine and aspartate, which is sometimes replaced (e.g. in lipases from *Geotrichum candidum* or *Candida rugosa*) by glutamate as an exception [24]. The esterification mechanism of lipases resembles that of serine proteases and involving two tetrahedral intermediates [25]. Scheme 1.4 illustrates the lipase catalysed esterification through the nucleophilic attack of serine forming a first tetrahedral intermediate, which is transformed to an acyl enzyme complex by the release of a water molecule. A second tetrahedral intermediate is formed through an alcohol acting as nucleophile, and eventually the enzyme returns to its original state by loosing the ester molecule. The intermediates carry an oxyanion, which is stabilised by hydrogen bonds with the amino groups on the main amino acid backbone of the protein. This stabilising oxyanion hole is another conserved structure commonly found in lipases [15]. Lipases can be distinguished from esterases by a phenomenon called interfacial activiation. Unlike esterases catalysing the hydrolysis of water-soluble esters, most lipases require an aqueous-lipid interface to be catalytically active [24]. The presence of a conformationally flexible lid, an amphiphilic α-helix peptide loop covering the active site [22], causes the interfacial lipase activity. The lid remains closed, if the hydrophobic side of the lid points towards the hydrophobic pocket of the enzyme, i.e. in the absence of a lipid-water interface. In contrast to that, the amphiphilic lid can rearrange in the presence of an aqueous-lipid interface to uncover the hydrophobic core of the active center. *Candida rugosa* lipase is an example for an interfacially activated enzyme [26, 27], which is widely used for

enzymatic esterification reactions.



Scheme 1.4: Reaction mechanism of lipase catalysed esterification through its serine-histidine-aspartate/glutamate catalytic triad (adapted from [25]).

1.5 Deep eutectic solvents

1.5.1 Origin and definition of deep eutectic solvents

'Deep eutectic solvents' (DES) are eutectic mixtures, but there is still a need to precisely define what a DES actually is. The term eutectic is derived from Greek εντηκτος, meaning easy or lowest melting [28, 29]. The expression eutectic was first introduced by Frederick Guthrie in 1884 "for bodies made up of two or more constituents, which constituents are in such proportion to one another as to give to the resultant compound body a minimum temperature of liquefaction – that is, a lower temperature of liquefaction than that given by any other proportion" [29, 30]. This early definition summarises the main properties of a eutectic, being a mixture of at least two components in a certain molar ratio that has a lower melting temperature than its starting materials. Today, the description of eutectics has evolved to define a eutectic reaction, which is an isothermal reversible reaction of a liquid phase that is transformed into at least two different solid phases during

cooling of the system [28, 29]. Describing eutectics with the help of a eutectic reaction reflects the changes in a solid-liquid phase diagram. Figure 1.1 represents a schematic phase diagram of a binary eutectic composed of two compounds A and B. If a liquid eutectic mixture is cooled to pass the melting curves, solids are formed within a liquid phase (grey area) or the mixture solidifies completely (dark grey area). As these phase changes are reversible, the mixture can be liquefied again (white area) by increasing the temperature. The eutectic point is an isobaric invariant of the system and describes the composition ($x_B{}^E$) and the minimum melting temperature (T^E) along the two intersecting solubility melting curves [28, 29].

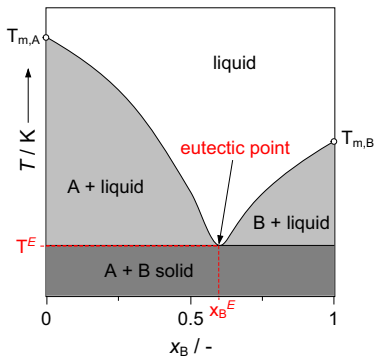


Figure 1.1: Schematic phase diagram of a binary eutectic mixture of A + B showing the eutectic composition $x_B{}^E$ and temperature T^E (i.e. eutectic point) of the mixture ($x_B{}^E$ was set arbitrarily in this example).

DESs were first described by Abbott and co-workers in 2003. The authors reported on eutectic solvents, that are liquid at ambient temperature, but formed by mixing solid quaternary ammonium salts (e.g. choline chloride, ChCl) with solid amides (e.g. urea, U) [31]. The 'deep' freezing point depression of the ChCl:U 1:2 mol/mol mixture was explained by the hydrogen bond interaction between the chloride anion and the urea molecule [31]. Thus, the term 'deep eutectic solvent' was born to describe eutectic mixtures that are liquid at room temperature and formed by hydrogen bond interactions between a hydrogen bond acceptor (HBA) and hydro-

gen bond donor (HBD) compound. Since 2003, many novel DESs were developed mainly based on ChCl, but also 'natural deep eutectic solvents' (NADESs) were defined based on the quality of the starting materials. It was even speculated whether NADESs play a role for metabolic reactions as third cellular phase beside aqueous and lipid phases present in a cell [32]. An overview on potential HBAs and HBDs to prepare DESs is illustrated by Figure 1.2. Due to the huge variety of HBA and HBD molecules that can be combined, there is an enormous flexibility to design novel DESs. In addition to that, DESs are often considered as biodegradable, non-toxic and inexpensive solvents, but as composite solvents their properties obviously depend on the starting materials.

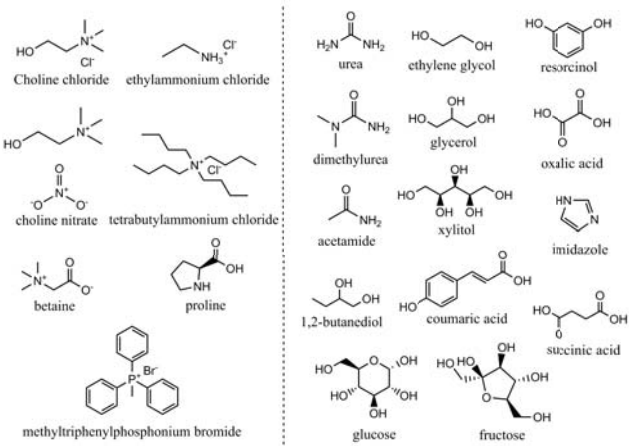


Figure 1.2: Overview on a few HBA (left) and HBD (right) molecules to prepare DESs (totality of HBA/HBDs is not claimed by this overview).

Although DESs are often compared to ILs or sometimes are even termed novel ILs, these solvent classes are different. The hydrogen bond interactions between the compounds distinguish DESs from ionic liquids (ILs). In contrast to ILs, DESs are not solely composed of ionic species, and additionally DESs can also be composed of non-ionic species [29, 33]. Hydrogen bond interactions were investigated in ChCl:U DESs and a strong interaction between the chlorine anion and the amino groups of urea were found [34]. In addition to that, the hydroxyl group of choline

was observed to also interact strongly with chloride through hydrogen bonding and these interactions and further weak hydrogen bonds cause a stable, radially layered sandwich structure to bind chloride with choline and urea [35]. The balance of the strong forces between all species in ChCl:U contributes to prevent the crystallisation of the DES at ambient temperature [35].

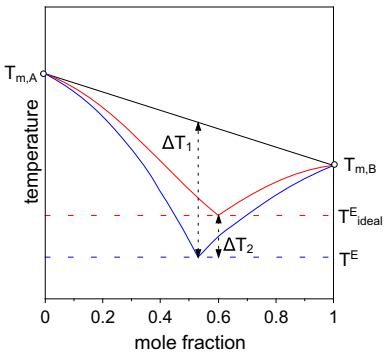


Figure 1.3: Schematic phase diagram of an ideal eutectic mixture (—) and a DES (—) (adapted from [29]).

A strict definition of what DESs are was long missing. Neither the presence of hydrogen bonds between the compounds of a mixture nor the existence of a eutectic point are sufficient to define a DES, as basically all mixtures of compounds that are immisicible in the solid phase have a eutectic point [29]. To differentiate DESs from other eutectic mixtures, it was proposed to define DESs as mixtures of pure components, which present a lower eutectic point temperature than an ideal liquid mixture [29]. Figure 1.3 depicts the difference of the ideal and the real eutectic point in a schematic solid-liquid phase diagram of a binary mixture. To define the temperature depression in a DES the difference of the ideal and the real eutectic temperatures (ΔT_2) should be used instead of the difference of the real and an interpolated eutectic temperature (ΔT_1), following the definition of Coutinho and co-workers [29]. To further define DESs, they should be liquid under operating conditions, which is not required to be conform with the eutectic composition and temperature of the mixture [29]. However, it is difficult to characterise the most

prominent DESs based on ChCl according to this definition due to the thermal decomposition of ChCl at higher temperature preventing the measurement of the melting properties of the pure compound. By approximating the melting curve based on estimating the fusion properties of ChCl, the ChCl:U mixture can be assumed to be a DES due to a negative deviation from ideal behaviour in the urea melting curve and an almost ideal ChCl solubility curve [29]. Terpene:fatty acid mixtures and tetra-n-ammonium salt:fatty acid mixtures are two examples for hydrophobic DESs. While terpene:fatty acid mixtures present quasi-ideal behaviour, tetra-n-ammonium salt:fatty acid mixtures (i.e. tetra-ethyl, -methyl or -propyl ammonium chloride as HBA) show deviations from ideality in the salt solubility curve [29]. This means that terpene:fatty acid are ideal liquid mixtures, whereas tetra-n-ammonium salt:fatty acid mixtures can be considered as DESs. By a strict definition, terpene:fatty acid mixtures are simply eutectic solvents, but should not be categorized as 'deep' eutectic solvents. However, since terpene:fatty acid mixtures are flexible and tunable fluids, presenting a homogeneous liquid phase at ambient temperature despite being prepared from solid starting materials, terpene:fatty acid eutectic mixtures are highly suited to be practically used as solvents.

1.5.2 Application of DESs

Since DESs were first described in 2003 many applications have emerged in various scientific fields. Although DESs can be prepared easily in the laboratory by heating and stirring of the starting materials in a proper molar ratio, different DESs were commercialised and put on the market by Scionix Ltd[2]. The relatively easy preparation of DESs probably has led to a widespread hype on DESs in different disciplines. As more sustainable technologies are needed for future chemical processes, NADESs have even been termed as "solvents for the 21st century" due to their 'greenness' and lower cost in comparison to ILs [36]. This chapter provides a brief overview on major DES applications, which are:

- Extraction, dissolution of complex molecules

[2]See company website: `http://scionix.co.uk`, accessed on 11/05/2019

- Analytical applications
- Separation
- Pharmaceutical applications
- Electrochemical applications
- Reaction medium for organic syntheses, biocatalysis

The extraction of natural substances is an important application of DESs. Some NADESs were reported to be capable of dissolving complex natural molecules, such as rutin, paclitaxel, gingkolide B, salmon DNA, albumin or amylase [32]. Extraction methods with DESs have been proposed for phenolic compounds from model oils [37] or plants [38, 39]. Hydrophobic DESs were investigated as extraction solvents for transition metal ions [40] or for the extraction of artemisinin from plant material [41].

The development of extraction methods is especially important in analytical chemistry. A growing number of DES-based micro-extraction methods is therefore reported for analytical applications [42]. Another example for the use of DESs in analytical techniques is the application in liquid-liquid chromatography. DESs have been investigated as a replacement of water in biphasic liquid-liquid chromatography systems composed of water and organic solvents to separate a complex mixture of natural compounds [43].

DESs were also investigated and determined as suitable fluids for specific separation tasks. For instance, ChCl:Gly was investigated to capture glycerol, a byproduct during biodiesel preparation [44]. As DESs were reported to be capable of solubilising gases like CO_2 or SO_2, gas separation is another interesting application of DESs to capture increasingly emitted gases through DESs as absorbing fluids [45].

Eutectic mixtures are also known in the pharmaceutical field to formulate drugs. The mixtures of the solid anesthetics lidocaine and prilocaine is an example for a

eutectic cream, which has a lower melting point than its individual constituents, and therefore the skin permeation of this eutectic mixture of local anesthetic (trade name EMLA) is enhanced [46]. Terpene-based eutectic mixtures (e.g. menthol/camphor among others) and their interaction with active pharamceutical ingredients (APIs) (e.g. thymol/ibuprofen among others) also play an important role for pharmaceutical preparations [29]. Recently, therapeutic DESs (THEDES) were defined as DESs comprising at least one API as one of the DES components (e.g. ChCl or menthol with acetylsalicylic acid, benzoic acid or phenylacetic acid as APIs) [47].

Another field of application is electrochemistry, where DESs attracted great attention as potential electrolytes in batteries due to their broad electrochemical window. In lithium-ion batteries DESs are potential replacements for hazardous organic compounds of the electrolyte. *N*-methylacetamide:lithium salt DESs were proposed as a suitable electrolytes for lithium-ion batteries [48]. Furthermore, DESs were also used as electrolyte in redox flow batteries [49, 50].

In terms of organic chemistry, DESs have been called the "organic reaction medium of the century" and many reactions (e.g. redox, esterification-type or condensation) are possible in DESs with their components acting either as reagents or catalysts (e.g. acidic or alkaline DESs) [51]. DESs are also interesting reaction media for coupled chemo-enzymatic reactions, combining organo- and enzyme catalysis. For example, the enzymatic step in the chemoenzymatic epoxidation of glyceryl trioleate was enhanced by the use of a DES [52]. Moreover, enantioselective coupled organo-enzymatic C−C bond formations have been reported in DESs, which facilitated product recovery and recycling of the organocatalyst [53, 54]. The role of DESs as alternative reaction media for biocatalysis is presented in the following chapter. Despite the numerous potential fields of application and the huge versatility of DESs in terms of their tunable physicochemical properties, no industrial application of DESs is known to date.

1.5.3 DESs and biocatalysis

DESs were recognised as novel reaction media for biocatalysis since the first successful hydrolase catalysed reactions were reported in DESs in 2008. Kazlauskas and co-workers investigated lipase catalysed transesterification and aminolysis reactions, for which some DESs enabled comparable or even higher initial specific activities than toluene or ILs [55]. Lipases are generally known as robust enzymes and, therefore, lipase catalysed reactions are the most frequently studied reactions in DESs. For instance, the enzymatic synthesis of biodiesel is an important field for the application of lipases in DESs [56, 57]. Also a promiscuous lipase reaction, i.e. an aldol formation, was reported using porcine pancreas lipase (PPL) in ChCl:Gly DES, which promoted an increased conversion of the substrate in comparison to toluene [58].

Although a majority of enzymatic reactions in DESs focusses on lipases, the use of DESs with other enzymes than lipases, and for biotransformations with wohle cell catalysts are currently under research. Recently, glucosidases, another type of hydrolases, were investigated in DESs for the deglycosilation of complex molecules [59, 60]. In aqueous buffer/DES mixtures the solubility of the glycosylated flavonoid hesperidin was improved along with the enzyme activity to obtain the deglycosylated flavonoid hesperetin with analgesic, anti-inflammatory and antioxidant properties [59]. Peptide and dipeptide syntheses have been accomplished in DESs involving the proteases α-chymotrypsin [61] or papain [62], respectively. The effect of DESs on oxidoreductases is also currently researched. ChCl-based DESs were reported to promote higher horseradish peroxidase activity in buffer/DES mixtures in comparison to choline acetate based DESs [63]. Cytochrome c is another oxidoreductase studied in aqueous DES reaction mixtures. Ethylammonium chloride based DESs with ethylene glycol or urea as HBDs were successfully recycled up to four times from buffer/DES reaction systems for an oxidative decolourisation reaction of pinacyanol chloride involving immobilised cytochrome c [64]. Benzaldehyde lyase was investigated in buffer/DES reaction mixtures for the enantioselective C−C bond formation between aldehydes. High conversion (95 %) and enantiomeric excess ($ee > 99\,\%$) was obtained in the

ChCl:Gly/buffer (60:40, v/v) mixture for the carboligation of benzaldehyde as a substrate [65].

Additionally, the application of DES reaction media is not limited to enzymatic reactions with isolated enzymes, but also whole cells were studied as catalysts in DESs especially for co-factor dependent enzymatic reactions (e.g. oxidations/reductions by dehydrogenases). Yeasts [66, 67] as well as recombinant *E. coli* [68, 69] are mainly researched for whole cell catalysis in DESs. For example, ChCl:Gly and ChCl:sugar DESs mixed with up to 50 % (w/w) water were suitable media for the yeast mediated reduction of 3-oxobutanoate with comparable yields to buffer, and the water content and the DES itself was found to influence the enantioselectivity [67]. Moreover, also bacterial and plant cells are used in DESs for the oxidation or reduction of complex molecules. For instance, the addition of a small amount of DES (1 % v/v) as co-solvent to an aqueous buffer improved the oxidation of isoeugenol to vanillin by *Lysinibacillus fusiformis* cells, for which ChAc-based DESs enhanced the bacterial cell membrance permeability and were therefore more effective than ChCl-based DESs [70]. Recently, enantioselective reduction and hydrolysis reactions were accomplished by carrot root cells in water/NADES mixtures [71]. These examples illustrate that DESs are relevant co-solvents for whole cell mediated biotransformations to tune the efficiency as well as the enantioselectivity of the targeted reactions.

1.6 The concept of 2-in-1 reaction media[3]

Many studies that deal with enzymes in DESs focus on the volumetric addition of DESs to buffers and consequently on the resulting effects of the DES addtion on enzyme activity or stability (e.g. most of the examples given in the previous chapter). However, especially in case of water soluble DESs, such as the commonly used ChCl-based DESs, the integrity of the DES in an aqueous solution can be doubted. Even though most studies report that adding the DES components individually would not promote the same stabilising effects than the addition of a DES, the DES hydrogen bond network is likely to be disrupted by mixing the DES

[3]The 2-in-1 concept has been presented by the author and co-workers in [72].

with aqueous buffers. For the ChCl:1,2-propanediol:water 1:1:1 mol/mol/mol DES the hydrogen bonding was observed to completely disappear upon the addition of 50 % (v/v) water [73].

Generally, three different application modes can be distinguished for using DESs as biocatalysis reaction media (see Figure 1.4):

- (1) DES used as co-solvent (water or buffer is the major solvent)
- (2) Pure DESs (e.g. replacing hazardous solvents/enabling high substrate solubility)
- (3) DES acts as solvent and substrate (2-in-1 concept)

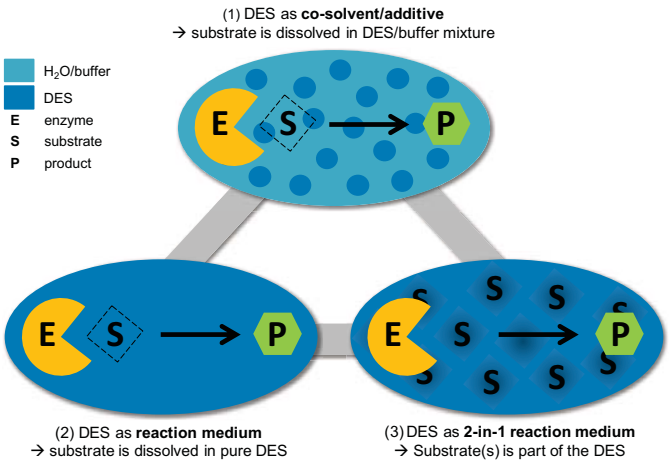


Figure 1.4: For biocatalysis DESs can be used as co-solvents or additives to an aqueous buffer (1), pure reaction solvents (2) or as 2-in-1 reaction media acting as combined solvent and substrate (3).

Instead of using pure DESs or DESs as co-solvents to obtain buffers with improved properties in terms of enzyme activity/stability, DESs might also act as combined solvent and substrate. Due to their composite character DESs can be designed to

comprise one or ideally all of the substrates as HBA/HBD compounds. With this approach, solid substrates become accessible for an enzymatic reaction as they are present liquid in a eutectic mixture. If the DES acts entirely as reaction medium and substrate, the use of a solvent in a classical sense is not necessary (i.e. for dissolving the substrates), and solvent-free reactions with an optimal atomic efficiency can be accomplished. Working with combined solvent and substrate eutectic mixtures also presents a strategy to achieve higher substrates concentrations (and thus higher product titres) than in conventional dilute aqueous solutions usually applied in enzymatic biotransformations. A DES, which is composed of the substrates for an enzymatic reaction, meets two functions–reaction medium and substrate pool at the same time–in one, and therefore this conception is termed 2-in-1 reaction medium concept.

Recently, a few studies exploited the potential of DESs as 2-in-1 reaction media. For example, ChCl:Gly was used for the lipase catalysed synthesis of α-monobenzoate glycerol [74], ChCl:sugar DESs supplied the sugar substrate for the lipase catalysed glycolipid synthesis [75–78], ChCl:U · H_2O_2 acted as reaction medium and H_2O_2 source for the chemo-enzymatic epoxidation of monoterpenes [79], and ChCl:Gly was also used as 2-in-1 reaction medium for the lipase catalysed synthesis of 1,3-diacylglycerol [80]. Even though ChCl was needed as an auxiliary to merge the targeted substrate in a eutectic, these examples illustrate the potential of using 2-in-1 DESs as non-aqueous reaction media for synthetic reactions. Moreover, the use of hazardous solvents is reduced or completely avoided, which contributes to a more sustainable and ecological reaction design. Therefore, eutectic substrate mixtures, acting as combined solvent and substrate source, are a smart way to develop efficient biotransformation solvents. Generally, the best solvent would be no solvent [81], and DESs certainly have much to offer to meet such a requirement. However, the 2-in-1 DES reaction medium concept has not yet been fully exploited, and ideally the use of an auxiliary DES compound such as ChCl should be replaced to obtain a pure substrate:substrate DES. Although choline is an inexpensive nutrition bulk chemical naturally occurring in many plants and organisms (e.g. lecithin, acetylcholin), its use for ChCl:substrate DESs is impractical from a reaction engineering perspective. Product purification becomes more difficult by

using auxiliaries, such as ChCl, which needs to be separated from the product after the reaction. In contrast to that, advantages in terms of reaction efficiency and product purification can be expected for entirely substrate-based DESs. In order to fully exploit the potential of DES as alternative reaction media for biocatalysis, the application of substrate:substrate DESs for non-aqueous, synthetic reactions was especially addressed in the present study.

2 Objective of the study

The increasing demand for more sustainable chemical processes has resulted in a growing interest in alternative technologies. Combining biocatalysis with novel 'deep eutectic solvents' (DES) reaction media is a promising approach to accomplish synthetic reactions under mild and environmentally acceptable conditions. The tunable properties of DESs may allow to engineer tailor-made and sustainable DES reaction media for a targeted reaction, while enzymes are beneficial catalysts due to their high specificity at relatively low reaction temperature or pressure. These special features of both enzyme catalysis and DESs would help to fulfil some of the principles of 'Green Chemistry' (e.g. use of catalysts, prevention of waste or avoiding auxiliary solvents) to achieve more sustainable processes.

Although DESs have been widely used as co-solvents in aqueous reaction media to stabilise enzymes, this application was not in the focus of the present study. DESs were rather regarded as powerful replacements for non-aqueous ILs or organic solvents in the biotransformation of sparingly water-soluble substrates. The biotransformation of hydrophobic substrates remains a challenging task, since the ideal solvent needs to compromise high enzyme stability/activity with a high solubility of the substrate at the same time. The choice of potential reaction media is however limited, as enzymes are usually less active in organic solvents than in water. Stripping off water molecules from the hydration shell of the enzyme in polar organic solvents even might prevent efficient catalysis. Often non-polar organic solvents are the preferred reaction media to maintain a proper enzyme flexibility. Therefore, DESs can add a novel non-aqueous reaction medium to the biocatalysis solvent toolbox for synthetic reactions.

The objective of the present study was to evaluate the potential of novel DESs as alternative, non-aqueous reaction media for enzyme catalysed reactions involving poorly water-soluble substrates. Therefore, the first goal of this study was to characterise different DESs by their physicochemical properties, which were considered relevant for biocatalysis, to better understand the nature of this novel solvent class.

In the second step, a suitable model reaction was to be determined in DESs, by screening different lipase catalysed reactions in DESs. A special focus was eventually set on developing 2-in-1 DES reaction systems for the biotransformation of the terpene alcohol menthol. Among various menthol isomers (–)-menthol is a characteristic minty aroma and flavour compound widely used in food, cosmetic or pharmaceutical products. Modified (–)-menthol extenuates the strong minty flavour, which is sometimes desired in cosmetic or pharmaceutical formulations. Once the lipase catalysed esterification of (–)-menthol was defined as model reaction, the next objective of this study was to optimise the reaction conditions and to demonstrate that an efficient biotransformation is feasible in a DES. As the recovery of products from DES reaction media is often not addressed, another scope of this study was to investigate the product purification and recycling of unconverted substrates. The potential of DESs reaction media was highlighted by characterising the properties of DESs and demonstrating their applicability for developing an integrated bioprocess, from a model reaction through reaction optimisation to product purficiation. Finally, DESs ought to be evaluated against other non-conventional solvents used in biocatalysis, which was attempted by benchmarking the results in comparison to published data and by value-benefit analysis.

3 Physicochemical properties of different DESs

3.1 Introductory words

In the quest for alternative solvents for enzyme-catalysed reactions, the physicochemical characterisation of such novel reaction media is essential. In order to use DESs as reaction solvents, they have to meet certain demands, such as being liquid in a temperature range acceptable for enzymatic reactions. In contrast to neat solvents, the physicochemical properties of DESs as composite solvents are directly linked to the DES starting materials. For example, DESs based on ChCl are rather polar solvents [82], whereas also hydrophobic DESs have been developed based on longer chain quaternary ammonium salts [83, 84] or on terpenes, such as menthol or thymol [85, 86]. The physical and chemical properties of a DES also vary depending on the mole fraction of the DES compounds. As an example, the alteration of the ChCl:Gly molar composition affected the density, viscosity and conductivity of the solvent [87]. In view of applying DESs as reaction media for biocatalysis, this chapter provides information on a few DES properties that are important for enzymatic reactions (see Figure 3.1).

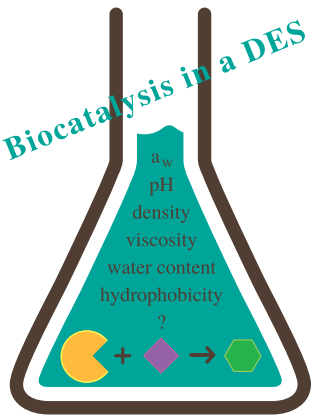


Figure 3.1: Unknown physical and chemical DES parameters influencing an enzymatic reaction.

The density is characterised as a basic solvent property, while the water content and water activity (a_w) in a DES solvent are essential factors influencing the enzyme performance in a non-conventional reaction medium. Characterizing the water saturation capacity also provides information on the DES hydrophobicity. Furthermore, the rheological behaviour and the viscosity of some DESs was assessed in terms of how the relatively high viscosity of some DESs can be reduced. Figure 3.1 gives an overview on different DES physical and chemical parameters, which might have an influence on an enzymatic reaction in this kind of non-conventional reaction medium.

3.2 Density

Solvent density is an important physical parameter in chemical engineering to calculate the Reynolds number characterizing the fluid dynamics in a reactor, for instance. The density of a substance is defined as its ratio mass to volume ratio

$$\rho = \frac{m}{V} \tag{3.1}$$

and is a pressure and temperature dependent parameter. While high pressure leads to an increasing density through molecular compression, high temperatures reduce the density by increasing the molecular movement.

As opposed to pure fluids, the physical and chemical parameters of composite solvents depend on the mole fractions of the starting materials. In the case of DESs, this means that phyicochemical parameters change with the variation of the mole fraction of one DESs compound (i.e. the HBD or HBA). It was shown that in comparison to pure glycerol, the density of the mixture of ChCl and glycerol is reduced upon increasing the ChCl mole fraction [87]. The addition of ChCl to glycerol modifies the density and therefore the formation of a eutectic mixture can be advantageous over using pure solvents to tune basic physical parameters.

The temperature dependent changes in the density of various types of DESs have been studied extensively [88–96]. In the case of water-miscible DESs, the addition of water also results in the modification of the density [73, 97–99]. While most of

Table 3.1: Density of different menthol-based DESs.

DES	Molar ratio mol/mol	Density $g\,mL^{-1}$	Standard deviation $n = 3$
(–)-Menthol:caprylic acid	1:0.82	0.916	± 0.001
(–)-Menthol:capric acid	1:0.54	0.910	± 0.001
(–)-Menthol:lauric acid	3:1	0.907	± 0.001
(±)-Menthol:caprylic acid	1:0.82	0.912	± 0.004
(±)-Menthol:capric acid	1:0.54	0.909	± 0.004
(±)-Menthol:lauric acid	3:1	0.905	± 0.002

the conventional ChCl-DESs are water-miscible, there are also a few hydrophobic DESs [83, 85, 86], which form biphasic solvent mixtures upon the addition of water. Terpene-based DESs are attributed as natural, hydrophobic DESs among the group of water-immiscible DESs. The density of different menthol:fatty acid DESs was characterised by a differential weighing method. Table 3.1 shows that the density decreases with an increasing fatty acid chain length, which was also reported by Martins et al. [85]. As the density of the hydrophobic menthol:fatty acid DESs is lower than the density of water (see Table 3.1), the DES forms the top layer when it is mixed with water. A possible application for hydrophobic DESs is the use as extraction solvents, which has been investigated in several studies [40, 41, 83, 85, 100].

3.3 DES pH

Enzymes usually work most efficiently at a certain pH optimum in an aqueous reaction medium, where the pH is defined as the negative decadic logarithm of the activity of the hydrogen ion [101]. In dilute solutions the pH can be approximated by the negative decadic logarithm of the molar concentration of the hydrogen ion [102].

$$\mathrm{pH} = -\lg\ c(\mathrm{H_3O^+}) \tag{3.2}$$

Since DESs can be applied as pure solvents or as additives to aqueous buffers in enzyme catalysed reactions, it is relevant to characterise the pH of neat DESs and

to answer the question on how the addition of DESs shifts the pH of an aqueous reaction medium. Although pH scales exist for organic solvents, they are not comparable to each other and attempts were made to define a unified pH scale valid for gases, liquids and solids [101]. However, there is a poor understanding of the proton activity and transfer mechanisms in novel solvents, such as DESs or ILs [103]. The pH values of pure ChCl:U, ChCl:Gly and ChChl:EG cover the alkaline, neutral and acidic range with pH values of 10.4 [104], 7.5 [104] and 4.7 [105] at 20 °C, respectively. However, the use of conventional pH electrodes with glass membranes can cause difficulties in anhydrous conditions [103] and therefore pH measurement in water-free solvents, such as neat DESs, is afflicted with uncertainty.

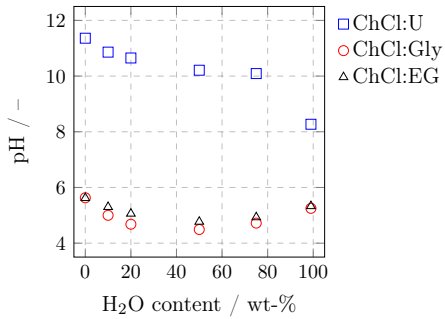
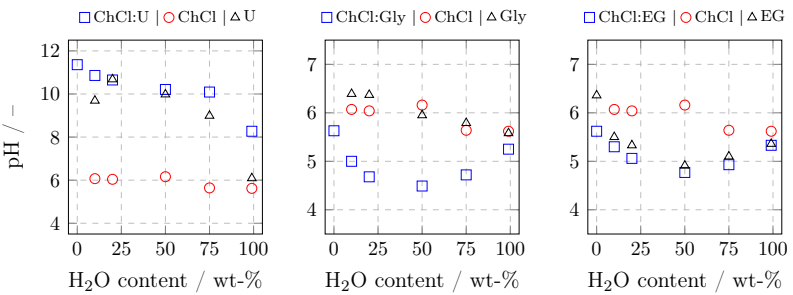


Figure 3.2: Effect of water addition on the pH of ChCl:U 1:2 mol/mol, ChCl:Gly 1:2 mol/mol and ChCl:EG 1:3 mol/mol at ambient temperature.

The use of DESs as additives or co-solvents for enzyme catalysed reactions in buffers is widespread and positive effects on enzyme stability [106], activity [107, 108] or selectivity [109, 110] have been reported. The application of DESs as co-solvents for biocatalysis requires the characterisation of the impact of the DES and the DES components on the pH of the reaction medium. Therefore, the pH of different water miscible DESs was measured as a function of the water content of the DESs (see Figure 3.2). While ChCl:U-aqueous mixtures exhibited alkaline pH values, the pH for ChCl:Gly and ChCl:EG water mixtures ranges from approximately 4 to 6. Moreover, the effect of the DES starting materials in aque-

ous mixtures was investigated in comparison to the the corresponding DES-water mixtures (see Figure 3.3). The variation of the pH with the addition of water to ChCl:U and ChCl:EG can be explained by the HBD compounds urea (see Figure 3.3a) and ethylene glycol (see Figure 3.3c), respectively, whereas ChCl appears not to contribute to the pH changes in the DES-water mixtures. In contrast to that, ChCl:Gly water mixtures resulted in pH changes, which are neither influenced by ChCl nor by glycerol (see Figure 3.3b). Durand et al. also studied the influence of the pH of different DES-water mixtures on a lipase catalysed alcoholysis reaction. They showed that the pH of DES-water mixtures is only of secondary importance and that the interplay of the DES HBD and the water content is important to efficiently perform the alcoholysis [111]. Due to the non-conventional character of DESs, the pH is obviously influenced by mixing DESs with water, but it is less significant than in water to perform enzyme catalysed efficiently in DESs.



(a) ChCl:U (b) ChCl:Gly (c) ChCl:EG

Figure 3.3: Effect of DES compounds on the pH of ChCl:U 1:2 mol/mol (a), ChCl:Gly 1:2 mol/mol (b) and ChCl:EG 1:2 mol/mol (c) at ambient temperature.

3.4 Water content and water activity[4]

In terms of biocatalysis in DESs, the water content and the thermodynamic activity of water (a_w) in a non-conventional reaction medium are parameters that are

[4]Some of the presented results have been published by the author and co-workers in [112, 113].

easier to approach than the pH. As mentioned previously, the physical and chemical properties depend on the starting materials of the composite solvent. DESs are frequently synthesized with the quaternary ammonium salt ChCl, which is a strongly hygroscopic solid. This property is transferred to composite ChCl:HBD DESs making them prone to take up water from the ambient air moisture. DESs cannot be generally accepted as stable solvents and the water content might be altered depending on the storage conditions. As it is often necessary to control the water content at the beginning or during an enzymatic reaction, the characterisation of the water content of DESs is essential both in terms of their storage stability and in terms of their acceptability as reaction media.

The water adsorption of different ChCl- and hydrophobic TbACl-DESs was studied by exposing the DESs to ambient air in comparison to keeping the DESs in closed vials (see Figure 3.4). Upon storage in open vials the ChCl-based DES absorbed significant amounts of water from initially less than 0.5 wt% to about 15 wt% after 9 days of storage (see Figure 3.4a). This demonstrates that the water-miscible ChCl-based DESs are sensitive to air humidity due to the hygroscopicity of ChCl. In contrast to that, the water-immiscible TbACl-based DESs absorbed less water from the atmosphere upon storage in open vials. However, a decreasing water content was observed for the TbACl:1-hexanol DES, which may be caused by evaporation of the long-chain alcohol compound (see Figure 3.4a). Figure 3.4b depicts the water content of the DESs that were stored in closed vials, which resulted in unaltered water contents. This indicated that the water content of all tested DESs is not changed, if the DESs are stored in closed containers.

In order to characterise the hydrophobicity of a DES, it is interesting to study to which extent water is miscible with hydrophobic DESs, when they are in contact with an aqueous phase. The saturation of hydrophobic (–)-menthol:fatty acid DESs with water was investigated by incubating the DESs with different volumes of water under constant agitation at 35 °C for 24 h. Under these conditions, the water content of all DESs was unchanged after 24 h, which rules out any water uptake from the atmosphere. If 1 wt% (relating to the DES weight) of water was added to (–)-menthol:fatty acid DESs, the water content of the DESs was increased

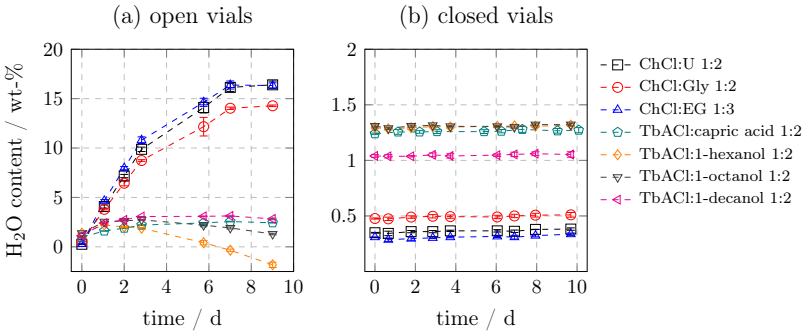


Figure 3.4: Water absorption of different DESs stored in contact with ambient atmosphere (a) and in closed vials (b) at 25 °C.

by about the same amount (see Figure 3.5). The addition of 1 wt% resulted in a transparent, homogeneous liquid. In contrast to that, two phases are formed by the addition of 5 or 10 wt% water to the hydrophobic (–)-menthol:fatty acid DESs. After the 24 h incubation, the water content in the DESs phase increased to a certain maximum, irrespective of the amount of water present (5 or 10 wt%). As deduced from Figure 3.5, the water saturation contents for (–)-menthol:caprylic acid (C8:0 fatty acid), (–)-menthol:capric acid (C10:0 fatty acid) and (–)-menthol:lauric acid (C12:0 fatty acid) were 2.6, 2.1 and 1.9 wt%, respectively, at a water addition of 10 wt%. The water saturation was observed to decrease with an increasing hydrophobicity of the HBD, i.e. an increasing carbon chain length of the fatty acid (caprylic acid C8:0; capric acid C10:0; lauric acid C12:0). Therefore, the water saturation content provided comparative information on the overall hydrophobicity of the DES mixtures.

In terms of applying DESs as reaction media for enzymatic biotransformations, the water content is an essential parameter to ensure enzyme activity, since a minimal amount of water is necessary to maintain a proper protein fold in a non-aqueous environment. The a_w is preferably used as a measure of the amount of water in organic reaction solvents, since the enzyme activity is affected by the relative amount of water with respect to the total water solubility [114]. In general, the a_w can be

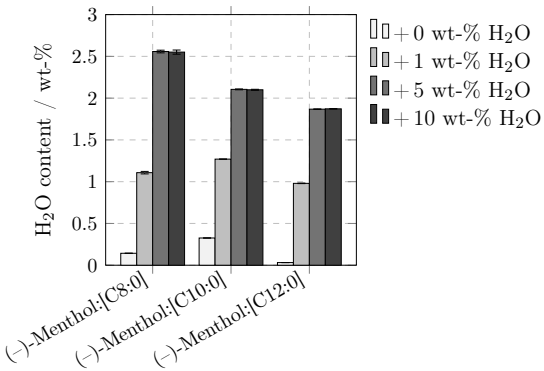


Figure 3.5: Water content of hydrophobic (–)-menthol-based DESs after incuabtion with different amounts of water (wt% relating to the DES weight) at 1100 rpm and 35 °C for 24 h ($n = 3$). The DESs were synthesized with saturated fatty acids of different carbon chain length (caprylic acid C8:0; capric acid C10:0; lauric acid C12:0).

interpreted as the amount of unbound, freely available water in a certain matrix. In order to provide sufficient hydration to an enzyme in a non-aqueous reaction medium and to control the a_w during a reaction, saturated salt solutions can be used to equilibrate the solvent to a defined a_w via the gas phase [115]. While adjusting the a_w through the water vapour in the gas phase is difficult for hygroscopic ChCl-DESs [116], it was possible to correlate the a_w with the water content in a hydrophobic (−)-menthol:lauric acid DES (see Figure 3.6).

The experimental data was fitted by a linear function using QtiPlot software (version Qt: 5.9.7) with instrumental weighing. The following equation (3.3) was obtained where y is the water content (expressed as $\mathrm{mg_{water}\,g_{DES}^{-1}}$) and x is the water activity:

$$y = 13.995\,x + 0.01 \tag{3.3}$$

The resulting equation can be interpreted as a linear sorption isotherm to describe the water uptake of the (–)-menthol:lauric acid DES from saturated salt solutions of different a_w. In analogy to Henry's law, which usually describes gas-

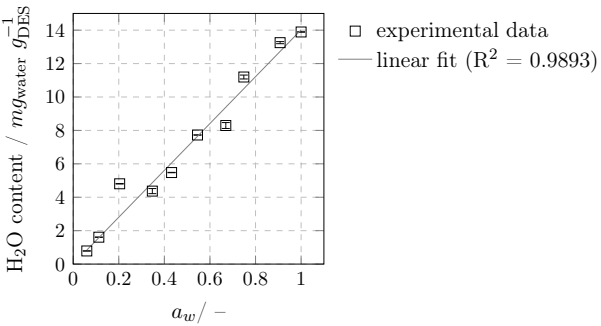


Figure 3.6: Correlation of a_w and the water content in (−)-menthol:lauric acid 3:1 mol/mol after incubation of the DES with different saturated salt solutions at 35 °C, ($n = 3$).

liquid dissolution or linear solid-solid adsorption phenomena, equation 3.3 can also be expressed in terms of the water partial pressure p and the water concentration q in the DES

$$q = H_{sorp}\ a_w = H_{sorp}\frac{p}{p_0} \tag{3.4}$$

with a_w characterising the ratio of the water vapour pressure of a substance compared to the vapour pressure of pure water at a constant temperature.

Therefore, Henry's constant H_{sorp} was determined for a_w from 0 to 1 by a slope of 13.995 $mg_{water}\ g_{DES}^{-1}$ for the water concentration q in the (–)-menthol:lauric acid DES after incubation at different water partial pressures (expressed through the a_w of the saturated salt solution). The incubation of the DES with pure water, corresponding to a_w 1, was expected to induce a similar water saturation content as previously determined by mixing the DES directly with water (1.9 wt%, see Figure 3.5). Through gas phase equilibration of the (–)-menthol:lauric acid DES at a_w 1, water saturation was obtained with 14 $mg_{water}\ g_{DES}^{-1}$, corresponding to 1.4 wt% of water in the DES phase. Both methods delivered similar results on the maximum amount of water that can be taken up by the (–)-menthol:lauric acid DES and a minor deviation might be explained by a day-to-day variation in the preparation of the DES.

3.5 Rheological characterisation and DES viscosity

Viscosity is a measure for the inner flow resistance of the molecules in a liquid upon the action of shear or tensile stress. As a constant of proportionality, viscosity (η) describes the correlation between shear stress (τ) and shear rate ($\dot{\gamma}$), if a fluid is deformed by a moving plate, for instance.

$$\tau = \eta \cdot \dot{\gamma} \tag{3.5}$$

As bioprocesses generally take place in a dilute aqueous environment, viscosity is often not an influential factor. However, viscosity becomes a limiting parameter across the potential use of DESs as reaction media for biocatalysis due to the honey-like texture of many DESs. Processing of viscous liquids is principally a difficult task in terms of an increased energy input, which is required for pumping or stirring. Beyond that, a high viscosity lowers the mass transfer in a reaction solvent. These challenges need to be considered, if viscous DESs are intended to be used as reaction media for biocatalysis. Therefore, the rheological characterisation of reaction solvents is an important task. Next to temperature dependent changes, the viscosity can also be altered in response to shear stress, if a fluid shows non-Newtonian flow behaviour. As the viscosity of a reaction medium has an impact on the mass transfer, it is essential to have knowledge about the viscosity of the reaction solvent and how it is affected, if external parameters are varied.

The flow behaviour of some ChCl-based DES was characterised as non-Newtonian, and shear-thinning characteristics were observed for some DESs [117–119]. The rheological flow behaviour of a few ChCl-based DESs (ChCl:Gly 1:2 mol/mol, ChCl:U 1:2 mol/mol, ChCl:EG 1:3 mol/mol) and some hydrophobic TbACl-based DESs (HBD: 1-hexanol, 1-octanol, 1-decanol or decanoic acid; TbACl:HBD 1:2 mol/mol) was investigated (see Figure 3.7).

Figure 3.7 A shows the flow curves of the ChCl- and TbACl-DESs over a gradually increasing shear rate. Viscosity corresponds to the slope of the flow curves in this plot. As the slope of the flow curves is constant, which refers to a constant viscosity over the tested shear rates, Newtonian flow behaviour can be assumed

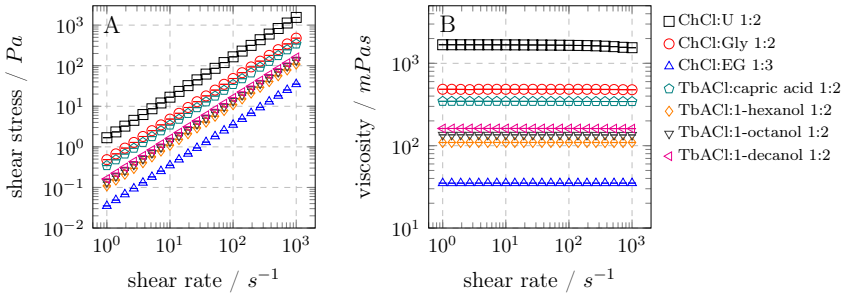


Figure 3.7: Flow behaviour of different DESs at 20 °C. Shear stress τ (A) and viscosity η (B) are shown as a function of the the shear rate $\dot{\gamma}$.

for the ChCl- and TbACl-DESs. Except for ChCl:U, the viscosity curves also visualise that the viscosity of the DESs remains unchanged within the tested shear rate ranges (see Figure 3.7 B). ChCl:U was slightly shear-thinning at high shear rates (100 to 1000 s^{-1}). Such a trend might not be a real shear-thinning effect, but might be ascribed to a loss of sample from the gap between the plates at high shear rates. However, slight shear-thinning properties were also reported for ChCl:U, ChCl:Gly and ChCl:EG at much lower shear rates of up to 100 s^{-1}, but the DESs were still considered as Newtonian fluids in the lower shear rate range [117]. In conclusion, ideal flow behaviour predominated for ChCl- and TbACl-based DESs for shear rates of up to 100 s^{-1}, and therefore these solvents were basically regarded as Newtonian fluids. The viscosity of the DESs ranged from approximately 1700 to 35 mPa·s for ChCl:U and ChCl:EG, respectively, which is 35 to 1700-times higher than for water. More specific details on the rheological flow behaviour of ChCl:U are presented in the supplementary section (see chapter A.1).

Since viscosity is a temperature dependent variable, the viscosity of a DES can be modified by increasing or decreasing temperature. The temperature dependent behaviour can be described by a logarithmic form of the Arrhenius equation, where η_0 is a constant, E_A is the activation energy, R is the ideal gas constant and T is the temperature.

$$\ln \eta = \ln \eta_0 + \frac{E_A}{R \cdot T} \tag{3.6}$$

It has been reported that the temperature dependent changes in the viscosity of various DESs can be approximated with an Arrhenius equation [90, 92, 93, 120]. For ChCl- and TbACl-based DESs a decrease of the viscosity was observed at elevated temperatures (see Figure 3.8). The viscosity of ChCl:U 1:2 mol/mol was drastically reduced from approximately 1800 to 40 mPa·s, if the temperature was increased from 20 to 80 °C. In contrast to that, the viscosity of hydrophobic (−)-menthol:fatty acid DESs is rather low at room temperature (below 30 mPa·s at 25 °C for (−)-menthol:capric acid, (−)-menthol:caprylic acid and (−)-menthol:lauric acid) and also decreases with an increasing temperature [85]. Temperature is thus a powerful parameter to lower the viscosity of both hydrophilic and hydrophobic DES reaction media in order to enhance mass transfer. However, this also indicates that thermally stable enzymes would be necessary to perform biotransformation reactions efficiently in DESs.

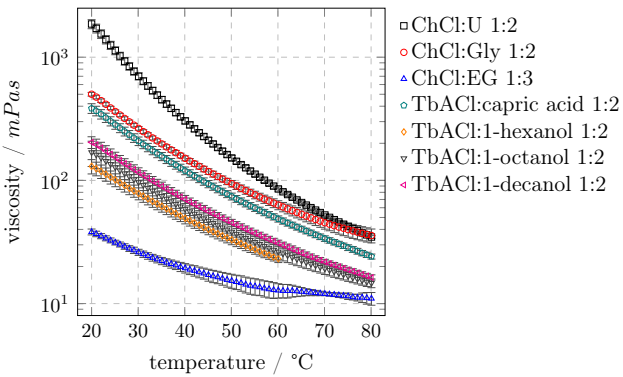
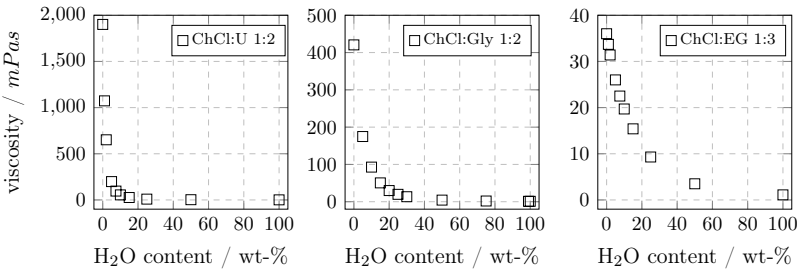


Figure 3.8: Temperature dependent changes of the viscosity of different DESs.

Another way to modify the relatively high viscosity of DESs is to mix them with water, although this is only efficient for water miscible (hydrophilic) DESs. The H-bond network of a neat DES is interfered through the addition of water, leading to a viscosity reduction. The addition of large quantities of water might even dissolve the DES compounds completely for DESs composed of water soluble compounds (e.g. ChCl, glycerol, urea, ethylene glycol). For instance, it has been re-

ported that the hydrogen bonding between in the ChCl:1,2-propanediol:water 1:1:1 mol/mol/mol DES completely disappeared by the addition of 50 % (v/v) water to the DES [73]. A strong reduction of the viscosity of ChCl:U and ChCl:Gly was observed by the addition of 10 and 30 % (w/w), respectively (see Figures 3.9a, 3.9b), which results from a weakening of the H-bond network between the DES compounds. The viscosity of ChCl:EG also decreased gradually with the addition of water (see Figure 3.9c). Although it is difficult to quantify solvent polarity with a single physical constant, ChCl:U was reported to be more dipolar than ChCl:Gly or ChCl:EG on a pyrene emission intensity scale [82]. Therefore, the higher dipolarity of ChCl:U in contrast to ChCl:Gly or ChCl:EG could explain, why the addition of water to this DES is more effective to reduce the viscosity than for the less dipolar DESs.



(a) ChCl:U (b) ChCl:Gly (c) ChCl:EG

Figure 3.9: Effect of water addition on the viscosity of ChCl:U 1:2 mol/mol (a), ChCl:Gly 1:2 mol/mol (b) and ChCl:EG 1:2 mol/mol (c).

3.6 Summary

Relevant physical and chemical properties of different hydrophilic and hydrophobic DESs have been characterised and the results can be summarised as follows:

- Hydrophobic menthol:fatty acid DESs have a lower density than water and, therefore, form the top layer in binary water-DES mixtures.

- The addition of water to hydrophilic DESs (ChCl:U, ChCl:Gly, ChCl:EG) modulated the pH of the binary water-DES mixtures.
- The pH of binary water DES-mixtures of ChCl:U and ChCl:EG can be explained by the HBD U or EG, respectively, while ChCl seems to be irresponsible for the pH change in the DES-water mixtures.
- Hydrophilic DESs (ChCl:U, ChCl:Gly, ChCl:U) are susceptible to air humidity and take up water easily, if not stored closed. In contrast to that hydrophobic TbACl-based DESs are less sensitive to accumulate water upon storage in open vials.
- The water content in the hydrophobic (−)-menthol:lauric acid 3:1 mol/mol DESs can be adjusted and controlled using a gas phase equilibration method with saturated salt solutions of defined water activity (a_w).
- ChCl:U, ChCl:Gly, ChCl:EG, TbACl:decanoic acid, TbACl:1-hexanol, TbACl:1-octanol and TbACl:1-decanol are Newtonian fluids and their viscosity can be significantly reduced by increasing the temperature.
- Moreover, the viscosity of water miscible DESs (ChCl:U, ChCl:Gly, ChCl:EG) can be decreased by the addition of water

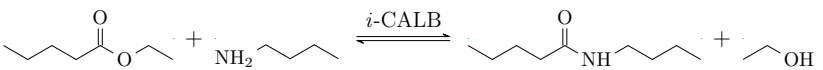
4 Lipase catalysed reactions in DESs

4.1 Introductory words

Since the 1980s it is well accepted that enzymes can be active under non-aqueous conditions, such as in organic solvents [17]. Lipases are especially robust biocatalysts and are active in a huge variety of solvent systems, e.g. in aqueous mixtures with water miscible organic solvents, biphasic reaction mixtures with water immiscible organic solvents, non-aqueous solvents, supercritical fluids, reverse micellar systems, solvent-free reaction systems, gaseous reaction media or in ionic liquids [121]. In 2008 the reaction medium toolbox for hydrolase-catalysed reactions has been expanded to DESs by the first proof-of-principle study on lipase-catalysed transesterification and aminolysis reactions in DESs [55]. Since then, many examples for a variety of lipase-catalysed reactions in DESs have followed involving hydrolysis reactions [108], (trans-)esterification reactions [74, 80, 112, 116], C−C-bond formations [58, 122] or chemo-enzymatic epoxidation reactions [52, 79, 123]. In this chapter several different lipase-catalysed reaction types are presented and the applicability of DESs to the targeted reactions is discussed.

4.2 Aminolysis

The lipase catalysed aminolysis of 1-butylamine with ethyl valerate to form the 1-butyl valeric acid amide and ethanol was one of the first reaction performed in ChCl-based DESs [55] and therefore, this type of reaction was studied in a first round of experiments. The aminolysis was performed using an immobilised *Candida antarctica* lipase B (*i*-CALB) as catalyst and the reaction is shown by Scheme 4.1.



Scheme 4.1: CALB catalysed aminolysis of ethyl valerate and 1-butylamine.

While the reaction was conducted in toluene as a reference, ChCl:Gly (1:2 mol/mol), ChCl:U (1:2 mol/mol) and ChCl:acetamide (1:2 mol/mol) were used for the aminolysis in DES reaction media. Figure 4.1 depicts the time course of the product

formation (1-butyl valeric acid amide) qualitatively by the peak area obtained after GC analysis. In comparison to toluene, the qualitatively expressed product formation was lower in all tested DESs. The liquid handling of viscous DESs did not allow to transfer a defined volume of the DESs to the reaction vials and the reaction was performed in a defined weight of DES instead. The difference in the density of the DESs and of toluene could also result in different reactant concentrations. However, in terms of the conversion of ethyl valerate after 6 h, the reaction was more efficient in ChCl:Gly (98.8%) than in ChCl:U (96%), toluene (90.5%) or ChCl:acetamide (83.4%), which is comparable to the published data.

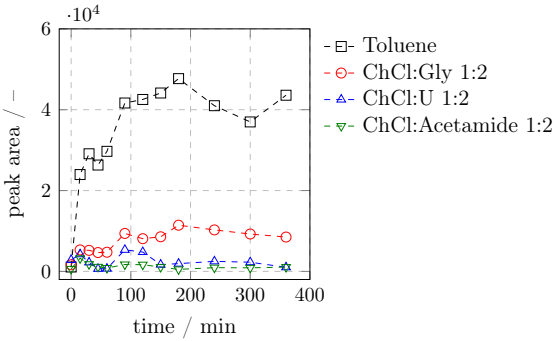


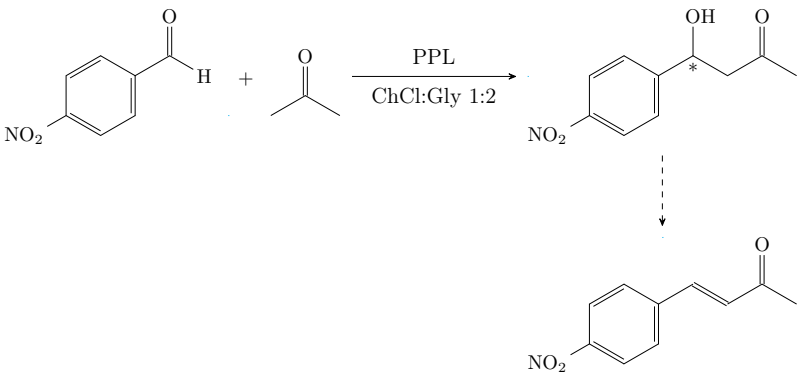
Figure 4.1: Time course of the 1-butyl valeric acid amide formation by *Candida antarctica* lipase B qulitatively expressed as peak area in toluene and three different DES.

4.3 Aldol reaction[5]

In organic chemistry aldol reactions refer to the C−C-bond formation between two carbonyl compounds (e.g. ketones, aldehydes) to create a β-hydroxycarbonyl compound (aldol addition) [125]. If water is eliminated from the β-hydroxycarbonyl compound, the reaction is termed aldol condensation [125]. Aldol reactions are tra-

[5]Some of the presented results are part of Natalie Müller's Master thesis entitled "Aldolreaktion in eutektischen Lösungsmitteln" [124]. The thesis was prepared at the DECHEMA's Research Institute (Industrial Biotechnology) between 01/2017 and 07/2017.

ditionally acid- or base-catalysed [125], but organocatalysis, involving proline as a catalyst for instance, is an important tool to achieve asymmetric C−C-bond formations [126]. In nature, enzyme catalysed aldol reactions occur in the metabolic pathways of living organisms to form or cleave C−C-bonds involving aldolases, i.e. enzymes belonging to the group of lyases (EC 4.-.-.-). For synthetic applications, DESs have been used as co-solvents for the benzaldehyde lyase catalysed C−C-bond formation of different aldehydes [65]. Furthermore, synthetic enzyme-catalysed aldol additions can also be performed with robust lipases showing a promiscuous activity to catalyse C−C-bond formations [127]. Reaction mechanisms for the lipase catalysed asymmetric aldol addition of acyclic as well as cyclic ketones with aromoatic aldehydes have been proposed recently [128].



Scheme 4.2: PPL catalysed aldol reaction of 4-nitrobenzaldehyde and acetone at 60 °C and 250 rpm in ChCl:Gly DES as reported by González-Martínez et al. [58].

An increasing interest in sustainable conditions for chemical reactions has lead to the use of neat ChCl-DES reaction media for a promiscuous lipase-catalysed aldol reaction [58]. The reported aldol addition (see Scheme 4.2) of 4-nitrobenzaldehyde (4-NBA) and acetone with porcine pancreas lipase (PPL) was selected as a starting point to study if hydrophobic DESs and substrate-based DESs are advantageous over ChCl-based DESs for the lipase catalysed aldol formation. In a preliminary screening, hydrophobic DESs were preapared and tested in comparison to

ChCl:Gly or water to increase the solubility of 4-NBA, which is only slightly soluble in water. Hydrophobic DESs were prepared with menthol, tetra-*n*-octylammonium bromide (ToABr) or tetra-*n*-butylammonium chloride (TbACl) as hydrogen bond donors and combined with HBD of different polarity. Table 4.1 gives an overview on the DES compositions.

Table 4.1: DESs prepared for the PPL catalysed aldol reaction.

DES	Molar ratio *mol/mol*	$T_{synthesis}$ *℃*	Reference
ChCl:Gly	1:2	50	[87]
Menthol:pyruvic acid	1:2	70	[86]
Menthol:lactic acid	1:2	70	[86]
Menthol:levulinic acid	2:1	70	[-]
Menthol:acetic acid	1:1	70	[86]
ToABr:1,5-pentanediol	1:3	70	[96][a]
ToABr:ethylene glycol	1:3	70	[96][a]
TbACl:4-nitrobenzaldehyde	2.2:1.5	80	[122]

[a] DESs in [96] were prepared using TbACl, whereas the HBD was replaced by ToABr for the present experiments.

The DESs were liquid at 60 °C and the solubility of the substrate 4-NBA was assessed at the same temperature. The 4-NBA solubility was lowest in water and in the hydrophilic ChCl:Gly DES among all tested solvents (see Table 4.2). The compound was more soluble in DESs consisting of a hydrophobic HBA and a hydrophilic HBD. The polarity of the HBD of the DESs possibly results in an improved solubility due to the polar residues (nitro- and aldehyde group) within the 4-NBA molecule [122]. For example, the solubility is significantly higher in ToABr:ethylene glycol than in ToABr:1,5-pentanediol, although the DESs only differ by three carbons in the HBD alkyl chain length. A similar result was obtained in a study on tailoring DESs for the extraction of artemisinin, where DESs composed of C_3−C_6 fatty alcohols probably had a similar polartiy and enhanced the extraction yield [84]. Therefore, the careful selection and combination of HBD

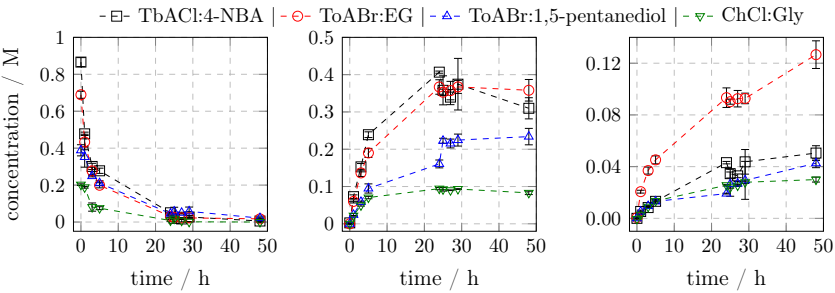
and HBA compounds enables the flexible design of tailor-made solvents that match the polarity of the reactant of interest at best. In terms of the PPL catalysed aldol addition, the reaction yield might be improved by the use of more hydrophobic DESs, enabling higher substrate loads.

Table 4.2: Solubility of 4-nitrobenzaldehyde (4-NBA) in water and different DESs at 60 °C.

Solvent	Solubility of 4-NBA M	Standard deviation $n = 3$
Water	0.014	± 0.001
ChCl:Gly (1:2)	0.25	± 0.02
Menthol:acetic acid (1:1)	0.18	± 0.08
Menthol:levulinic acid (2:1)	0.64	± 0.15
Menthol:lactic acid (1:2)	0.33	± 0.09
Menthol:pyruvic acid (1:2)	1.87	± 0.27
ToABr:1,5-pentanediol (1:3)	0.70	± 0.03
ToABr:ethylene glycol (1:3)	1.25	± 0.21

The PPL catalysed aldol reaction (see Scheme 4.2) was studied in different hydrophobic DES reaction media (see Table 4.1), which should give rise to an enhanced reaction yield due to an improved solubility of the substrate 4-NBA. Since acids can function as catalysts for the aldol reaction, the menthol:acid DESs were excluded from further experiments. Instead, a substrate based DES, composed of TbACl and 4-NBA, was prepared to act as reaction solvent and substrate pool at the same time for the aldol reaction. Furthermore, ToABr:1,5-pentanediol and ToABr:EG were tested as reaction media, whereas the reaction in ChCl:Gly was used as a reference for a published aldol addition in a DES. The reactions were performed with different starting concentrations of 4-NBA depending on the maximum solubility of the substrate in each DES and the reactant concentrations were measured over time (see Figure 4.2). After 24 h the substrate was almost fully converted in all DESs (see Figure 4.2a), indicating that PPL is not only

active in ChCl:Gly, but also in the hydrophobic ToABr-based DESs and in the TbACl:substrate DES. Aldol formation was observed in all DESs and reached saturation after approximately 24 h (see Figure 4.2b). As the condensation further converts the aldol to a more unsaturated olefinic compound, secondary product formation was also observed in all tested reaction media (see Figure 4.2c). However, the formation of the secondary olefinic product cannot explain the deviation between product concentration and substrate conversion. This difference might be partially ascribed to the evaporation of acetone at 60 °C reaction temperature and to an inefficient extraction method from the inhomogeneous reaction system. More interestingly, the hydrophilic ChCl:Gly DES promoted the secondary aldol condensation to form the olefinic side product, whereas this step was less favoured in the hydrophobic DESs (see Figure 4.2c). If this is expressed as ratio of the aldol to the condensation product, the aldol was the preferred product of the reaction in ToABr-1,5-pentanediol (6.4:1) and in the TbACl:substrate DES (6.1:1), whereas the reaction was less specific towards the aldol in ChCl:Gly and ToABr:ethylene glycol (2.8:1, respectively).



(a) 4-Nitrobenzaldehyde (b) Aldol (c) Condensation product

Figure 4.2: Time course of the substrate 4-nitrobenzaldehyde (a), aldol formation (b) and aldol condensation product (c) of the PPL catalysed aldol reaction in different DESs. Reaction conditions: 9, 20, 40 and 50 mmol in ChCl:Gly, ToABr:1,5-pentanediol, ToABr:EG and TbACl:4-NBA, respectively; V_{DES} = 30 mL, 4-NBA:acetone 1:5 (mol/mol), PPL:4-NBA 1:1 (w/w), V_{H_2O} = 1.5 mL, T = 60 °C, 400 rpm, $n = 3$.

Since proline can act as an organocatalyst for aldol reactions [126], it was investigated whether the C−C-bond formation also proceeds in the hydrophobic DESs in the presence of inactivated enzyme. The aldol product was synthesized after 24 h in all DESs with PPL inactivated either by thermal degradation in the presence of 12 M urea, by a serine-protease inhibitor (PMSF = phenylmethylsulfonyl fluoride) or by autoclaving (see Figure 4.3). Qualitative activity assays showed that PPL was inactivated by all denaturing treatments. The results point towards an unspecific acid/base catalysed aldol addition by the amino acids within the protein, which has been reported for an aldol reaction catalysed by bovine serum albumin (BSA) as a non-enzyme protein [122]. However, the specificity of the enzyme catalysed aldol reaction was not compared to the reactions with the inactivated PPLs in our experiments. While BSA-catalysed aldol additions performed in organic solvents yielded low amounts of the aldol product [127, 129], Milker et al. have discussed the effect of BSA-catalysed aldol additions on the product specificity in comparison to PPL-catalysed reactions in different DESs. In particular, hydrophobic DESs promoted the formation of the targeted aldol product, whereas BSA-catalysed reactions were rather unspecific [122]. Similar investigations would be necessary to further interpret the results obtained from the aldol additions with inactivated PPL in different DESs.

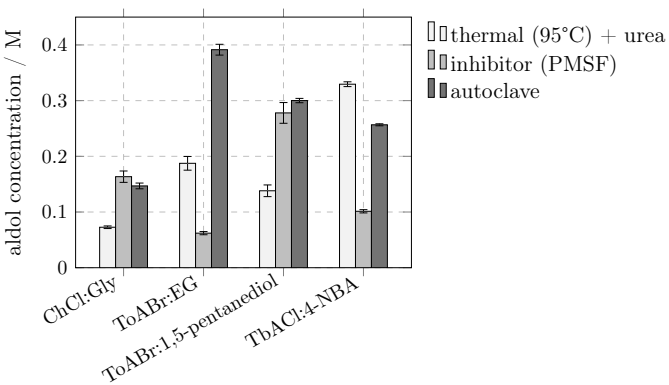


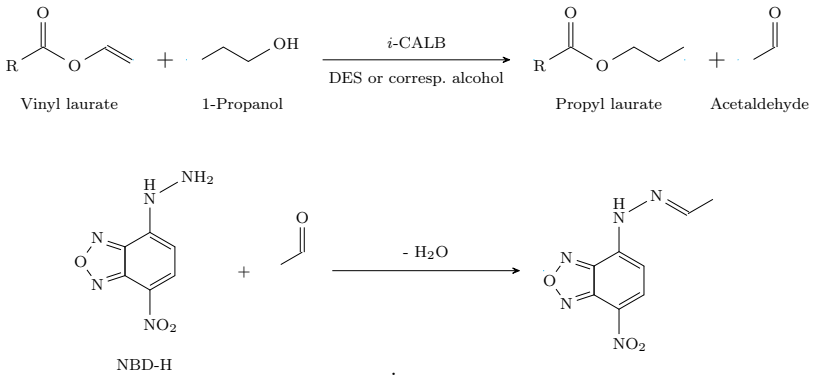
Figure 4.3: Aldol concentration after 24 h in different DESs with inactivated PPL ($n = 3$).

In conclusion, hydrophobic DESs are more beneficial reaction media in contrast to the hydrophilic ChCl:Gly DES in terms of an improved substrate solubility. The substrate based TbACl:4-NBA DES is especially suited to perform the PPL-catalysed aldol reaction, as it enables very high substrate loads and reduces solvent waste due to its 2-in-1 character. Although the initial reaction velocity was faster in the hydrophilic ChCl:Gly than in hydrophobic ToABr:EG or ToABr:1,5-pentanediol [122], the lipase catalysed aldol addition might be further improved in the hydrophobic DESs by adjusting and controlling the a_w during the reaction, which has not yet been addressed. However, Milker et al. concluded that despite the high substrate solubility in the TbACl:4-NBA DES, the aldol addition is still faster and more efficient in the co-solvent acetone, resulting in a solvent-less, more productive and "greener" reaction approach compared to using DESs [122]. Therefore, the application of DESs has to be critically evaluated for each specific reaction system, especially if one of the substrates is liquid and can be used as reaction solvent, as it has been shown in the context of the PPL-catalysed aldol addition of 4-NBA and acetone.

4.4 Transesterification in hydrophobic DESs

Lipase-catalysed transesterification reactions have been studied in hydrophilic DESs in many cases [78, 111, 116], but reports on biotransformations in hydrophobic DESs are rare. However, if unpolar substrates are involved in transesterification reactions, the application of hydrophobic DESs might be interesting to dissolve and convert high substrate loads. As a model reaction the transesterification of vinyl laurate and 1-propanol was selected involving two substrates of different polarity (see Scheme 4.3). Additionally, the course of the reaction was analysed photometrically by a fluorescence dye (NBD-H), which is activated by the release of the transesterification product acetaldehyde, as reported by Konarzycka-Bessler and Bornscheuer [130].

Hydrophilic ChCl:Gly, ChCl:EG and ChCl:U were compared to two hydrophobic fatty acid DESs, ToABr:decanoic acid and TAbCl:decanoic acid [83], for a pre-



Scheme 4.3: Transesterification of vinyl laurate with 1-propanol catalysed by immobilised CALB in different DESs with acetaldehyde activation of a fluoresence dye (NBD-H, 4-hydrazino-7-nitro-2,1,3-benzoxadiazole) for photometric analysis of the reaction

liminary reaction medium assessment. It was observed that the substrate (vinyl laurate) was insoluble in ChCl:Gly and that the reaction proceeded in the organic solvent layer formed on top of the hydrophilic DES. With ChCl:U and ChCl:EG the reaction efficiency was generally low. Moreover, the reaction was promoted without CALB in the hydrophobic DESs, indicating that the transesterification might be acid catalysed at a reaction temperature of 40 °C due to the fatty acid in the DES.

For a second set of experiments, novel non-fatty acid hydrophobic DESs were prepared with medium chain alcohol HBDs and TbACl as alternative DES reaction media (see Table 4.3). Vinyl laurate was miscible with the hydrophobic TbACl:alcohol DESs at a concentration of 0.15 M. Even if the alcohols can act as competing reactants for the desired conversion of vinyl laurate and 1-propanol, these DESs were considered as suitable reaction media, since the reactivity of a competing DES constituent is reduced due to the strong hydrogen bond interactions within the DES [55]. The transesterifications were also performed in the alcohols for better comparison. Based on the fluorescence measurement, the CALB catalysed transesterification reaction appeared to proceed inefficiently in

the TbACl:alcohol DESs (see Figure 4.4 B, D, F) compared to using the corresponding alcohols as reaction media (see Figure 4.4 A, C, E). However, this result can be explained by the interference of the fluorescence dye with the DESs, in which the dye changed its colour and therefore its emission behaviour.

Table 4.3: DESs prepared for the CALB catalysed transesterification reaction.

DES	Molar ratio mol/mol	$T_{synthesis}$ $℃$
TbACl:1-hexanol	1:2	45
TbACl:1-octanol	1:2	45
TbACl:1-decanol	1:2	45

Since the NBD-H dye exhibited an absorption maximum at 480 nm in both reaction media, the transesterification reaction in TbACl:1-octanol and in the corresponding alcohol (1-octanol) was compared based on an absorption measurement. With this method the transesterification was observed to proceed equally well in the DES medium as well as in the organic solvent (see Figure 4.5), showing that the DES has no negative impact on the immobilised enzyme.

However, the use of TbACl:alcohol based DESs for the studied transesterification reaction needs to be evaluated critically due to the health hazards coming along with TbACl [131]. Moreover, the TbACl:alcohol DESs are characterised by a high viscosity (100 to 200 mPa·s (20 °C), see Figure 3.7 B), which makes their application impractical in terms of liquid handling and mass transfer limitations. Certainly, a solvent-free reaction system with the alcohol acting as substrate and solvent would be a better alternative to the hydrophobic TbACl:alcohols with regard to waste reduction and solvent toxicity.

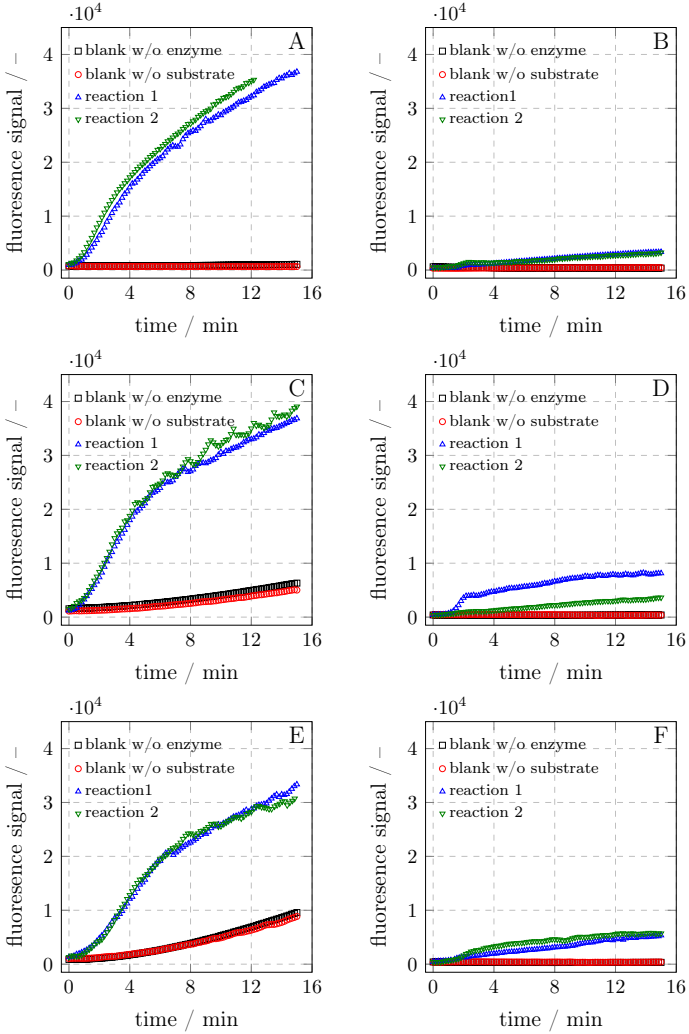


Figure 4.4: CALB-catalysed transesterification in hydrophobic alcohol-based DESs (B = TbACl:1-hexanol, D = TbACl:1-octanol, F = TbACl:1-decanol) in comparison to pure alcohols (A = 1-hexanol, C = 1-octanol, E = 1-decanol) monitored by fluoresence intensity of a coupled dye activating reaction ($\lambda_{ex} = 480\,\text{nm}$; $\lambda_{em} = 545\,\text{nm}$). Reaction conditions: 15 µmol vinyl laurate, 665.5 µmol 1-propanol, 0.011 µmol NBD-H, 0.825 mg immobilised CALB, $V_{total} = 150\,\mu\text{L}$, 432 rpm, $T = 40\,^{\circ}\text{C}$.

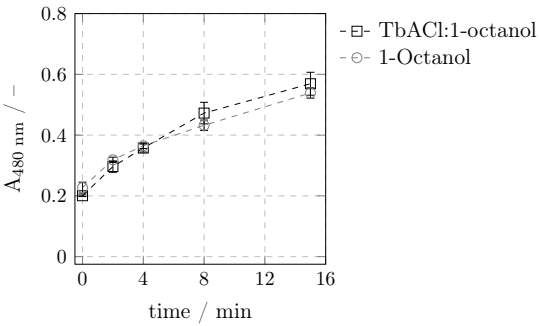


Figure 4.5: CALB-catalysed transesterification in TbACl:1-octanol and in 1-octanol monitored by absorption ($\lambda = 480\,nm$). Reaction conditions: 900 µmol vinyl laurate, 40 mmol 1-propanol, 0.66 µmol NBD-H, 49.5 mg immobilised CALB, $V_{total} = 9\,mL$, 1300 rpm, $T = 40\,°C$, $n = 3$.

4.5 Transesterification of terpenes in terpene-based DESs

Terpenes are a diverse class of natural compounds with broad applications in the food, chemical, cosmetic or pharmaceutical industries. Due to the high economic relevance, the chemical modification of terpenes is sometimes necessary to achieve the desired properties of a terpene in a product. Monoterpenoid alcohols such as geraniol, citronellol or menthol and their short-chain esters are important fragrances with applications as flavour compounds in food or cosmetic products [132]. The biotechnological modification of terpenes is particularly interesting for these industries to consider the modified agents as "natural" additives. Therefore, lipase catalysed esterifications of monoterpenoid alcohols were attempted in terpene-based DESs, avoiding the use of an organic solvent to dissolve the unpolar monoterpenoid substrates. Geraniol, citronellol and (−)-menthol were chosen as HBDs, which were combined with ChCl or TbACl as HBAs at three different molar ratios. After heating and stirring at 60 °C, the formation of a DES was visually evaluated at room temperature. If no solids were present in the mixtures after they cooled down to room temperature, the mixtures were considered as a DES. At the studied molar composition and temperatures ChCl was not able to interact with the terpenes, whereas DESs were obtained for the mixtures of TbACl:geraniol (1:1) and TbACl:citronellol (1:1 and 1:2), but not for TbACl:(−)-menthol (see

Table 4.4). The lipase catalysed transesterification of geraniol or citronellol with vinyl acetate was tested in TbACl:geraniol 1:1 mol/mol and TbACl:citronellol 1:1 mol/mol, respectively. Three different lipases (immobilised CALB from c-LEcta, Novozyme N435 or PPL powder) were applied to study the formation of geranyl acetate or citronellyl acetate in the DESs. The ester products were detected qualitatively in both DESs with all studied lipases, indicating that the enzymatic synthesis of geranyl and citronellyl acetate is generally possible in the TbACl:terpene based DESs. However, the application of TbACl:terpene DESs for the lipase catalysed flavour synthesis is unattractive due to the previously mentioned hazards associated with TbACl. Solvent-free reaction systems were established and other novel reaction media, such as ionic liquids or supercritical fluids, appear to be more efficient and promising in the field of the lipase catalysed flavour and fragrance synthesis [20]. In conclusion, developing substrate-based DESs for the biotransformation of terpenes is not yet fully exploited and requires more detailed differential scanning calorimetric studies to determine the eutectic point of substrate DESs. Furthermore, DES reaction systems need to be critically compared to existing protocols involving novel reaction media for the synthesis of flavour compounds.

Table 4.4: Preparation of terpene-based DESs. The formation of a DES at a certain molar ratio is denoted by a plus sign (+), whereas a minus sign indicates that no DES was obtained (–).

DES	Molar ratio *mol/mol*	$T_{synthesis}$ *°C*	DES formation
	2:1		–
ChCl:geraniol	1:1	60	–
	1:2		–
	2:1		–
ChCl:(±)-β-citronellol	1:1	60	–
	1:2		–

Continued on next page

Table 4.4 – *(Continued)*

DES	Molar ratio *mol/mol*	$T_{synthesis}$ *°C*	DES formation
	2:1		–
ChCl:(–)-menthol	1:1	60	–
	1:2		–
	2:1		–
TbACl:geraniol	1:1	60	+
	1:2		not tested
	2:1		–
TbACl:(±)-β-citronellol	1:1	60	+
	1:2		+
	2:1		–
TbACl:(–)-menthol	1:1	60	–
	1:2		not tested

4.6 Summary

In this chapter, the experimental reproduction of two published reactions involving lipases in DES reaction media was described [55, 58] and the use of DESs for the particular reaction systems was discussed. Moreover, novel hydrophobic DESs were developed for a lipase-catalysed transesterification reactions. The results can be summarised as follows:

- A lipase-catalysed (CALB N435) aminolysis reaction, which was one of the first published reactions in DESs [55], was reproduced and the results were comparable to the reported data with higher conversions achieved in ChCl:Gly and ChCl:U than in toluene.

- The PPL-catalysed aldol reaction of 4-NBA and acetone was investigated [58] and it was attempted to improve the yield by using hydrophobic DESs with a higher substrate dissolution capacity.

- Except for the menthol:acetic acid DES, all studied hydrophobic DESs enhanced the solubility of 4-NBA in relation to ChCl:Gly by a factor of 1.3 (menthol:lactic acid) up to 7.5 (menthol:pyruvic acid).
- Menthol:acid DESs were excluded as reaction media for the lipase-catalysed aldol addition due to the risk of a competing acid-catalysis. A substrate-based TbACl:4-NBA DES was designed to act as combined reactant and solvent, enabling high substrate loads.
- As expected, performing the aldol reactions in DESs with an improved substrate solubility yielded higher product concentrations. The DESs affected the product specificity, as the preferred product in ToABr:1,5-pentanediol and TbACl:4-NBA was the aldol, while the reaction was less specific in ChCl:Gly and ToABr:EG, which promoted the secondary aldol condensation step more than the more unpolar DESs.
- The formation of aldol products with inactivated PPL indicated that an unspecific acid/base catalysis is possible by amino acid residues from the denatured enzyme.
- The application of DESs for the enzyme catalysed aldol addition of 4-NBA and acetone is inferior to the solvent-free reaction system involving acetone as solvent and substrate [122].
- Investigating the applicability of different DESs for the CALB catalysed transesterification of vinyl laurate and 1-propanol showed that hydrophilic ChCl-based DESs and fatty acid based hydrophobic DESs are not suited for this type of reaction.
- Novel TbACl:alcohol DESs were developed, but were associated with a few drawbacks; (1) The fluorescence dye properties are changed in the TbACl:alcohol DESs in a way that the fluorescence measurement becomes inapplicable. (2) TbACl:alcohol DESs are 100 to 200-times more visous than water. (3) Although absorption measurement indicated that the reaction is catalysed equally in TbACl:1-octanol and in 1-octanol, the health and environmental hazards linked to the TbACl HBA make the use of the DES unattractive.

- The lipase catalysed synthesis of terpene esters in TbACl:terpene based DESs is possible, but unattractive due to the hazards associated with TbACl and a large variety of efficient alternative reaction media used in this field.

5 DESs as 2-in-1 reaction media[6]

5.1 Introductory words

One major benefit of using DESs as reaction media for biocatalysis is their flexibility in terms of the large number of compounds that can be combined to form a eutectic mixture. It is therefore possible to design DESs in a way that they consist only of the substrates of a desired enzymatic reaction. This approach is particularly appealing for two reasons: (1) solid reactants can be transformed to their fluid state in a eutectic mixture, which makes solid substrates accessible for enzymes; and (2) the DES contributes to reduce the solvent consumption and waste accumulation during a reaction, since the DES simultaneously acts as substrate pool and reaction solvent (solvent-free reaction systems). The particular feature of DESs to work as combined solvent and substrates is described by the term 2-in-1 reaction media. There are also a few publications exploiting DESs as 2-in-1 reaction media for biocatalysis. For instance, the lipase-catalysed sugar fatty acid ester synthesis, [77, 78], the chemo-enzymatic epoxidation of monoterpenes [79], the synthesis of 1,3-diacylglycerol [80] or α-monobenzoate [74] were successfully accomplished by using 2-in-1 DES reaction media. However, in these investigations only one DES compound functioned as substrate, while an appropriate additive (HBA or HBD) was necessary to prepare the DES, and the other substrate was added to the DES. In contrast to that, this chapter is focussed on introducing true 2-in-1 DES reaction media for the lipase catalysed esterification of terpenes. In such reaction systems, no additional solvent or auxiliary is necessary, as the liquid DES is already composed of both substrates. Various steps were performed to develop an enzymatic terpene esterification route relying on 2-in-1 DES reaction media. Initially, different DESs were prepared and tested for their potential application as combined reaction substrates and solvents, and menthol:fatty acid DESs were determined as suitable candidates. By the addition of a lipase from *C. rugosa* (CRL), which was most efficient among the screened lipases, (–)-menthyl fatty acid esters with different acylic carbon chain length (C8, C10 or C12) were yielded. The esterification of (–)-menthol with long-chain fatty acids has been described in organic

[6]Some of the presented results have been published by the author and co-workers in [112, 113]

solvent-free systems to modify (–)-menthol for cosmetic applications [133]. The enzymatic synthesis of medium chain (–)-menthyl fatty acid esters, presented in this chapter, is another example of how (–)-menthol can be modified using 2-in-1 DES reaction media. The CRL catalysed esterification of (–)-menthol:fatty acid DESs was investigated in biphasic water-DES reactions systems and at controlled water activities (a_w). The esterification was optimised in terms of statistical design of experiments and the potential application of a DES reaction system for the resolution of (±)-menthol is discussed.

5.2 Preparation of different 2-in-1 DESs

Several terpene alcohol:acid DESs have already been published consisting of two possible substrates for a lipase catalysed esterification of both DES compounds (see Table 5.1) and can therefore be regarded as true 2-in-1 reaction media.

Table 5.1: 2-in-1 DESs for the lipase catalysed esterification of terpene alcohol containing both substrates.

DES	Molar ratio *mol/mol*	$T_{synthesis}$ *°C*	Reference
(–)-Menthol:caprylic acid	1:0.82	37	[85]
(–)-Menthol:capric acid	1:0.54	37	[85]
(–)-Menthol:lauric acid	3:1	37	[85]
(–)-Menthol:acetic acid	1:1	25	[86] (±)-menthol
(–)-Menthol:L-lactic acid	1:2	35	[86] (±)-menthol
(–)-Menthol:DL-lactic acid	1:2	35	[86] (±)-menthol
(–)-Menthol:benzoic acid[a]	2:1, 4:1, 9:1	55	[-]

[a] The DESs are liquid at T $\geq$ 55 °C and become solid at room temperature. No liquids were obtained with equimolar amounts or with benzoic acid in excess.

It has been attempted to prepare a novel (–)-menthol:benzoic acid DES and different molar ratios were tested to obtain a eutectic mixture. The 2:1, 4:1 and 9:1 eutectic mixtures were liquid at an elevated temperature and became solid when they cooled down to room temperature, irrespective of the molar composition. Except (–)-menthol:acetic acid, all other DESs required moderate heating to ob-

tain a liquid. In contrast to that, (–)-menthol:acetic acid formed a transparent liquid at room temperature without the need of any energy input. (–)-Menthol:capric acid, (–)-menthol:lauric acid and (–)-menthol:benzoic acid are DESs composed of solids, whereas the other DESs consist of solid (–)-menthol and a liquid HBD (caprylic acid, acetic acid, L-lactic acid and DL-lactic acid).

5.3 DES and lipase screening

5.3.1 Esterification of (–)-menthol:benzoic acid DES

The applicability of the substrate based DESs (Table 5.1) for the lipase catalysed esterification of the DES compounds was screened using different lipases. Since the (–)-menthol:benzoic acid DES was only liquid at high temperatures, this DES was studied individually at a reaction temperature of 55 °C with immobilised *Candida antarctica* lipase B (CALB, c-LEcta) due to the enzyme's high thermal stability in non-polar organic solvents.

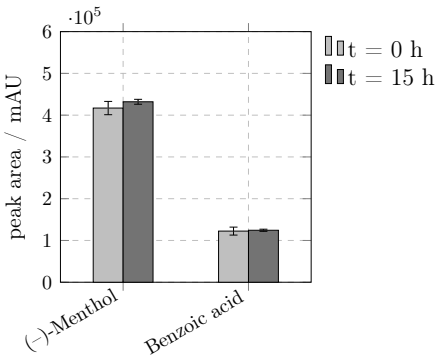


Figure 5.1: Qualitative substrate depletion of (–)-menthol and benzoic acid of the CALB-catalysed esterification in (–)-menthol:benzoic acid DES ($n = 3$). Reaction conditions: 2.3 mmol (–)-menthol, 1.15 mmol benzoic acid, 5 mg CALB, $T = 55$ °C.

The esterification reaction was performed in the (–)-menthol:benzoic acid 2:1 mol/mol DES to avoid an unnecessary excess of (–)-menthol. The reaction was started simply by the addition of the immobilised lipase, but no conversion

of (–)-menthol or benzoic acid was observed after 15 h (see Figure 5.1). Therefore, the (–)-menthol:benzoic acid DES was excluded from further experiments and its limited liquid range further complicates the application as reaction medium.

5.3.2 Esterification of (–)-menthol:acetic and lactic acid DESs

The enzymatic synthesis of (–)-menthyl acetate or lactate, which are used as flavour compound [134] or cooling agent [135] respectively, is interesting due to the consumers' preference for "natural" products and a developing market for biotechnoloigcally produced flavours [19]. In order to evaluate the potential of the (–)-menthol:acid DESs to act as 2-in-1 reaction media, the possible self-esterification of the DESs was studied and compared to lipase catalysed esterifications. Since the organic acids are used in equimolar amounts or in excess to form the DESs, the high acid content might trigger the proton catalysed esterification. Therefore, control experiments were performed incubating the neat (–)-menthol:acid DESs for 24 h at 35 °C without lipases. Although no enzyme was present, it was observed that ≥10 % of (–)-menthol was converted in all (–)-menthol:acid DESs (see Figure 5.2). The following screening experiments were thus focussed on (–)-menthol:fatty acid DESs. These DESs were reported to be stable against self-esterification after their preparation (48 h) [85].

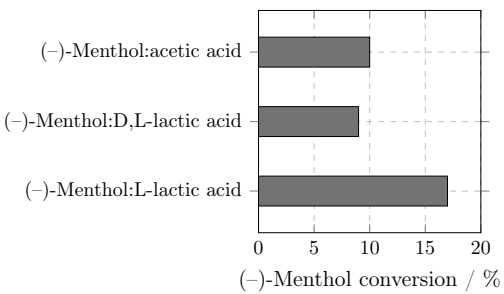
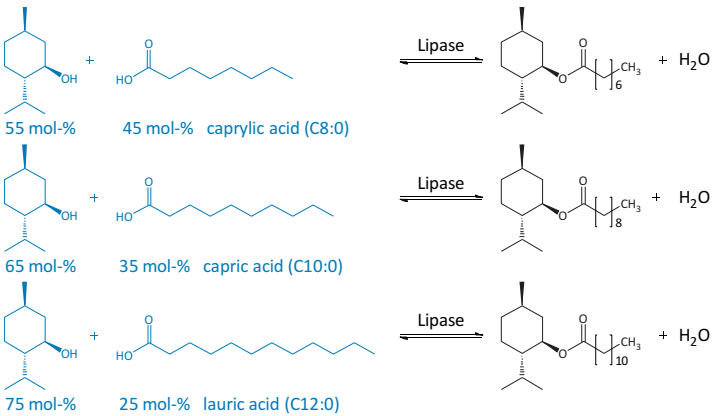


Figure 5.2: Conversion of (–)-menthol in different DESs without lipase added. Reaction conditions: $m_{DES} = 0.5\,\text{g}$, 400 rpm, $T = 35\,°\text{C}$, $n = 1$.

5.3.3 Esterication of (–)-menthol:fatty acid DESs

The enzymatic modification of (–)-menthol through esterification with long-chain fatty acids was described in an organic solvent-free reaction system to obtain modified (–)-menthol for cosmetic applications [133]. In this context, the (–)-menthol:fatty acid DESs can be regarded as new 2-in-1 reaction media to achieve the enzymatic conversion of (–)-menthol. The targeted esterification reactions for the enzymatic synthesis of (–)-menthyl fatty acid esters, relying on the corresponding DESs as reaction media, are depicted by Scheme 5.1.



Scheme 5.1: Lipase catalysed synthesis of (–)-menthyl fatty acid esters with DESs containing both substrates. The blue colour (—) indicates that the substrates form liquid eutectic mixtures, acting as combined substrates and reaction solvents.

The enzyme screenings were performed with commercially available lipases, which were two immobilised CALB enzymes (N435 and c-LEcta immo Plus) and four powder formulated lipases from *Candida rugosa* (CRL), *Pseudomonas fluorescens* (PFL), *Burkholderia cepacia* (BCL, Amano lipase PS) and *Pseudomonas cepacia* (PCL, today renamed as *Burkholderia cepacia*). If immobilised CALB lipases were utilised for the DES esterifications, the fatty acid conversion was rather low ($< 10\,\%$) or not measurable (see Table 5.2). Although no conversion was detected in the (–)-menthol:lauric acid DES (Ment:[C12:0]) with N435, the product ester

occurred in this DES in a small amount. For the powder formulated lipases BCL, PCL and PFL no or a slight activity was observed, whereas CRL outperformed these enzymes in terms of fatty acid conversion (see Table 5.2).

Table 5.2: Fatty acid conversion in neat DESs with six lipases and negative control without an enzyme. Reaction conditions: $m_{DES} = 0.5\,\text{g}$, $T = 35\,^{\circ}\text{C}$, $m_{enzyme} = 5\,\text{mg}$, 400 rpm, $t = 5\,\text{d}$ ($n = 1$).

	Fatty acid conversion / %		
Lipase	Ment:[C8:0]	Ment:[C10:0]	Ment:[C12:0]
Novo435 CALB	6	6	–[a]
c-LEcta CALB	8	8	–
BCL	–	–	1
PCL	3	–	2
CRL	11	24[a]	73[a]
PFL	0.1	–	–
Without lipase	–	0.1	–

[a] The product peak was observed in the chromatograms.

If the (–)-menthol:fatty acid DESs were incubated without any lipase, no ester formation had been observed (see Table 5.2). Thus, (–)-menthol:fatty acid DESs were not prone to self-esterification at an elevated incubation temperature of 35 °C and the DESs were regarded as stable solvents. The ester formation with CRL was confirmed in two of the three DESs ((–)-menthol:capric acid and (–)-menthol:lauric acid) based on the respective product peaks in the chromatograms. The powder lipases were compared according to their specific hydrolysis activity. Although CRL exhibited the lowest specific activity towards cleavage of a *p*-nitrophenyl ester among the tested lipases (see Figure 5.3A), high fatty acid conversions were observed with CRL for the esterification of the DESs. Moreover, the lipase powders also differed in the amount of protein and the highest protein content was measured for the CRL powder (13 wt%) followed by the PFL (8.5 wt%), BCL (1.5 wt%) and PCL (1.2 wt%) powder (see Figure 5.3B). Since the lipase powders were utilised with the same weight for the DES esterifications, the esterifications with CRL were performed with a higher amount of protein and

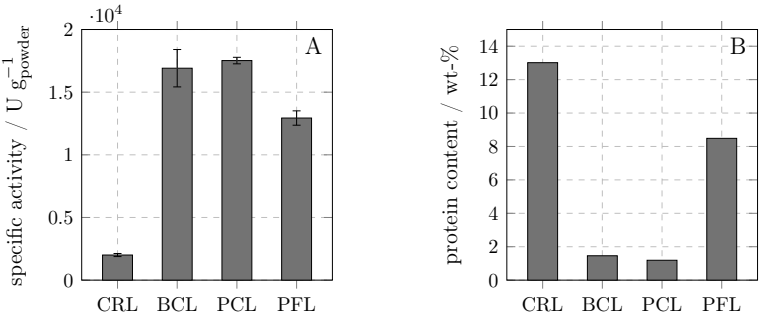


Figure 5.3: Specific hydrolytic activity (A) of lipases in 50 mM Tris-HCl buffer at pH 8 and 35 °C (1 U = 1 µmol min^{-1}) and protein content (B) of the lipase powders.

therefore higher fatty acid conversions can be expected. However, the difference in the protein content cannot completely explain, why only an insignificant conversion was observed with PFL, BCL or PCL. A better performance would have been expected especially for PFL, which had the second highest protein content. CRL has been studied for the enzymatic conversion of menthol in non-aqueous reaction media [136, 137] and in biphasic reaction mixtures [138]. Based on this and on the experimental screening results, CRL can be considered as a suitable biocatalyst for the esterification of (–)-menthol:fatty acid DESs. The experimental results also show that the application range of CRL can be expanded to DESs and more specifically to (–)-menthol:fatty acid DESs, in which CRL was active and capable of catalysing the ester synthesis of the DES compounds.

5.4 Esterification of (–)-menthol:fatty acid DESs with CRL

5.4.1 Biphasic DES-water reaction systems

Lipase catalysed esterification reactions in non-aqueous reaction media are influenced by the presence of water. High water contents would shift the reaction equilibrium from ester synthesis to an undesired ester hydrolysis, but at the same time water is essential to maintain enzyme activity. Water content is a key parameter for CRL catalysed reactions, since the lipase is activated at phase interfaces due to its lid structure [26]. The hydrophobic part of the lid shielding the active side is

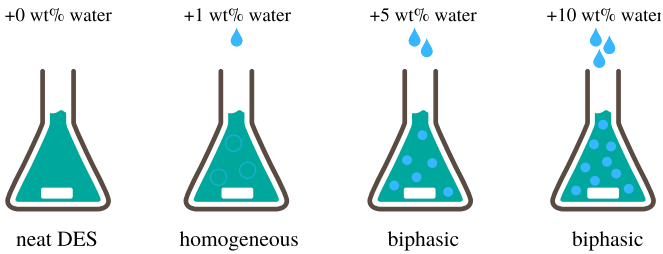


Figure 5.4: Illustration of water additions to the hydrophobic (–)-menthol:lauric acid DES resulting in homogeneous or biphasic reaction mixtures.

stabilised in the presence of a solvent through hydrophobic interactions, while the hydrophilic outer part of the lid interacts with the hydrophilic protein surface [27]. For instance, the CRL catalysed transesterification of racemic 1-phenyl-propan-2-ol with vinyl acetate in n-hexane/THF reaction mixtures was improved by increasing the water content up to a certain optimum [139]. In order to study the effect of water on the CRL catalysed esterification in (–)-menthol:fatty acid DESs, the reaction was performed in biphasic DES-water mixtures under the expectation that the presence of a phase interface would enhance the CRL lid flexibility and thus improve the reaction yield. The aqueous phase does not act as solvent in terms substrate dissolution and is an additive necessary to provide a hydrophilic phase in the hydrophobic DESs. The esterification was studied with three water addition levels (1, 5 or 10 wt% relating to the DES weight). The reactions in neat (–)-menthol:fatty acid DESs (0 wt% water addition) served as reference reaction systems. While a homogeneous reaction system was obtained through the addition of 1 wt% water, the addition of 5 or 10 wt% resulted in biphasic reaction mixtures, which is illustrated in Figure 5.4. Since the water saturation capacity of (–)-menthol:lauric acid was 1.9 wt% (see chapter 3.4), the addition of large quantities of water exceed this solubility limit and two phases were obtained.

In terms of fatty acid conversion, the homogeneous reaction mixtures with a water addition of 1 wt% were generally less efficient than the neat or the biphasic DES reaction systems (see Figure 5.5). The addition of water to the (–)-menthol:caprylic

acid or (–)-menthol:capric DES accelerated the initial esterification rates in comparison to the neat DES reaction systems (see Figure 5.5a, b).

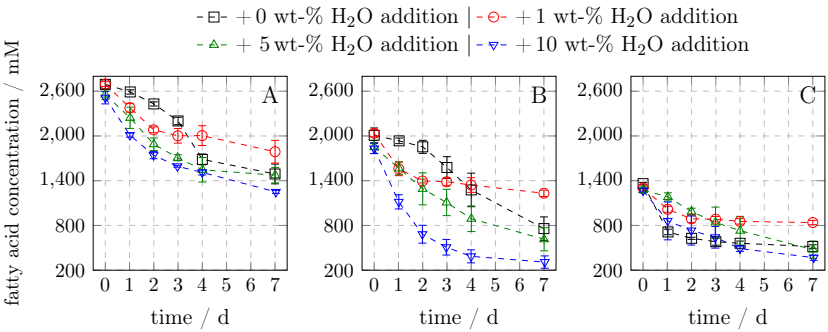


Figure 5.5: Caprylic acid (A), capric acid (B) and lauric acid (C) concentrations during CRL catalysed esterifications in the respective (–)-menthol:fatty acid DESs with different amounts of water added. Reaction conditions: m_{DES} = 0.5 g [(–)-menthol:caprylic acid 1:0.82mol/mol (A), (–)-menthol:capric acid 1:0.54 mol/mol (B), (–)-menthol:lauric acid 3:1 mol/mol (C)], 5 mg CRL, 700 rpm, T = 35 °C, n = 3.

The addition of water to the (–)-menthol:caprylic acid or (–)-menthol:capric DES accelerated the initial esterification rates in comparison to the neat DES reaction systems (see Figure 5.5a, b). Especially for (–)-menthol:capric acid, an improved esterification was observed with a water addition of 10 wt% (see Figure 5.5b). The reaction equilibrium was apparently not negatively affected by a water addition of up to 10 wt% to the (–)-menthol:caprylic acid and (–)-menthol:capric acid DESs. A similar observation was made for the CRL catalysed solvent-free synthesis of (–)-menthyl oleate, for which optimal esterification rates were achieved with a water addition of 20 to 30 % (percentage information not specified) [133]. For the (–)-menthol:lauric acid DES reaction system, water addition appeared to be inefficient and similar fatty acid concentrations were observed after 7 d for the esterification in the neat and the biphasic DES reaction systems (see Figure 5.5c). The ester products were identified by LC-MS (see chapter A.3, Table A.2). Esters were detected in all DES reaction systems and the amount of (–)-menthol lauric

acid ester was quantified. In terms of the (–)-menthyl laurate concentration, there was no distinguishable difference in the initial product formation rate of the neat and biphasic DES reaction system with 10 wt% water added based on high standard deviations (see Figure 5.6). The final product concentration after 7 d was higher using the biphasic reaction mixture with 10 wt% water addition (957 mM) than with the neat DES reaction system (784 mM).

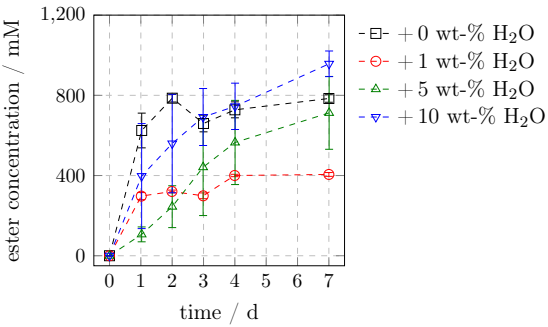


Figure 5.6: Time course of the (–)-menthyl laurate ester concentration of the CRL catalysed esterification in (–)-menthol:lauric acid 3:1 mol/mol. Reaction conditions: $m_{DES} = 0.5$ g, 5 mg CRL, 700 rpm, $T = 35$ °C, $n = 3$.

Moreover, the influence of the stirring speed was studied in terms of different water additions. An increase of the aqueous phase at constant agitation would reduce the hydrophilic-hydrophobic interface, whereas increasing the stirring speed for the same amount of water would enhance the interfacial surface area. The overlapping effect of water addition and stirrer speed were studied in the (–)-menthol:lauric acid DES reaction systems by increasing the water addition from 10 to 40 wt%, while the agitation rate was adjusted at 500, 800 or 1500 rpm. When the esterification was performed in neat (–)-menthol:lauric acid DES, higher stirring speeds (800, 1500 rpm) led to slightly lower ester product concentrations compared to a more gentle agitation of the reaction system at 500 rpm (see Figure 5.7). At a water addition of 10 wt%, the product concentration was observed to gradually increase with higher agitation rates (see Figure 5.7). This might be explained by an

interfacial activation of CRL, which was possibly provided with a larger inferfacial surface area by more vigorous mixing of the binary DES-water reaction mixture at high agitation rates. For a 40 wt% addition of water to the DES reaction system, the stirring speed appeared to have no influence on the ester concentration (see Figure 5.7). Adding large quantities of water (up to 70 wt%) to the DES resulted in two separate liquid layers, even at the highest possible agitation of 1500 rpm. Therefore, appropriate mixing of the biphasic DES-water reaction mixtures was considered as a limiting factor in the experimental set-up.

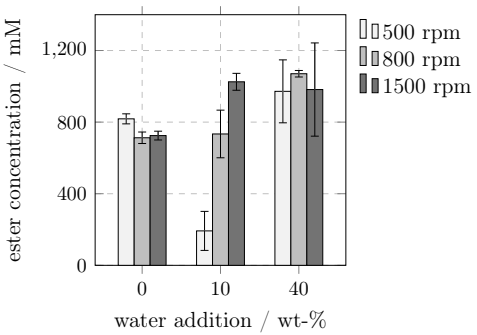


Figure 5.7: Effect of stirrer speed and amount of water addition on (–)-menthyl laurate ester concentration. Reaction conditions: $m_{DES} = 0.5\,\text{g}$, 5 mg CRL, $t = 48\,\text{h}$, $T = 35\,^{\circ}\text{C}$, $n = 3$.

In the next set of experiments, the enzymatic esterification of the (–)-menthol:lauric acid DES was further investigated in terms of improving the experimental set-up for biphasic DES-water mixtures to enable the conversion of a larger amount of DES. Double-walled glass reactors were used to perform the esterification with 25 g DES. Mixing of the two phases was improved by using the a different type of reaction vessel and an appropriate magnetic stirrer. The reaction was performed in the neat DES and in biphasic mixtures with 20 or 50 wt% water added (relating to the DES weight). The formation of two separate liquid layers was avoided by the new experimental set-up. As depicted by Figure 5.8, the esterification in biphasic DES-water mixtures outperformed the neat (–)-menthol:lauric acid DES in the beginning of the reaction. With the addition of water, the esterification

proceeded faster than in the neat DES within the first three days. At this time point, the esterification reached about 84 % lauric acid conversion in the biphasic DES-water reaction mixtures, whereas only 67 % of lauric acid were converted in the neat DES. However, the fatty acid concentration reached the same level after five days in all reaction systems. With ongoing esterification, water is released as a side product in the neat DES reaction system. Once a critical water concentration might be reached in the DES for CRL to become fully active, the neat DES reaction systems performs as well as the biphasic DES-water reaction systems on the long term. In summary, stirring was not a limiting factor with the new set-up. In fact, the addition of water promoted faster conversion of the DESs, which has also been reported for a solvent-free enzymatic esterification of (–)-menthol and oleic acid [133]. But since a conversion of approximately 84 % was accomplished over time with both the neat and biphasic DES reaction systems, the addition of large amounts of water might not be necessary to activate CRL. Instead of generating macroscopic hydrophilic/hydrophobic interfaces, further experiments focussed on developing DES reaction systems with microscopic water supply to accomplish higher fatty acid conversions.

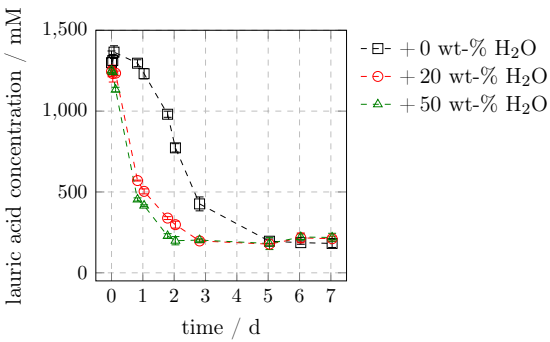


Figure 5.8: Time course of lauric acid concentrations in neat (–)-menthol:lauric acid 3:1 mol/mol and in biphasic DES-water mixtures with 20 wt% or 50 wt% water added. Reaction conditions: $m_{DES} = 25\,g$, 250 mg CRL, 700 rpm, $T = 35\,°C$, $n = 3$.

5.4.2 DES esterification at controlled water activity

The thermodynamic activity of water (a_w) is a measure for the amount of water present in non-aqueous reaction media. The a_w can also influence the reaction rate, enantioselectivity and equilibrium conversion of lipase catalysed reactions in non-conventional media [121]. Many lipase catalysed reactions were successfully performed in different types of non-aqueous media at controlled a_w [140–142], but only limited information is available on the effect of the a_w in DESs utilised for enzymatic reactions [111, 116]. Since the conversion of the DES compounds was limited with biphasic DES-water reaction systems, the impact of a controlled a_w was studied for the CRL catalysed esterification of (–)-menthol:fatty acid DESs. The conversion of the (–)-menthol:lauric acid DES was selected as a model reaction system.

In order to control the a_w during an enzymatic reaction, saturated salt solutions are commonly used to adjust and fix the a_w via the gas phase. Figure 5.9 shows the experimental set-up for screening the impact of different a_w values on the DES esterification by using saturated salt solutions in a double jacketed reaction vessel with an external heating circuit. The DESs were separated from the saturated salt solution by a glass insert.

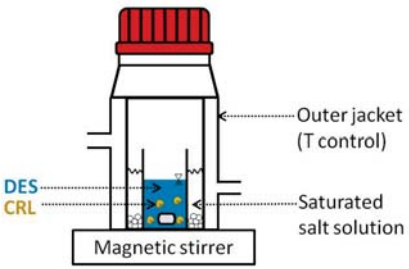


Figure 5.9: Experimental set-up for screening the CRL catalysed esterification under a_w controlled conditions.

In a preliminary experiment, the effect of different a_w values on the CRL catalysed esterification of the (–)-menthol:lauric acid DES was investigated and compared to a neat DES reaction medium, which was not treated or incubated. As illustrated

by Figure 5.10, the a_w had an influence on the lauric acid conversion (2.25 d). The conversion of the fatty acid reached over 90 % for a_w values ranging from 0.22 to 0.55, which is a clear improvement in contrast to the neat DES reaction system. Increasing the a_w further to 0.67 caused a slight decrease of the lauric acid conversion. Opposed to that, a low a_w of 0.12 restricted the enzymatic esterification of the DES. After three days, lauric acid was even fully converted for the esterifications with an a_w of 0.22 and 0.32. Similar observations were made for a CRL catalysed transesterification in an organic solvent, for which the highest reaction rate occurred at an a_w of 0.33, while the rate decreased at lower or higher a_w [141].

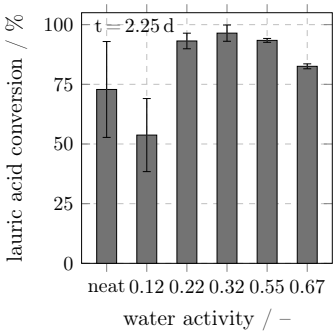


Figure 5.10: Lauric acid (LA) conversion after 2.25 days of the CRL catalysed esterification in neat (–)-menthol:lauric acid DES in comparison to different water activity controlled reaction systems (DESs and CRL were preincubated for 3 d with the respective salt solution at 35 °C). Reaction conditions: $m_{DES} = 2$ g, 20 mg CRL, 300 rpm, $T = 35$ °C, $n = 3$.

5.4.3 Comparison of reaction systems

To better characterize the performance of the enzymatic synthesis of (–)-menthyl laurate in different DES-based reactions systems, conversion and productivity were compared to a reference reaction published by Babali and co-workers (see Table 5.3). They reported the CRL catalysed esterification of (–)-menthol and lauric acid in an organic reaction solvent using molecular sieves for water removal [143]. Under these conditions 97 % lauric acid were converted after 24 h and a

productivity of $33\,g\,L^{-1}\,d^{-1}$ was accomplished. In comparison to that, the fatty acid conversion (32 %) was poor upon esterification in neat (–)-menthol:lauric acid DES, but an enhanced productivity of $205\,g\,L^{-1}\,d^{-1}$ was achieved (see Table 5.3). An improved productivity can be explained by a high substrate load in the DES. Instead of removing water, water was added on purpose to the DES reaction system at first place. It was expected that a biphasic DES-water reaction mixture would outperform the esterification in neat DES due to an interfacial activation of CRL. The addition of water sightly improved conversion, however at the cost of a decreased productivity of only $133\,g\,L^{-1}\,d^{-1}$ (see Table 5.3). In comparison to neat or biphasic water-DES reaction mixtures, the esterification performance was enhanced under a_w controlled conditions both in terms of conversion and productivity, reaching 65 % and $238\,g\,L^{-1}\,d^{-1}$, respectively. Even though the esterification conditions were not optimal to obtain high conversions, Table 5.3 shows that the DES generally enabled a high productivity at extremely low reaction volumes as opposed to the esterification in *i*-octane. Since promising results were obtained with a_w controlled DES reaction systems, the enzymatic esterification of

Table 5.3: Comparison of organic solvent and DES reaction systems for the enzymatic synthesis of (–)-menthyl laurate.

	Reaction systems			
Parameters	Organic[a]	neat[b]	biphasic[b]	a_w[b]
Solvent	*i*-octane	DES	DES	DES
Volume (mL)	10	0.55	0.55	2.2
Substrate ratio[c] (mol/mol)	2:1	3:1	3:1	3:1
'Additive'	mol. sieves	–	10 wt% H_2O	a_w 0.32
Temperature (°C)	35	35	35	35
CRL amount (mg)	47	5	5	20
Conversion[d] (%)	97	32	48	65
Productivity[d] ($g\,L^{-1}\,d^{-1}$)	33	205	133	287

[a] Data from Babali et al. [143]
[b] See chapters 5.4.1 (Figure 5.5, Figure 5.6) and 5.4.2
[c] Molar substrate ratio of (–)-menthol:lauric acid
[d] Lauric acid conversion and productivity calculated after 24 h

the (–)-menthol:lauric acid DES was further optimised focussing on a_w.

5.4.4 Reaction optimisation by response surface methodology

Statistical design of experiments (DoE) was applied for optimising the CRL catalysed esterification of the (–)-menthol:lauric acid DES in terms of accelerating the reaction velocity. The response surface methodology (RSM) has been reportedly used to optimise CRL catalysed esterifications of (–)-menthol in organic solvent-free reaction systems [144, 145] and was therefore considered as a suitable method. A three factorial central composite design (CCD) was selected to study the effect and the interaction of a_w (A), enzyme amount (B) and reaction temperature (C) on the ester formation velocity, which was chosen as model response (Y) to reduce the reaction time. Table 5.4 provides an overview on the the coded and actual levels of the factors, where ±1, 0 and ±α represent the respective corner, center and axial points of the design space.

Table 5.4: Coded and actual levels of factors used for the central composite design.

		Coded levels				
Factor	Symbol	−α	−1	0	+1	+α
a_w (–)	A	0.02	0.11	0.39	0.67	0.76
Enzyme amount (mg)	B	4.4	12	36	60	67.6
Reaction temperature (°C)	C	16	20	32.5	45	49

While the corner points (±1) of the design space were selected on the basis of previous experimental results, the axial design points (±α) and the center point (0) were calculated by the DesignExpert8 software. Since high conversions were achieved at a reaction temperature of 35 °C and at water activities from about 0.22 to 0.32, the a_w and temperature corner design points (±1) were spanned in a way to cover these values in the design space. Thus, the corner points for the a_w and temperature were set to range from 0.11 to 0.67 and temperatures from 20

to 45 °C, respectively. Regarding the enzyme amount used for the reactions, the corner points of the design space were set at 12 and 60 mg of biocatalyst input. Although it can be expected that a higher amount of enzyme would speed up the reaction, the amount of lipase was limited to 60 mg for economical reasons to avoid waste of the biocatalyst.

Since the aggregate state of eutectic mixtures strongly depends on the temperature, the variation of the reaction temperature is visualised in the phase diagram of the (–)-menthol:lauric acid DES (see Figure 5.11). The red arrows represent the ±α (dashed arrow) and ±1 (solid arrow) temperature levels of the selected design space. While the DES becomes solid at certain compositions and temperatures (grey area of the phase diagram), the reaction temperature variation was set in a range that ensures the DES reaction medium would be in its liquid state (white area of the phase diagram). The variation of the molar composition of the DES was not considered for the DoE based optimisation of the reaction conditions. Altering the DES composition was investigated in the following stage having the optimised reaction conditions in terms of temperature, enzyme amount and a_w at hand.

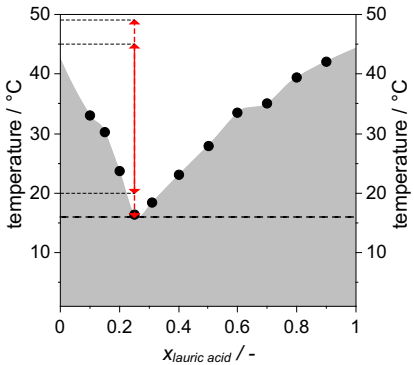


Figure 5.11: Phase diagram of (–)-menthol:lauric acid DES adapted from [85] indicating the temperature variation applied in the DoE. White area represents the liquid range of the DES. Dashed arrow is ±α level, solid arrow is ±1 level.

As the a_w is a temperature-dependent parameter, the experimental set-up for the

DoE was further improved in comparison to the set-up, which was used for the screening experiments. The experimental set-up was modified by using an external incubating hood. The reaction vessels were put a into an additional incubating hood to better control the gas phase temperature in the reactors, while the outer jacket of the vessels was also kept connected to a heating circuit to control the temperature of the liquid phases. Figure 5.12 illustrates the improved experimental set-up.

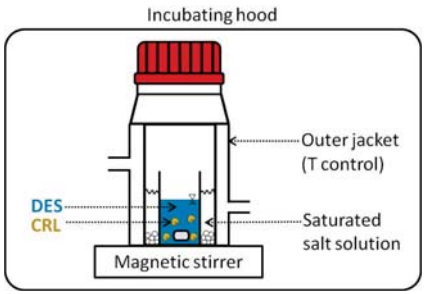
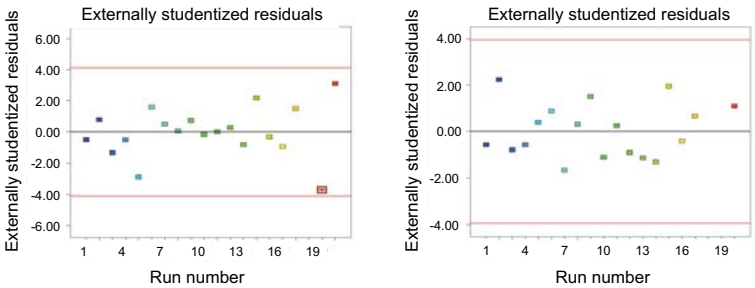


Figure 5.12: Experimental set-up for the CRL catalysed esterification under a_w controlled conditions with double temperature control.

After selecting the corner points of the design space for each factor and modifying the experimental set-up, the CCD experimental plan was generated with the DesignExpert8 software, comprising a total number of 20 experiments. The entire CCD experimental plan, the resulting data and details on the modelling process are presented in the supplementary section of this study (see chapter A.5, Table A.4). It should be noted that two experimental runs were excluded for the model building process. As the DES unexpectedly became solid under the experimental conditions of run 18 ($a_w = 0.39$, $m_{CRL} = 36\,\mathrm{g}$, $T = 16\,^{\circ}\mathrm{C}$), no experimental data was obtained at a reaction temperature of 16 °C, which represents the boundary of the design space in terms of the factor of temperature. For another experimental run (run 19), the externally studentized residuals plot indicated that the data was close to being an outlier (see Figure 5.13a). Since outlying data can create an exceptionally high influence on the model, run 19 was excluded for the modelling to obtain a

more robust model. As figure 5.13b depicts the residuals were distributed more evenly after exclusion of run 19 (see Figure 5.13b).



(a) Run 19 included.

(b) Run 19 excluded.

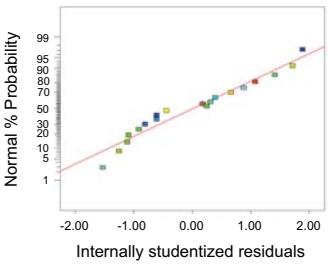
Figure 5.13: Externally studentized residuals of the quadratic regression of the experimental data including and excluding run 19.

For a valid regression model the residuals (error terms) have to follow a normal distribution with constant variance and a mean of zero, which was verified by several diagnostic plots (see Figure 5.14). The residuals follow a straight line in the normal probability plot and thus normality can be expected (see Figure 5.14a). It can also be assumed that the errors follow a normal distribution with constant variance, as the residuals are randomly scattered when they are plotted against the predicted values (see Figure 5.14b). The Box-Cox plot indicated that a mathematical power transformation of the data was not necessary as λ (green line) was close to 1 (see Figure 5.14c).

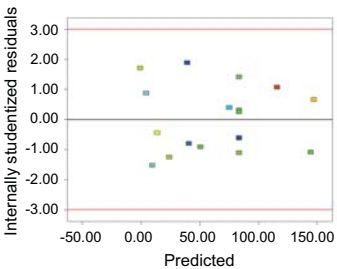
A quadratic model function was obtained by approximating the experimental data of 18 runs using a stepwise regression with a significance level of 0.1 ($\alpha_{\text{in/out}} = 0.1$). The model equation is shown by equation 5.1 in terms of actual factors.

$$\begin{aligned} Y = 60.49 + 469.37A + 1.54B - 9.53C \\ +0.04BC - 526.29A^2 - 0.02B^2 + 0.14C^2 \end{aligned} \tag{5.1}$$

(a) Normal plot of residuals



(b) Residuals vs. predicted



(c) Box-Cox plot for power transformation

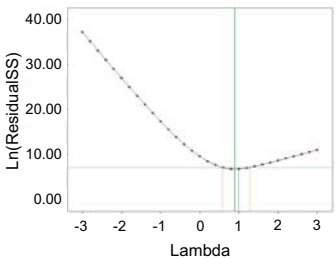


Figure 5.14: Diagnostic plots to evaluate the quadratic model obtained by stepwise regression without runs 18 and 19.

The model was significant to describe the experimental data, as the adjusted R^2 (adj. $R^2 = 0.9564$) and the predicted R^2 (pred. $R^2 = 0.8883$) were in reasonable agreement and close to 1. The lack of fit was insignificant ($p = 0.2529$), indicating that the model accurately represented the experimental data. All factors (a_w , T, m_{CRL}) were identified as significant influential factors with p-values of less than 0.05 ($p_{a_w} = 0.0004$; $p_{m_{CRL}} < 0.0001$; $p_T = 0.0015$). Moreover, the interaction of the enzyme amount and reaction temperature ($p = 0.006$) had a significant impact on the ester synthesis rate. Therefore, it is important to consider the interaction of temperature and enzyme amount to maximise the esterification performance. A

high enzyme load and an elevated reaction temperatures at a constant a_w of 0.55 resulted in the highest ester synthesis rates (see Figure 5.15). If the reaction was performed at a_w values below or above 0.55 under otherwise identical conditions, the product formation was observed to decrease (data not shown).

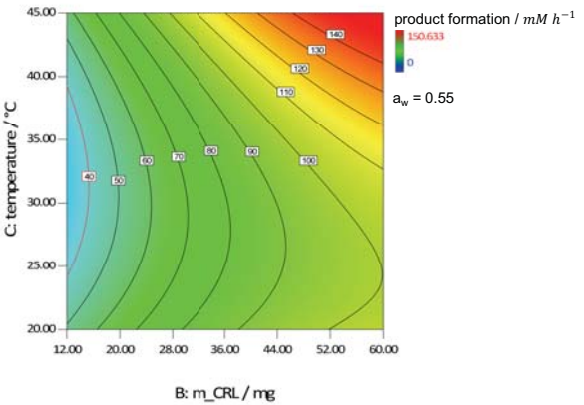


Figure 5.15: Heat map of the ester synthesis rate depending on the interaction of enzyme amount and reaction temperature of the CRL catalysed esterification in (–)-menthol:lauric acid DES at $a_w = 0.55$. Auxiliary lines with numbers help to visualise the course of the product formation.

Since the product formation rate is maximised at the boundaries of the design space (45 °C, 60 mg CRL), another experimental run was performed under the optimised reaction conditions to confirm the ester synthesis rate predicted by the model under these conditions. The desirability function of DesignExpert8 was used to determine the factor combinations that maximise the ester synthesis rate (range from 80 up to 600 mM h^{-1}). As extrapolation is not permitted, the factors were varied only within the design space (factor levels ±1). The highest predicted ester synthesis rate (161.5 mM h^{-1}) was calculated for a a_w of 0.55, 60 mg CRL and a reaction temperature of 45 °C. For the confirmation experiment sodium bromide (NaBr) was used as saturated salt solution, exhibiting a water activity close to 0.55 ($a_w = 0.52$ at 45 °C [146]). The experimental results of the esterification under optimised conditions are shown by Figure 5.16A. The experimentally observed

ester synthesis rate was 174 mM h^{-1} and was therefore slightly higher than the predicted rate of 161.5 mM h^{-1}. This deviation was still within the 95 % confidence interval for the product formation rate (134.2 to 188.8 mM h^{-1}). Thus, the model was capable of predicting a maximum ester synthesis rate accurately within the selected design space. Moreover, the relative humidity (RH) was monitored in one of the reaction vessels as a measure for the a_w (a_w = RH/100 %). The relative humidity reached a constant value of approximately 50 % (corresponding to a_w = 0.5) after 1 d, while higher humidities were measured initially (see Figure 5.16B). This was explained by temperature fluctuations caused through a higher sampling rate in the beginning of the experiment (see Figure 5.16B). On a long term, the gas phase equilibration of the DESs to a certain water content was a suitable method to control the a_w during the esterification reaction.

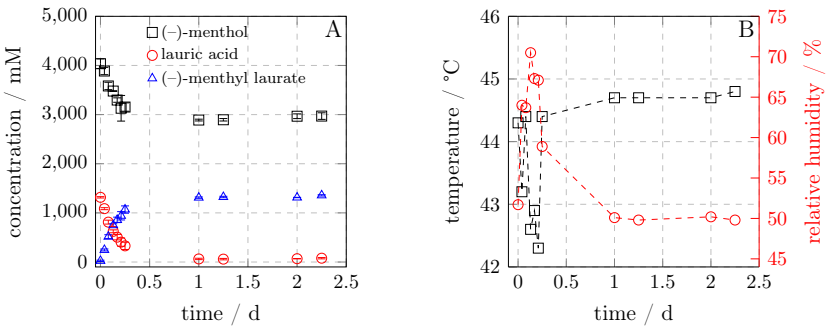


Figure 5.16: Time course of reatanct concentrations (A) and relative humidity and temperature (B) during the CRL catalysed esterification of (–)-menthol:lauric acid DES with NaBr saturated salt solution. Reaction conditions: m_{DES} = 2 g, 60 mg CRL, 900 rpm, T = 45 °C, n = 3.

In order to exclude that the esterification is acid-catalysed under the optimised reaction conditions a control experiment was performed without CRL. If the esterification was performed in the presence of CRL at a water activity of 0.5, 45 °C, product formation was observed rapidly after the reaction was started. In contrast to that, no conversion of the substrates and only a small amount of ester was detected in the absence of CRL (see Figure 5.17). It was therefore concluded that

the esterification of the (–)-menthol:lauric acid DES is catalysed by the enzyme under the selected conditions, whereas a reaction temperature of 45 °and a a_w of 0.5 do not induce an acid-catalysed reaction mechanism.

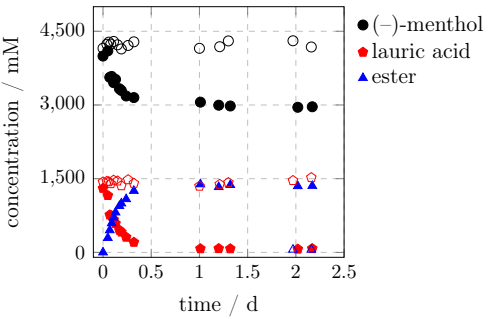


Figure 5.17: Time course of reatanct concentrations with CRL (closed symbols) and without CRL (open symbols) in the (–)-menthol:lauric acid DES. Reaction conditions: $m_{DES} = 2\,\text{g}$, 60 mg CRL, 900 rpm, $T = 45\,°\text{C}$,$a_{w,NaBr} = 0.52$, $n = 1$.

With the optimised DES esterification conditions, the lauric acid conversion reached 95 % after 1 d and a (–)-menthyl laurate ester concentration of up to $1.36 \pm 0.04\,\text{M}$ was accomplished at the end of the reaction (2.25 d). In comparison to the a_w screening experiments (see Figure 5.10) the reaction time was reduced by one half. In contrast to the esterification in biphasic DES-water mixtures (71 % conversion, 7 d, 10 wt% water addition; see chapter 5.4.1), the reaction was significantly accelerated under the a_w optimised conditions, reaching higher lauric acid conversions and reducing the reaction time by 7-fold. A detailed comparison of the DES reaction systems can be found in the supplementary section (see chapter 6.6, Table 6.2). The improved ester formation might be ascribed on the one hand to a suitable water supply to the lipase, which is activated at a molecular DES-water interface. Moreover, water produced throughout the esterification, is removed via gas phase equilibration as soon as the water partial pressure in the reaction mixture exceeds the water partial pressure induced by the saturated salt solution. Therefore, controlling the water content of the DES during the reaction by saturated salt solutions can be regarded as a beneficial method to compromise

enzyme activity and ester synthesis in a hydrophobic DES reaction medium. On the other hand, an increased temperature of 45 °C contributes to the reduction of the viscosity of the DES reaction medium, which can improve the mass transfer between the enzyme and the substrates. Furthermore, it was observed that the esterification reaction was also enhanced increasing the enzyme amount. The interaction of the reaction temperature and the amount of CRL had a high influence on the CRL catalysed esterification of the (–)-menthol:lauric DES. This strong interacting effect was underestimated when the CCD was designed and augmenting the experimental design would be necessary to obtain more information on the optimal combination of reaction temperature and enzyme amount. However, a cost-efficient process design would focus on balancing productivity with energy and biocatalyst costs and limiting the reaction temperature and enzyme amount might be beneficial to minimise the process costs. The economic aspect was out of scope of this optimisation, but might be important for future optimisations and process development of CRL catalysed esterifications in DESs.

5.4.5 Variation of DES molar ratio

The DES molar ratio is especially important, if DESs are intended to act as 2-in-1 reaction media. On the one hand, the molar composition determines the liquid range of a DES. For instance, altering the mole fraction of one compound at a constant temperature could result in an inhomogeneous mixture by crystallisation. On the other hand, the initial reaction rate is affected by the molar substrate ratio in a solvent-free enzymatic reaction system [147]. In case of the enzymatic esterification of the (–)-menthol:lauric acid DES, the effect of different molar compositions on the ester formation velocity was studied under the optimised reaction conditions (60 mg CRL, 45 °C, $a_w = 0.55$). The (–)-menthol:lauric acid molar ratio was varied from 1.5:1 up to 4:1 at a constant amount of lauric acid (3 mmol), corresponding to lauric acid mole fractions of 0.4 to 0.2, respectively. The molar ratios were limited to mixtures forming liquids at room temperature (23 °C) to avoid solidifying of the DES upon sampling and to enable a reproducible sampling process. Figure 5.18 visualises the variation of the molar ratio in a phase diagram.

Based on single experiments for each molar ratio, an enhanced esterification ve-

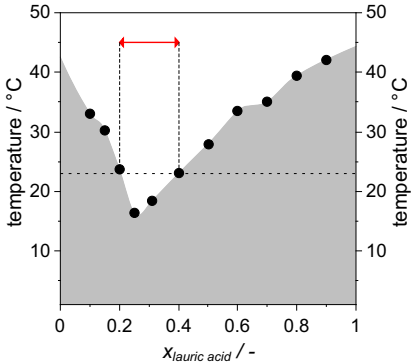


Figure 5.18: Phase diagram of the (–)-menthol:lauric acid mixture (adapted from [85]) showing the variation of the lauric acid mole fraction at an experimental temperature of 45 °C (red arrow). White area represents the liquid range of the DES. The molar ratios were limited to liquids at room temperature (23 °C, horizontal dashed line) to enable a reproducible sampling process.

locity was observed with the reduction of the (–)-menthol mole fraction (see Figure 5.19A). Performing the esterification in a 1.5:1 mol/mol mixture increased the ester formation velocity from 236 mM h^{-1} (3:1 mol/mol) to 368 mM h^{-1}. The conversion of lauric acid was not affected by different substrate ratios and approximately 95 % fatty acid conversion was reached after 23 h in all tested DESs with compositional variation (see Figure 5.19B). Thus, an excess of (–)-menthol for the esterification of the (–)-menthol:lauric acid DES is not necessarily required. Reducing the (–)-menthol mole fraction of the DES contributes to a more efficient biotransformation in terms of waste reduction and in terms of improving the (–)-menthol conversion. Future research is recommended to focus on further optimising the interaction of temperature and substrate molar ratio to obtain an 'ideal' DES reaction system consisting of equimolar substrate amounts.

5.4.6 CRL selectivity

The use of CRL for the enzymatic resolution of racemic (±)-menthol has already been described in the 1980s [148]. Since then, the resolution of (±)-menthol enan-

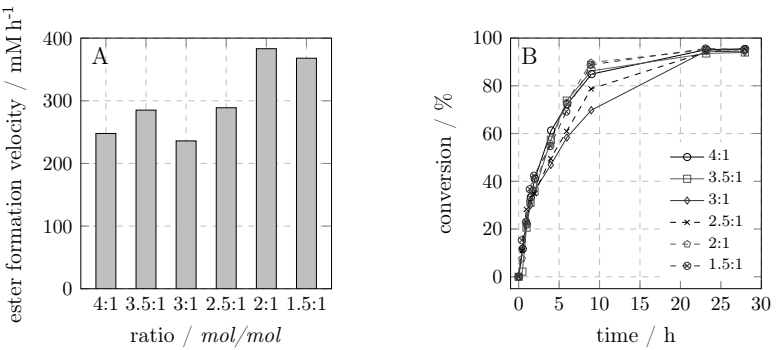


Figure 5.19: Esterification velocity (A) and lauric acid conversion (B) in (–)-menthol:lauric acid DESs at different molar compositions. Reaction conditions: 3 mmol lauric acid, 60 mg CRL, 900 rpm, $T = 45\,°C$, $a_{w,NaBr} = 0.52$, $n = 1$.

tiomers with CRL was studied intensively in various reaction media, such as organic solvents or ionic liquids [136, 149–152]. The selective hydrolysis of (±)-menthol benzoate, an intermediate of the (–)-menthol production process, is an example for the industrial relevance of CRL [153]. However, the potential of CRL as selective catalyst in DESs has not yet been exploited. CRL was therefore initially used for the esterification of pure (+)- or (–)-menthol based DESs to evaluate the enantioselectivity of the enzyme in this novel solvent type. The reference reaction in the (–)-menthol:lauric acid DES was completed after approximately one day, reaching 94 % conversion of lauric acid at an esterification velocity of 234 mM h^{-1} (see Figure 5.20A). The ester concentration only slightly increased over time in the (+)-menthol:lauric acid DES indicating that CRL did not prefer the esterification of (+)-menthol (see Figure 5.20B). Based on previous experimental results and based on the fact that CRL has been used successfully for the conversion of (–)-menthol [133, 143], it was expected that CRL catalyses the esterification of (–)-menthol in the respective DES. Since it has been reported that (–)-menthol was preferred by a non-immobilised CRL if racemic (±)-menthol was used as substrate [149], it could be also expected that CRL would not catalyse the esterification of the (+)-menthol:lauric acid DES, which was experimentally confirmed (see Figure 5.20B). The results also indicate that the resolution of (±)-menthol us-

ing CRL would also be possible starting from a racemic DES reaction medium. A subsequent experiment with a racemic (±)-menthol:lauric acid (3:1 mol/mol) mixture showed that the esterification was efficiently catalysed in this DES with an esterification velocity (164 mM h^{-1}) and lauric acid conversion (89 %) only slightly lower than in pure (–)-menthol:lauric acid DES (see Figure 5.20A, C). Although the characterization of the enantiomeric excess in the (±)-menthol:lauric acid DES reaction system is an outstanding task, which was not considered in this work, the results underline that the CRL catalysed resolution of (±)-menthol is most likely possible with a DES reaction medium (see Figure 5.20A, B) and might become a relevant application of a DES-based biotransformation.

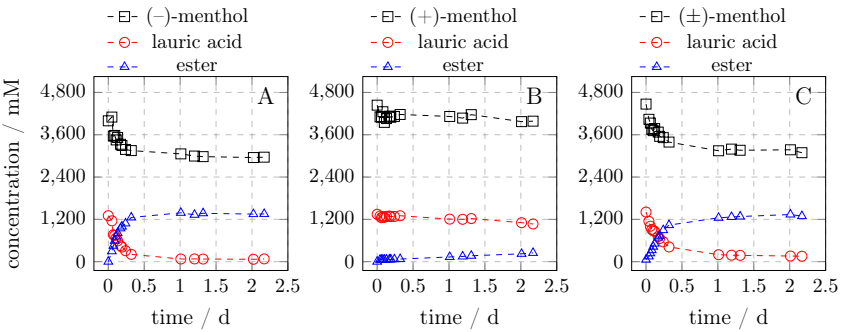


Figure 5.20: Comparison of the CRL catalysed esterification of (–)-menthol:lauric acid DES (A), (+)-menthol:lauric acid DES and (±)-menthol:lauric acid DES (C) with a menthol:lauric acid molar ratio of 3:1 mol/mol, respectively. Reaction conditions: 3 mmol lauric acid, 60 mg CRL, 900 rpm, $T = 45\,°C$, $a_{w,NaBr} = 0.52$, $n = 1$.

5.5 Summary

In this chapter, developing a biotransformation route for menthol, an important flavour and fragrance compound, was presented relying on 2-in-1 DES reaction media and the results can be summarised as follows:

- (–)-Menthol:fatty acid DESs were considered as suitable 2-in-1 DESs, as no self-esterification was observed when these DESs were incubated without

any lipase. Moreover, the resulting (–)-menthyl fatty acid ester products can be regarded as examples for modified (–)-menthol compounds for cosmetic applications.

- Screening different commercial lipases revealed that CRL was capable of catalysing the esterification of (–)-menthol with caprylic, capric and lauric acid in the respective neat DESs.

- Since CRL has a lid and is activated in the presence of hydrophobic interphases, the esterification was first studied in biphasic DES-water reaction systems. In comparison to a reference reaction in *i*-octane [143], quite high productivities were accomplished with the neat (–)-menthol:lauric acid DES reaction system and a biphasic DES-water reaction mixture with 10 wt% of water added to the DES.

- However, the fatty acid conversion was fairly poor in biphasic DES-water mixtures. Therefore, the a_w was considered as a suitable parameter to study the supply of water to the DES reaction systems. A preliminary experiment showed that the conversion in a_w controlled DES reaction systems was improved in comparison to the neat (–)-menthol:lauric acid DES reaction system.

- A central composite design was selected to optimise the esterification of the (–)-menthol:lauric acid DES in terms of a_w, enzyme amount and reaction temperature. The interaction of the reaction temperature and enzyme amount was determined as a significant factor combination influencing the ester synthesis rate. The obtained model was capable of accurately predicting the product formation, which was maximised at $a_w = 0.55$, $m_{CRL} = 60\,\text{mg}$ and $T = 45\,^\circ\text{C}$ in the chosen design space.

- As opposed to the biphasic aqueous DES reaction system with 10 wt% of water, the lauric acid conversion was improved under the optimised reaction conditions reaching 95 %, while the reaction time was reduced by 7-fold at the same time.

- The esterification of the (–)-menthol:lauric DES is expected to become more efficient by decreasing the initial molar ratio, as higher esterification velocities were observed for (–)-menthol:lauric acid 2:1 and 1.5:1 mol/mol mixtures.
- The resolution of (±)-menthol would be another possible application for 2-in-1 DES reaction media, as CRL catalysed the ester synthesis in the (–)-menthol:lauric acid DES, but not in a (+)-menthol:lauric acid DES.

6 Product purification[7]

6.1 Introductory words

The application of DESs as co-solvents, solvents or as combined substrate-reaction media is widely researched for various biotransformations, whereas there is often a lack of information on how the obtained products might be purified from a DES reaction mixture. Only a few studies deal with developing integrated DES-based bioprocesses including product purification. The recovery of monoterpene epoxides [79] and the refinement of biodiesel from DES mixtures [57] can be regarded as the only DES-based process outlines with product separation. The separation of unconverted compounds is especially important, if the DES acts as substrate and solvent and is not composed of equimolar amount of substrates. This is the case for the CRL catalysed synthesis of (–)-menthyl fatty acid esters in 2-in-1 DES reaction media, which was described in chapter 5.4. The eutectic point of the (–)-menthol:lauric acid DES occurs at a mole fraction of 0.29 of lauric acid at room temperature (290.73 K) [85] and (–)-menthol was present in excess for the CRL catalysed esterifications of this DES. The separation of (–)-menthol is addressed in this chapter to obtain the product with high purity and to recycle the unconverted (–)-menthol. Initially, the DES esterification was further optimised to reach high fatty acid conversion. The thermal separation of the DES reaction mixture requires a difference in the boiling points of the involved compounds, which is given for the DES constituents and the product ester (see Table 6.1). Therefore, a vacuum distillation step was applied to accomplish the separation of (–)-menthol from (–)-menthyl laurate and the recovered (–)-menthol was used to prepare a new DES for a subsequent enzymatic esterification cycle.

[7] Some of the presented results have been published by the author and co-workers in [154].

Table 6.1: Boiling temperatures of substrates (experimental data at atmospheric pressure) and product ester (predicted data at 760 Torr (1 bar) atmospheric pressure.

Compound	T_{boil} $^{\circ}C$	Reference
(–)-Menthol	212[a]	[155]
Lauric acid	298.9[a]	[155]
(–)-Menthyl laurate	379.1±10[b]	[155]

[a] experimental data at atmospheric pressure (i.e. 1.01 bar)
[b] predicted data at 760 Torr (i.e. 1.01 bar)

6.2 CRL catalysed synthesis of (–)-menthyl laurate

Preliminary distillation experiments with ternary mixtures containing unconverted substrates (–)-menthol and lauric acid as well as the (–)-menthyl product ester showed that it was difficult to separate both unreacted substrates from the ester without creating high losses of (–)-menthol at the same time. Thus, it the first objective was to achieve full conversion of the limiting substrate lauric acid during the esterification step. As a result, the reaction mixture would then consist only of excess (–)-menthol and the ester, which would simplify the separation step. For this reason, also reaction times of more than 24 h were accepted to achieve conversions as high as possible, and hence to obtain a binary instead of a ternary separation mixture. It was expected that the removal of water, which is formed as side product during the direct esterification, would shift the reaction equilibrium further towards ester synthesis. The DES was therefore pre-equilibrated at a reduced a_w using a saturated potassium acetate solution (a_w = 0.22 at 30 °C [146]) and the reaction was performed in the presence of the saturated salt. If the esterification had run to complete conversion of lauric acid without any water adjustments, the water content would have been theoretically increased up to 27 mg g_{DES}^{-1}. Through the gas phase equilibration of the DES with saturated potassium acetate, the water content was however adjusted and maintained at 3 mg g_{DES}^{-1} (calculated based on equation 3.3) and water was removed to the saturated salt solution. Furthermore, the enzyme powder was pre-incubated in the presence of

saturate potassium acetate to avoid water input by the addition of the enzyme powder and to provide a constant water content at the start of the reaction. A loss of enzyme activity was not observed when CRL was incubated for three days at 35 °C with saturated potassium acetate (see Figure 6.1).

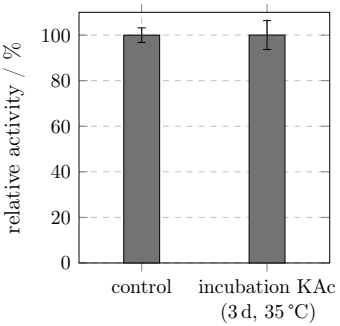


Figure 6.1: CRL activity after incubation of the enzyme powder over saturated potassium acetate solution at 35 °C for 3 d (n = 4) compared with a non-incubated control (1 U = 1 µmol$_{pNP}$ min^{-1}) at 35 °C and pH 8).

Two individual esterifications were carried out with 2 g (–)-menthol:lauric acid DES (3:1 mol/mol) for each reaction to obtain enough material for the purification step. After three days, both esterifications were completed and 100 % conversion of lauric acid was reached (see Figure 6.4A). For the following purification step, a fraction of each reaction mixture was harvested and combined for the separation by vacuum distillation.

6.3 Separation of (–)-menthyl laurate

The DES mixture, which was obtained after the esterification reactions, contained no residual lauric acid and therefore the purification task was reduced to separating a binary mixture. A DES mixture consisting of (–)-menthol and (–)-menthyl laurate (m = 1.4957 g, combined fractions from two esterification reactions) was used to separate the substrate (–)-menthol as low-boiling substance from the ester by a vacuum distillation step. Due to its higher boiling point the ester was accumulated as bottom product, whereas (–)-menthol was collected as overhead

product (see Figure 6.2).

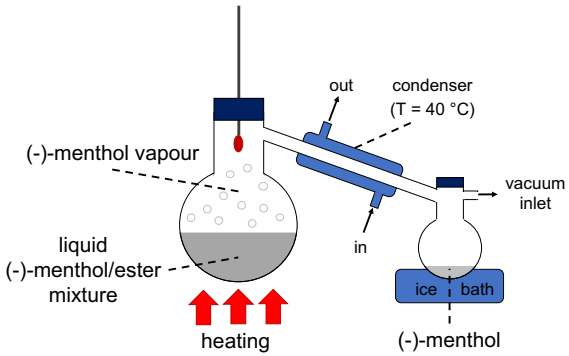


Figure 6.2: Schematic drawing of the vacuum distillation to separate a (–)-menthol/ (–)-menthyl laurate mixture. (–)-Menthol is the overhead product and the ester accumulates as bottom product.

After the separation, 735 mg product and 622.5 mg (–)-menthol were obtained. HPLC analysis showed that pure (–)-menthol was recovered as overhead product, while the bottom ester fraction contained a small (–)-menthol impurity (see Figure 6.3). A purity of 94 % was accomplished for the ester, as a small amount of (–)-menthol remained in the bottom fraction. A further purification of the product fraction was only possible by increasing the temperature above the boiling point of the ester, which would cause high product losses. The distillation was therefore stopped to recover pure (–)-menthol for recycling purposes and to obtain the ester in still acceptable purity. The distillative purification process was generally restricted by the use of a small scale-distillation apparatus without any refluxing fixtures, which would contribute to achieve better purities. A total loss of approximately 9 % was observed, probably due to a loss of (–)-menthol, which is highly volatile, and the evaporation of small quantities of residual water from the reactant mixture.

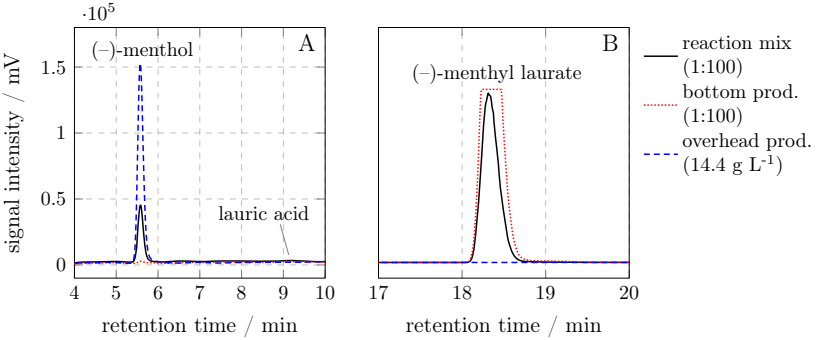


Figure 6.3: RID chromatogram (A) of (–)-menthol (t_R = 5.6 min) and lauric acid (t_R = 9.3 min) and ELSD chromatogram (B) of the (–)-menthyl laurate ester (t_R = 18.3 min). Samples were taken from the reaction mixture before distillation (—) and after the separation from the bottom (—) and overhead (—) fractions.

6.4 Re-use of recovered (–)-menthol

After the distillative separation, a new (–)-menthol:lauric acid (3:1 mol/mol) DES batch was prepared with the recovered 4 mmol (–)-menthol (m = 622.5 mg) by adding 1.33 mmol fatty acid (m = 266.9 mg). Another esterification was performed with 836.3 mg of the DES consisting of recycled (–)-menthol and fresh lauric acid. Fresh CRL powder was added to start the reaction, since the recycling of the enzyme was not considered at this stage. The reaction was run under identical conditions as the previous esterification batches, including the pre-incubation of the DES and the enzyme powder. An initial a_w of 0.16 was measured at the start of the reaction, corresponding to a calculated water content of 2.2 mg g_{DES}^{-1} of the DES mixture (see equation 3.3). Figure 6.4 shows the reactant concentrations and fatty acid conversion of the starting esterification batches (see Figure 6.4A) in comparison to the reaction carried out with the DES composed of recycled (–)-menthol (see Figure 6.4B). Similar to the starting esterifications, a high conversion of lauric acid ($\geq$ 98.7 %) was accomplished with the recycling (–)-menthol DES. These results showed that the separation method was efficient to recover (–)-menthol in high purity to enable its subsequent re-use for an equally well performing enzymatic reaction. At the example of the enzymatic esterification of a (–)-menthol:lauric acid

DES, the distillative separation of the unconverted DES compound was especially well suited to compromise high product purity with recovering pure (–)-menthol to facilitate its efficient re-use. Therefore, the enzymatic esterification of a 2-in-1 DES and the subsequent recycling and re-use of the excess DES compound can be regarded as a process intensifying approach to make the use of DESs as solvents for biocatalysis more attractive.

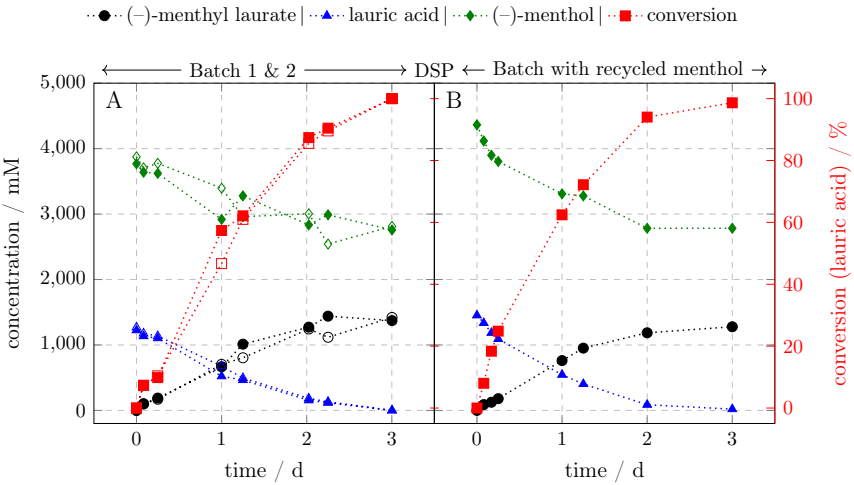


Figure 6.4: Reactant concentrations (left y-axis) and fatty acid conversion (right y-axis, red) of the CRL catalysed esterification in (–)-menthol:lauric acid of batch 1 (closed symbols) and batch 2 (open symbols) (A) and in the DES composed of the recycled (–)-menthol of combined fractions of batch 1 and 2 (B). The DES and CRL was equilibrated with saturated potassium acetate at 35 °C for 3 d. Reaction conditions: 1 wt% CRL (m_{CRL}/m_{DES}) , $T = 35\,°C$, potassium acetate saturated salt solution, 300 rpm.

6.5 Process outline

In order to evaluate the potential of DESs as alternative reaction solvents for enzymatic reactions two basic requirements should be met: (1) The use of a DES is advantageous over a comparable solvent for the desired reaction. (2) The DES enables product purification and recovery of recyclables to obtain an integrated

process design. DESs can be regarded as efficient alternative reaction media for enzymatic reactions, in particular, if the DES is composed of reaction substrates and acts as combined substrate and solvent. Beyond the fact that certain DESs might be characterized by an increased environmental compatibility, the 2-in-1 approach can help to create an advantage with using DESs, since the targeted reaction can be performed at high substrate loads under virtually solvent-free conditions. The CRL catalysed esterification of the (–)-menthol:lauric acid DES can be regarded as a promising example for the application of a DES reaction medium. Due to its eutectic point the DES was not composed of equimolar amounts of substrates, and therefore, the recovery of the excess substrate was addressed to develop a (–)-menthol recycling strategy. Even if the fatty acid was converted completely, (–)-menthol was present in excess and its separation from the product ester was necessary to provide an integrated process design. Figure 6.5 shows a schematic outline for a potential DES-based process to enzymatically synthesize a (–)-menthyl lauric acid ester. The (–)-menthol:lauric acid DES is prepared by heating and stirring in a receiving tank.

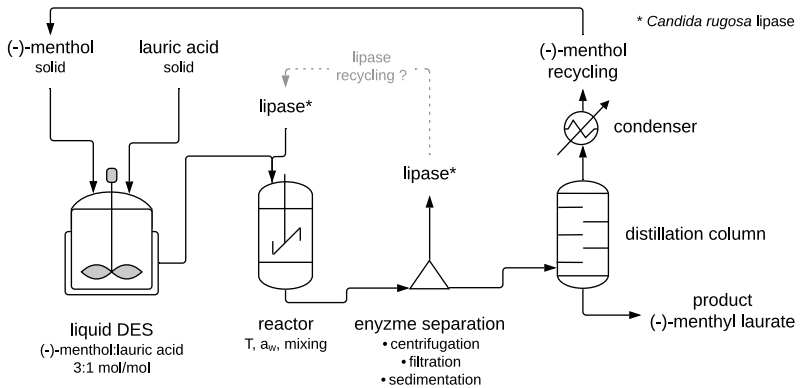


Figure 6.5: Schematic process outline for the enzymatic synthesis of a (–)-menthyl lauric acid ester in a 2-in-1 DES acting as combined substrate and reaction solvent.

The DES compounds are esterified in a stirred tank reactor by the addition of a

suitable lipase (e.g. CRL) under temperature and water activity controlled reaction conditions to reach 100 % fatty acid conversion. Different reactor concepts have been suggested for controlling the a_w during enzymatic reactions [140, 156], and even scalable packed-bed reactor systems have already been established for in situ a_w control [157, 158]. After the reaction, the enzyme is separated from the (–)-menthol-ester mixture (e.g. by filtration, sedimentation or centrifugation). It has been demonstrated in this study that excess (–)-menthol can be recovered via distillative separation and re-used for another esterification batch without loss of the process performance. The recycling of the enzyme was out of scope of this study, but would certainly contribute to further intensify the process. In summary, the outlined enzymatic process relying on a 2-in-1 DES reaction medium shows how DESs can be potentially applied for an efficient bioprocess design.

6.6 Summary on DES reaction systems and benchmark

The performance parameters of the (–)-menthol:lauric acid DES reaction systems, which were investigated in this study (i.e. esterification in neat DES, biphasic DES-water reaction mixtures or in a_w controlled DES reaction media), are summarised in Table 6.2. The environmental factor (E factor) can be used to determine the resource efficiency of syntheses and is a measure for the waste generated per product according to the following equation [159]:

$$E\ factor = \frac{m_{waste}}{m_{product}} \tag{6.1}$$

For the calculation of the E factor waste can be defined as everything except the product, whereas water is typically not considered as waste [159]. Regarding other process solvents than water, an optimistic assumption can be made that 90 % of the solvents can be reused, if real solvent losses are unknown [159]. Generally, a low E factor implies less waste, and therefore, better environmental compatibility, whereas a high E factor reflects a more negative environmental impact. If a synthesis produces ideally zero waste (e.g. by high atom efficiency and 100 % recycling of auxiliaries), the ideal E factor would be zero.

The E factor was calculated (at the endpoint of esterification reactions) for each

DES reaction system of this study, in addition to common process parameters such as conversion and productivity (which were calculated after 24 h for comparative reasons). Table 6.2 also presents a published CRL catalysed esterification in an organic reaction medium [143] to benchmark the results of the DES esterifications of this study with a conventional solvent reaction system. Due to higher substrate concentrations present in the DES, the productivity was enhanced in neat as well as in different biphasic and a_w controlled DES reaction systems compared to the reaction in *i*-octance. However, the organic solvent reaction system reached 97 % conversion of lauric acid after 24 h. A comparably high conversion of 95 % was only achieved with the a_w optimised DES reaction system. The reactions for the (–)-menthol recycling experiments were performed at a lower a_w to reach complete conversion after a prolonged reaction time of 3 d, and thus, lauric acid was only converted by 51 % after 24 h in this reaction set-up. In comparison to the organic solvent reaction systems with an E factor of 4, this parameter was improved by the use of DESs reaction media in any of the differen DES reaction set-ups (see Table 6.2). Considering that excess (–)-menthol from the 3:1 mol/mol (–)-menthol:lauric acid DESs is recyclable after a distillative separation step, an excellent E factor of 0.1 was obtained (see Table 6.2), which is even better than the desired E factor of 5 - 50 for the production of fine chemicals [159]. Usually, products that are manufactured in large scale, such as bulk chemicals or oil refinement products, should be characterised by E factors between 1 and 5 or less than 0.1, respectively [159]. Therefore, the DES reaction systems of the present study benefit from high substrate loads, the absence of any auxiliary, hazardous solvent and from excellent E factors, if a recycling loop is used to reuse the excess DES compound (i.e. (–)-menthol).

Table 6.2: Comparison of organic solvent and DES reaction systems for the enzymatic synthesis of (–)-menthyl laurate.

		Reaction systems					
Parameters	unit	Organic solvent[a]	neat DES, small set-up[b]	biphasic, small set-up[b]	biphasic, large set-up[c]	a_w optimised[d]	a_w controlled & (–)-menthol recycling[e]
Solvent	–	*i*-octane	DES	DES	DES	DES	DES
Volume	mL	10	0.55	0.55	27	2.2	2.2
(–)-Menthol:lauric acid	mol/mol	2:1	3:1	3:1	3:1	3:1	3:1
'Additive'	–	mol. sieves	–	10 wt% H_2O	50 wt% H_2O	a_w 0.55	a_w 0.22
Temperature	$°C$	35	35	35	35	45	35
CRL amount	mg	47	5	5	250	60	20
Conversion[f]	%	97	32	48	67	95	51
Productivity[f]	$g\,L^{-1}\,d^{-1}$	33	205	133	n.d.	443	231
E factor	–	4	2.2	1.8	n.d.	1.1	0.1

[a] Data from Babali et al. [143]
[b] See chapter 5.4.1, Figure 5.6
[c] See chapter 5.4.1, Figure 5.8
[d] Data from CCD optimisation (see Figure 5.16A)
[e] See chapter 6.4, Figure 6.4
[f] Lauric acid conversion and productivity after 24 h

6.7 Summary

In this chapter, a distillative purification approach was presented for the separation of a DES reaction mixture containing an enzymatically synthesized (–)-menthyl lauric acid ester and (–)-menthol. At the start of the esterification reaction (–)-menthol was present in 3-fold excess to form a DES with lauric acid, the second substrate for the targeted reaction. Even though the fatty acid was converted completely, the separation of the excess substrate (–)-menthol was necessary, which was accomplished by a vacuum distillation step. This approach enabled the reuse of (–)-menthol for another esterification reaction and the results can be summarised as follows:

- The CRL catalysed esterification of (–)-menthol:lauric acid DES was optimised to achieve 100 % fatty acid conversion. Controlling a_w during the reaction with saturated potassium acetate promoted full conversion after 3 d. A prolonged reaction time was accepted to reduce the number of reactants to be separated from a ternary (menthol, lauric acid, ester) to a binary (menthol, ester) mixture.

- Incubating the CRL powder at 35 °C for 3 d with saturated potassium acetate did not result in a loss of activity. This method was applied to enable a constant water content of the DES reaction medium at the reaction start.

- (–)-Menthol was separated overhead as low-boiling compound from the ester by vacuum distillation and the product was accumulated as bottom fraction. The ester (735 mg) was obtained with 94 % purity, as some residual (–)-menthol was still present in the product fraction when the distillation was stopped. Pure (–)-menthol was recovered as overhead product for recycling purposes.

- The recovered (–)-menthol (622.5 mg, 4 mmol) was used to prepare a new (–)-menthol:lauric acid (3:1 mol/mol) batch, which was used for a second esterification reaction with fresh CRL. After 3 d the esterification reached more than 98.7 % lauric acid conversion, showing that the reaction can be carried out efficiently with recovered (–)-menthol.

- A potential DES-based process was outlined for the enzymatic synthesis of (–)-menthyl laurate (see Figure 6.5).

- Comparing the DESs reaction systems of this study with a published CRL catalysed esterification of (–)-menthol and lauric acid, higher productivities and improved E factors were achieved with each of the DES reaction systems (e.g. neat DES, biphasic DES-water reaction mixtures or a_w controlled DES reaction conditions). A nearly ideal E factor of 0.1 was achieved by the distillative separation of excess (–)-menthol and its reuse for another DES esterification.

7 Discussion and outlook

This study presented different lipase catalysed reactions in DESs and focussed on evaluating the potential of these novel solvents for biocatalysis. The results were discussed in the respective chapters, and therefore this section provides a general evaluation of the potential application of DESs as alternative media for enzymatic reactions. Moreover, some future perspectives are given for the biotransformation of menthol in DESs, which was developed in this study.

7.1 Biotransformation of menthol with 2-in-1 DESs

Bioprocesses differ from chemical processes due to the specific nature of biocatalysts, and bioprocess development usually requires a few iterative cycles of biocatalyst selection, characterisation, engineering and application to establish efficient process including product recovery [160]. This study focussed on reaction medium engineering as one important stage during bioprocess development to evaluate the potential of DESs as novel nonaqueous reaction media for biocatalysis. Different reactions, e.g. aldol reaction (see chapter 4.3) or transesterification (see chapter 4.4), were investigated in different DESs to screen for possible applications of these novel solvents. The DESs were attempted to solubilise high substrate loads in some cases. With the application of DESs as 2-in-1 reaction media, acting as combined solvent and substrate for the esterification of (–)-menthol:lauric acid DES, different enzymes were screened to suit the solvent rather than determining a solvent that suits the enzyme (see chapter 5.3). With CRL the conversion of (–)-menthol in DESs composed of the monoterpene alcohol and fatty acids to form a corresponding (–)-menthyl fatty acid esters was successfully accomplished in this study. Developing a suitable DESs based reaction system, a_w controlled DES reaction systems were found to more efficient than binary DES-water systems (see chapter 5.4.3). Therefore, the CRL catalysed synthesis of (–)-menthyl laurate was studied in depth and optimised using a statistical design of experiments (see chapter 5.4.4). Moreover, a recycling loop was established for (–)-menthol (see chapter 6.4), which was used in excess due to the eutectic composition of the DES. The process is not only very efficient in terms of high productivity, but also resulted

in an excellent E factor close to 0 (see Table 7.1). Since CRL was not recycled, it was assumed as waste for calculating the E factor. However, with an appropriate enzyme recycling strategy the E factor would become practically 0. Comparing different DES reaction systems from this study with a published reaction to synthesize (–)-menthyl laurate demonstrates the enormous potential of DESs as alternatives to organic solvents. As opposed to the same reaction in an organic solvent, the enzymatic synthesis of (–)-menthyl laurate in a neat DES reaction system was enhanced by a factor of 6.2 in terms of batch productivity, while the E factor decreased by about one half from 4 to 2.2 (see Table 7.1). Optimising the DES reaction system by adding water or controlling the a_w further decreased E factor. These examples underline how DESs are able contribute to both more efficient and less waste intense synthesis routes by smart reaction medium engineering.

Table 7.1: Comparison of productivities and E factor for the enzymatic synthesis of (–)-menthyl laurate using different reaction systems.

Reaction systems	Productivity[a] $g\,L^{-1}\,d^{-1}$	E factor –
Organic solvent [b]	33	4
Neat DES [c]	205	2.2
Binary DES-water (+10 wt%)[c]	133	1.8
DES with optimised a_w [d]	443	1.1
DES with controlled a_w and (–)-menthol recycling[e]	231	0.1

[a] Productivity after 24 h
[b] Reaction in *i*-octane, data from [143]; E factor assumes 10 % solvent loss [159]
[c] See chapters 5.4.1 and 5.4.2
[d] Data from CCD optimisation (see Figure 5.16A)
[e] See chapter 6.4, Figure 6.4

For the CRL catalysed esterification of (–)-menthol:lauric acid DESs, the effect of varying temperature was studied by means of a statistical design of experiments (see chapter 5.4.4), while the variation of the molar composition of the DES was

investigated in another set of experiments (see chapter 5.4.5). However, the combined effect of temperature and compositional variation has not been assessed. The process window for these parameters can easily be derived from the phase diagram (see Figure 7.1). As a future perspective, the reaction might be further optimised by increasing the temperature, of course considering the enzyme's temperature optimum. By augmenting the experimental design with enzyme stability as another factor, it would be possible to also analyse the interaction of enzyme stability with temperature and/or a_w in the DES. Since a liquid mixture of (–)-menthol and lauric acid is obtained at temperatures above 45 °C regardless of the molar fraction of the DES compounds, there is no upper limit for elevating the reaction temperature. The optimum temperature would therefore be determined by the thermal stability of CRL in the DES. Since CRL was reported to be more stable in ILs than in organic solvents at temperatures of up to 50 °C [161], it might be possible to perform the enzymatic esterification of (–)-menthol:lauric acid also at higher temperatures. Moreover, increasing the reaction temperature also decreases the DES viscosity (see chapter 3.5, Figure 3.8), which could further enhance the mass transfer in the reaction system. The lowest possible reaction temperature is determined by the eutectic point occurring at approximately 16 °C at a mole fraction of 0.25 of lauric acid (see Figure 7.1).

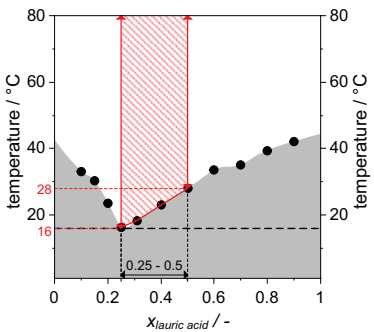


Figure 7.1: Process window for the (–)-menthyl laurate ester synthesis showing the molar DES composition and temperature range on the basis of the (–)-menthol:lauric acid phase diagram (adapted from [85].

In order to develop an atom efficient esterification process, it is recommended to vary the DES composition at a mole fraction of 0.25 to 0.5 of lauric acid (see Figure 7.1), corresponding to (–)-menthol:lauric acid mixtures of 3:1 to 1:1 mol/mol, respectively. Lauric acid mole fractions below 0.25 are not recommended, since it increases the (–)-menthol content to an unnecessary excess. From an economic perspective, the reaction would be performed ideally in the equimolar eutectic mixture to avoid substrate excess. In this case, the lowest possible reaction temperature is 28 °C to obtain a liquid mixture (see Figure 7.1). If the reaction conditions are adapted to reach full conversion, it would be possible to obtain pure product without the need for any purification step. Therefore, lauric acid mole fractions above 0.5 are not recommended. Reducing the DES composition from a 3:1 to a 1.5:1 mol/mol mixture gave already good results in terms of an enhanced ester formation velocity and conversion (see chapter 5.4.5, Figure 5.19). Therefore, the potential of DES reaction medium engineering has not been fully exploited for the CRL catalysed esterificaion of (–)-menthol:lauric acid. Further optimisations towards reaction temperature and DES composition seem to be very promising parameters to achieve an even more efficient process design in terms of atom economy, waste reduction and no need for product separation.

Beyond reaction medium engineering, process engineering is another approach to enhance the efficiency of a biocatalytic reaction. Generally, three different reactor types, which are stirred tank reactors (batch operation), continuous stirred tank reactors or plug flow reactors (continuous operation), are used for enzymatic reactions, where fed-batch operation can be regarded as modified batch process in stirred tank reactors [162]. Various examples for fed-batch enzymatic syntheses are found in literature with positive effects on the process performance [163, 164]. It was reported that the selective esterification of menthol performed better in a fed-batch process than in conventional batch mode due to better controlling the water content in the fed-batch system [165]. More recently, fed-batch and continuous process concepts have been presented for the esterificaiton of glycerol with benzoic acid in a ChCl:Gly DES [166]. If the esterification of (–)-menthol:lauric acid cannot be accomplished in an ‘ideal’ equimolar eutectic mixture, operating the enzymatic esterification in fed-batch mode with a constant feed of lauric acid

would be a valuable approach to compensate for excess (–)-menthol inherent to the eutectic composition of the DES. The fed-batch esterification of benzoic acid in ChCl:Gly is a similar example to the enzymatic synthesis of (–)-menthyl laurate, since both DESs act as combined solvent and substrate. In case of synthesizing α-monobenzoate glycerol, feeding benzoic acid in combination with using a buffer as co-solvent helped to overcome lipase deactivating effects associated with high acid concentrations (i.e. low pH). The effect of pH also needs to be studied to design a fed-batch process of the (–)-menthol:lauric acid esterification. However, for the enzymatic synthesis of (–)-menthyl laurate no aqueous co-solvent phase is needed, and the water content, adjusted by the a_w of saturated salt solutions is very low in the DES (see chapter 3.4, Figure 3.6). The definition of pH to predict the protonation state of an enzyme becomes more problematic when the dilute aqueous phase gets close to zero, and it is questionable whether pH is the right measure in this case [167]. Moreover, by varying the molar DESs composition, enhanced esterification velocities were observed for the (–)-menthol:lauric mixtures with a higher fatty acid content (see chapter 5.4.5, Figure 5.19). Therefore, feeding lauric acid in a fed-batch process might be not interfere with the protonation state of CRL due to an extremely low water environment in the hydrophobic DES. The fed-batch enzymatic esterification of (–)-menthol:lauric acid could be another strategy to obtain pure ester and to cut down on expensive downstream operations to separate the product. Since lauric aicd is a solid at room temperature, the fatty acid needs to be heated above its melting point (44 °C) to be dosed via a pump. Therefore, further investigations are necessary to evaluate whether the feed stream temperature is suited to the reaction temperature or whether temperature gradients might interfere with the enzymatic esterification. As a future perspective, the (–)-menthol:lauric acid DES esterification process, which was outlined in chapter 6.5 (see Figure 6.5) might therefore be modified to a fed-batch process to go without laborious and expensive product purification operations, and a simplified scheme is depicted by Figure 7.2. Guajardo and co-workers have reported that the reuse of immobilised CALB in a 2-in-1 DES reaction medium was possible for five batches at an activity loss of 37 % between the first two batches [74]. Recycling the CRL for the esterification of (–)-menthol:lauric acid DES was not investigated in this study, but modifications like enzyme immobilisation and recycling might

contribute to further intensify the process.

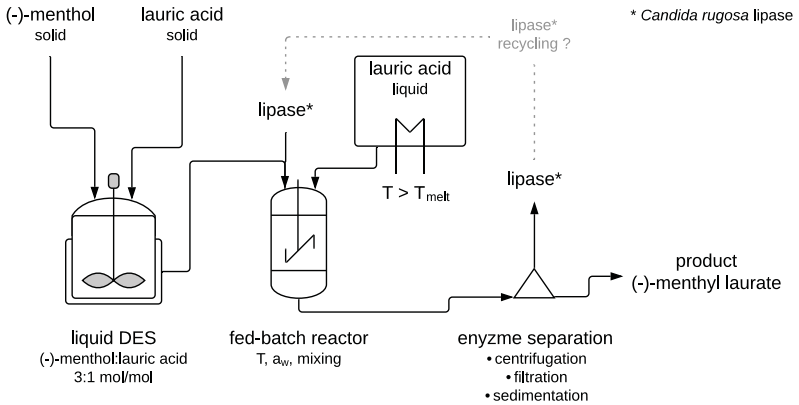
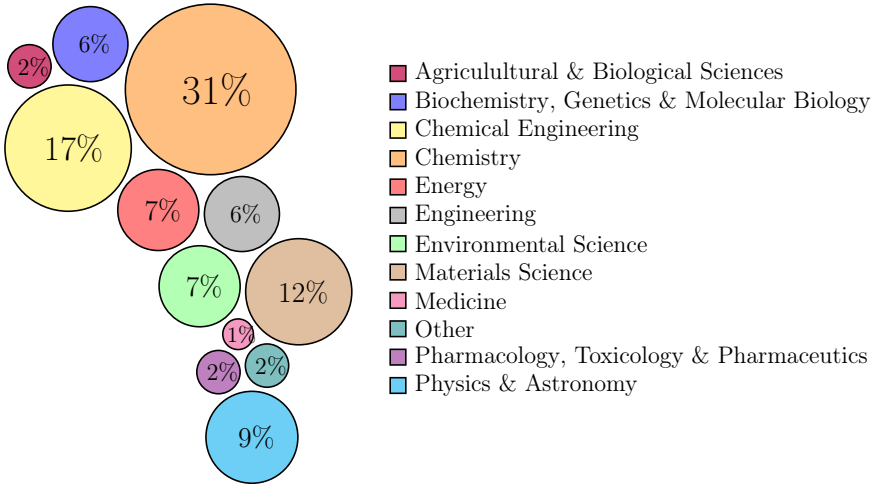


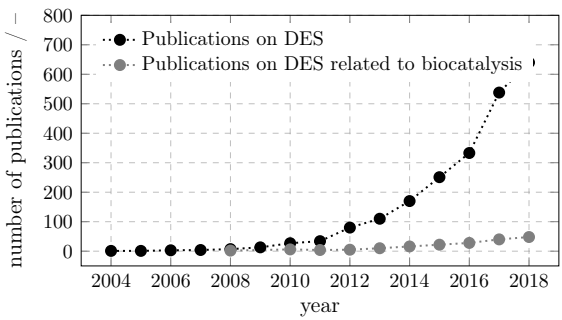
Figure 7.2: Schematic process outline for a lauric acid fed-batch process to synthesize (–)-menthyl lauric acid ester in a 2-in-1 DES acting as combined substrate and reaction solvent.

7.2 Evaluation of DESs for enzymatic reactions

The research interest in DESs is huge and these novel solvents are currently investigated in many different disciplines of natural sciences, e.g. from chemistry over materials science to pharmacology (see Figure 7.3a). The interest in the application of DESs in various fields is also reflected by a steadily increasing number of publications on DESs (see Figure 7.3b). Since the first paper on hydrolase catalysed reactions in DESs was published in 2008 [55], the number of scientific reports on DESs referring to biocatalysis has been increasing constantly. Although many different enzymatic reactions have already been successfully established with DESs [168], the interest in DESs for biocatalysis is rather low compared to the number of total publications dealing with DESs in general (see Figure 7.3b). Only a few investigations focus on developing an integrated DES bioprocess design, such as for the synthesis of monoterpene epoxides [79] or the production of biodiesel [57], and to



(a) Studies on DESs by subject area.



(b) Publications on DESs per year in general and relating to biocatalysis.

Figure 7.3: Publications on DESs (data retrieved from Scopus Abstract and Citation Database `www.scopus.com`[a]; accessed on 30/03/2019).

[a]Search query 1: 'deep eutectic solvent' in abstract, title or keywords;
Search query 2: 'deep eutectic solvent' AND 'biocatalysis' OR 'biotransformation' OR 'enzyme' OR 'whole cell' OR 'lipase' in abstract, title or keywords

date DESs have not yet been applied in an industrial bioprocess. Due to these reasons, the application of DESs for biotransformations must be regarded as an infant technology. However, the combination of biocatalysis and DESs has many features to fulfil some of the 12 Principles of Green Chemistry, such as preventing waste (1), increasing atom economy (2), using safer solvents (3), renewable feedstocks (7) or catalysts (9) [169, 170]. Biocatalysis is especially suited in the context of Green Chemistry, since it offers mild reaction conditions, environmentally compatible catalysts and solvents at high activities, chemo-, regio- and stereoselectivities in complex molecules [171].

DESs are an extremely flexible solvent class, and generally three different application principles can be distinguished for their use as biocatalytic reaction media (see chapter 1.6, Figure 1.4). DESs can be used as co-solvent or additive to aqueous buffer reaction systems to enable or modify certain solvent properties. Homogeneous reaction mixtures are often obtained due to the frequent use of hydrophilic, water-miscible DESs (e.g. most ChCl-based DESs). Also biphasic reaction mixtures are possible, if hydrophobic DESs are mixed with water or buffers, although so far enzymatic reactions in hydrophobic DESs have only been rarely investigated. Since water is the 'natural' environment of enzymes, the concept of using DESs as co-solvents is widely researched. For example, the effect on the enzymatic activity or stability caused by DES addition was studied for *Penicillium expansum* lipase [106], CRL [107] or a potato epoxide hydrolase (StEH1) [109]. In all cases, a certain DES addition improved the enzyme activity and stability. For the epoxide hydrolysis with StEH1, the enantiomeric excess (*ee*) was enhanced by the addtion of certain DESs in comparison to a buffer reaction system, but the *ee* was generally low and therefore inappropriate for biocatalytic usefulness [109]. The molecular interaction of DES co-solvents with proteins has also been investigated. For example, a higher activity of horseradish peroxidase induced by a DES co-solvent was correlated with a more flexible tertiary structure and a higher α-helix content of the secondary protein structure [63]. It was hypothesized that pure DESs enable partially folded proteins, but reduce enzyme activity through interplaying specific binding and solvophobic effects, while hydrated DESs (e.g. used as co-solvents in aqueous systems) would promote a water shell around the active site enabling

a proper protein fold, and thus retain activity to a certain extent [172]. Beyond affecting the protein structure and the activity of an enzyme, DESs were also used as performance additives to trigger certain physical properties of a reaction medium. For a chemo-enzymatic cascade reaction in a water-oil reaction mixture, DESs have recently been exploited as additives to reduce the surface tension of the aqueous phase [173]. As a result the conversion of soybean oil was considerably improved by adding various DESs due to an increased interfacial surface area of oil droplets dispersed in the DES-modified aqueous phase [173]. This DES property is particularly interesting to enhance mass transfer in multiphase enzymatic reactions. Using hydrophilic DESs as co-solvents with buffers/aqueous reaction media generally implicates the question whether the DES hydrogen bond network can be maintained in such mixtures. Diluting a ternary 1,2-propanediol:ChCl:water DES mixture with 50 % water disrupted the hydrogen bonds between 1,2-propanediol and ChCl completely [73]. This indicates that likely a solution of DES and buffer compounds is obtained after exceeding a certain level of water addition, which should be considered for the application of DESs as co-solvents.

Enzymatic reactions can also be performed in 'pure' DES reaction media (see chapter 1.6, Figure 1.4). However, reactions in 'pure' DESs are difficult to accomplish. Water is often essential to retain enzymatic activity, and therefore it was suggested to keep the water content as low as possible to prevent hydrolysis reactions and to avoid decomposition of the eutectic solvent [116]. In this context, the term 'pure' DES refers to only slightly modified, homogeneous solvent systems. Examples for syntheses in 'pure' DESs are lipase [55, 116], phospholipase [174], or protease [175] catalysed reactions. DESs represent alternatives to other nonaqueous media, such as organic solvents or ionic liquids, to accomplish synthetic reactions that are not possible in the presence of water. In contrast to ILs, DESs are widely recognised as less hazardous solvents. ILs and DESs should be considered as valuable alternatives to organic solvents due to their low volatility and flammability. However, DESs must not be generalised as 'green' solvents. ILs cannot be principally seen as 'green' or 'toxic' solvents due to the quality of their starting materials [159], which certainly applies to DESs as well. As composite solvents their properties are dependent on the selected DES constituents just like ILs. ChCl:organic acid DESs were

recently found to be even more toxic than their corresponding [Ch^+][carboxylate$^-$] ILs, although overall the DESs were assigned with moderate toxicity [176]. ChCl-based DESs (glycerol, glucose or oxalic acid as HBDs) were characterised as low to moderately cytotoxic and non-toxic towards plants [177]. It was further reported that the toxicity on fungi and fish cells varied with the composition of the DESs. DESs based on organic salts and alcohols, acids or amides were less toxic than organic salt:metal salt DESs [178]. While biodegradability is often ascribed to DESs *per se* due to their preparation with 'natural' starting materials, it was reported that some ChCl and especially ChAc-based DESs cannot be assumed to be readily biodegradable [179]. The question whether DESs are benign or toxic cannot be generally answered, and the (cyto-)toxicity of different DESs was found to vary with the structure of the components [120]. Therefore, generalisations describing DESs as non-toxic, biodegradable and benign solvents must be interpreted cautiously. However, the attention given to DESs over the past decade is still justifiable. As opposed to ILs, DESs can easily be prepared from relatively low cost starting materials [180], which explains why today DESs are often more attractive than ILs. Moreover, DESs are highly flexible solvents, their physical properties can be tailored to suit a specific reaction, and high substrate solvation capacities might be achieved with designer DESs. The potential to tailor a DES to an enzymatic reaction was assessed at the example of a lipase catalysed aldol reaction (see chapter 4.3). Hydrophobic DESs were tailored to enable high substrate concentrations (especially the substrate-based TbACl:4-NBA DES), and thus promoted high product titres. Nonetheless, Milker and co-workers were able to show that the aldol addition was faster and more efficient in the co-solvent acetone [122]. Tailoring DESs to dissolve a substrate of interest could carry a risk to use chemically similar DES compounds, which can act as competitive substrates in the desired reaction. Although side reactions were not investigated for the lipase-catalysed transesterifications of vinyl laurate and 1-propanol performed in TbACl-alcohol DESs (see chapter 4.4), competitive reactions between the substrate(s) of interest and DES compounds are certainly a drawback for the use of DESs as reaction media. However, it was reported that the reactivity of a competing substrate is reduced due to strong hydrogen bond interactions of the DES compounds [55, 116]. Moreover, DESs are designed and widely used for extractions of various bioactive

compounds [181]. Cao and co-workers recently gave an example for the design of a DES to extract artemisinin with a higher efficiency than petroleum ether [41]. Moreover, some complex biomolecules are better soluble in DESs than in water, and it was hypothesized that DESs may be a third liquid phase (beyond lipids and water) for cellular bioreactions [32]. The application of 'pure' DES reaction media might therefore become more important for the biotransformation of complex biomolecules well solubilised in DESs. Unfortunately, most studies only provide proof-of-principle that a certain enzymatic reaction can be performed in a DESs without clearly stating the advantages of the DES reaction systems over conventional reaction media. To fully exploit the potential of DESs, future investigations should focus on presenting 'real' benefits of DESs rather than quoting generalised arguments like enhanced 'greenness' or reduced toxicity.

Another application principle is to use DESs as combined substrate and solvent, performing as 2-in-1 reaction media (see chapter 1.6, Figure 1.4). This is a promising approach to enable reactions with minimal waste, or ideally with zero waste, if all involved substrates form a liquid DES. This 2-in-1 concept has already been applied to several lipase catalysed reactions, such as for the epoxidation of monoterepnes [79], the synthesis of glycolipids [77, 78], 1,3-diaclyglycerol [80] or α-monobenzoate glycerol [74, 166]. For all reactions, ChCl was paired with the substrate of interest to form a DES (e.g. ChCl:U $\cdot$ H_2O_2 for the chemoenzymatic epoxidation of monoterpenes, ChCl:sugar for the glycolipid synthesis, or ChCl:Gly for the synthesis of 1,3-diacylglycerol or α-monobenzoate glycerol). While in these reactions only one of the DES compounds acted as substrate, the synthesis of (–)-menthyl fatty acid esters, investigated in the present study (see chapter 5.3.3), represents the first enzymatic conversion using both DES compounds as substrates (menthol and fatty acid forming menthol:fatty acid DES). Chapter 7.1 gave a detailed outlook on the enzymatic conversion of (–)-menthol in combined substrate and solvent DESs. 2-in-1 DES reaction media, composed of substrates only, are advantageous, because

(a) solid substrates become liquid through forming a DES, and therefore substrates are made accessible for enzymatic action,

(b) reactions can be performed virtually solvent-free,

(c) high substrate concentrations can be accomplished,

(d) waste can be minimised or completely avoided.

The latter is also the major benefit over DES reaction systems involving only one substrate, since an auxiliary HBA or HBD compound (e.g. ChCl) is not necessary to complex the targeted substrate in a DES. If ChCl is used as an auxiliary HBA to form a DES, the solvent will come along with high chlorine concentrations. This should be considered for bioprocess development due to the corrosive effects of chlorine, e.g. on stainless steel parts. In general, the best solvent would be no solvent [81], promoting more efficient, ecological and less hazardous processes. Designing DESs to comprise the substrate(s) represents a powerful approach to achieve solventless reactions, which would help to get close to the 'no-solvent' scenario. In terms of the Principles of Green Chemistry, combining biocatalysis and DESs as 2-in-1 reaction media certainly discloses a vast potential to realise atom efficient and more sustainable processes.

So far, mostly lipase catalysed reactions have been investigated and successfully established in DESs with both free or immobilised enzymes. Since lipases are very robust enzymes, which are able to withstand even extreme reaction conditions (e.g. high temperature, organic solvents, low water contents), it is not surprising that lipases were the first enzymes to be used for biotransformations in DESs. While the main focus is on lipase catalysed (trans-)esterifications in DESs, there are also some examples for lipase catalysed aldol [58, 122], nitroaldol [182], hydrolysis [108], or perhydrolysis [52, 123] reactions in DES solvent systems. Reactions with other enzymes than lipases include dehalogenation with dehalogenases [110], deglycosylation of flavonoids with glucosidases [59, 60], oxidation reactions with horseradish peroxidases [63, 64, 183], C–C bond formation with lyase [65], or peptide bond formation with chymotrypsin [61] or papain [62]. Various whole cell catalysed biotransformations have also been reported mostly relying on DESs as co-solvents [66–71, 184]. The potential of DES reaction media is therefore constantly expanded to non-lipase catalysed reactions. However, the influence of DESs

on less robust enzymes has not yet been sufficiently addressed, especially under high substrate concentrations like in 2-in-1 DES reaction media. Another outstanding question is whether the design of 2-in-1 DESs can be rationalised to expand the concept to a broader range of substrates, in particular more complex substrates like large biomolecules. To better predict whether substrates of interest form eutectics more screening research using thermodynamic modelling tools would be necessary to understand the solid-liquid phase behaviour of potential 2-in-1 DES substrate mixtures.

Although it is difficult to generalise on the properties of different solvent classes, especially in terms of composite solvents, a general comparison of ILs, organic solvents, and DESs was yet attempted to answer the question how DESs would be evaluated as alternative solvents for biocatalysis. For this purpose, a subjective value-benefit analysis was performed by the author to compare different solvent classes in a measurable way. The value-benefit analysis tool has been selected, since it is one of the methods used in technology assessment [185]. In this context, the different solvents classes can be considered as different technology alternatives for biocatalysis in non-aqueous reaction media, whereupon a value-benefit analysis might be one tool to decide for the most efficient solvent. For comparison with DESs, ILs and organic solvents were selected as common reaction media applied in non-aqueous biotransformations, whereas supercritical fluids were not considered. Their aggregate state is neither liquid nor gaseous, and therefore supercritical fluids were excluded from comparison with liquid solvents. Seven general categories were subjectively selected, weighted and rated by the author (see Table 7.2). Analysing the benefits of each solvent class, physical properties, such as hazardousness, toxicity, viscosity and stability (weighted at 20 %) were regarded as more important than soft factors, such as availability, cost and sustainability (weighted at 6.66 %). Enzyme compatibility was not included in the analysis, since solvent-specific effects on the protein structure are too complex to be analysed in general. In fact, enzymatic reactions can be successfully performed in DESs, ILs and organic solvents, but DESs obtained the highest overall score of all alternative solvents in the general assessment (see Table 7.2). ILs were evaluated less beneficial than organic solvents and DESs due to a few drawbacks, such as high costs

or low availability. However, DESs and organic solvents were only rated average at 3.27 and 3.07, respectively (out of a maximum of 5). This indicates that there is no perfect non-conventional solvent. However, DESs have much to offer in the quest for novel solvents. DESs certainly have already many positive properties, which justifies their increased application as alternative reaction media for biocatalysis. DESs will hopefully contribute to meet certain requirements of Green Chemistry. As a conclusion, DESs should be regarded as a valuable extension to the toolbox of non-conventional solvents for biocatalysis, especially when they are used as 2-in-1 combined substrate and solvents, as designer solvents to well solubilise complex biomolecules, or as enzyme stabilising co-solvents due to the above mentioned benefits associated with such reaction systems.

Table 7.2: Evaluation of non-conventional reaction media.

		Organic solvents		ILs		DESs	
	Weight	Points	Score	Points	Score	Points	Score
Hazard	20 %	1	0.20	3	0.60	3	0.60
Toxicity	20 %	3	0.60	2	0.40	4	0.80
Viscosity	20 %	5	1.00	2	0.40	2	0.40
Stability	20 %	3	0.60	2	0.40	3	0.60
Availability	6.67 %	5	0.33	2	0.13	4	0.27
Cost	6.67 %	4	0.27	1	0.07	5	0.33
Sustainability	6.67 %	1	0.07	2	0.13	4	0.27
Sum			3.07		2.13		3.27

Points: very high (1), high (2), medium (3), low (4), very low (5).
Score is obtained by multiplication of $weight \times points$.

8 Summary

In this study, the physical and chemical properties of several DESs were characterised and the application of DESs to different lipase catalysed reactions was investigated to evaluate the potential of DESs as novel reaction solvents for biocatalysis. The main results of this study are summarised as follows:

Most DESs are characterised by a relatively high viscosity, and therefore the flow behaviour and viscous properties of different DESs were studied in more detail. ChCl- (U, EG and Gly as HBDs) and TbACl-based DESs (capric acid, 1-hexanol, 1-octanol and 1-decanol as HBDs) were identified as highly viscous Newtonian fluids. The viscosity ranged from 1800 (ChCl:U) to 35 mPa·s (ChCl:EG). The viscosity of the DESs was decreased by increasing the temperature. The addition of water to miscible ChCl-based DESs also decreased their viscosity.

The pH of a reaction medium is an important parameter for biocatalytic reactions, as the pH affects the enzyme fold in a solvent and hence its catalytic activity. As composite solvents, the pH of pure DESs depends obviously on the starting materials. Furthermore, the starting materials can also modify the pH of aqueous reaction media (buffers), if the DESs are used as co-solvents. Therefore, the changes of the pH in DES-water mixtures was investigated and water-DES mixtures with ChCl:U and ChCl:EG, the pH variations were explained by the influence of the HBDs urea and ethylene glycol, respectively. However, no such correlation could be detected for ChCl:Gly-water mixtures, and pH variations did not coincide with variations induced by the HBA or HBD alone.

Since DESs are often composed of hygroscopic starting materials, the water absorption of several DESs was studied under different storage conditions. ChCl:U, ChCl:Gly and ChCl:EG were characterised as strongly hygroscopic DESs absorbing up to 15 wt% of water, when these DESs were stored openly at ambient conditions. Hydrophobic TbACl-based DESs (TbACl:capric acid, TbACl:1-hexanol, TbACl:1-octanol, TbACl:1-decanol) only absorbed up to 2.5 wt% under the same conditions.

The water content of a solvent is a relevant parameter for enzyme catalysis particularly in non-aqueous reactions media, such as hydrophobic DESs. The maximum water content in hydrophobic (–)-menthol:caprylic acid, (–)-menthol:capric acid and (–)-menthol:lauric acid DESs was determined at 2.6, 2.1 and 1.9 wt%, respectively. The water saturation content was observed to increase with an increasing HBD carbon chain length of the fatty acid. The density of these (–)-menthol-based DESs was measured and is generally lower than the density of water. In order to adjust different water contents in a DES, gas phase equilibration with saturated salt solutions was used. The water uptake of (–)-menthol:lauric aicd DES followed a linear sorption isotherm upon equilibration with different saturated salt solutions of defined a_w. Henry's constant (H_{sorp}) was 13.995 $\mathrm{mg_{water}\,g_{DES}^{-1}}$ for the absorption of water in the (–)-menthol:lauric acid DES at 35 °C.

In terms of applying DESs as enzyme reaction media, the lipase (PPL) catalysed aldol reaction between 4-NBA and acetone was studied exemplarily in DESs. With hydrophobic DESs (ToABr:1,5-pentanediol, ToABr:EG, TbACl:4-NBA) higher substrate concentrations, and thus higher aldol product concentrations were achieved than with ChCl:Gly. The preferred product in ToABr:1,5-pentanediol and TbACl:4-NBA was the aldol, whereas the reaction was less specific in ChCl:Gly and ToABr:EG, which promoted the secondary aldol condensation step to a higher extent. The enzymatic transesterification of 1-propanol and vinyl laurate was explored as another exemplary reaction in DESs composed of TbACl:alcohol DESs (1-hexanol, 1-octanol, 1-decanol used as HBDs). However, the DESs interfered with NBD-H fluorescence dye to measure the course of the reaction. Beyond that, the DESs were not considered as suitable solvents for the targeted reaction system due to possible side reactions with the alcohol HBDs of the DES.

If DESs are composed of potentially competing reactants, this drawback could also be turned into an advantage by designing the DES to comprise the targeted reactant as HBD or HBA compound. In this context, (–)-menthol:fatty acid DESs were regarded as efficient 2-in-1 reaction media for the lipase catalysed esterification of the DES compounds, as the substrates essentially form the solvent for the reaction.

CRL was suited to synthesize (–)-menthyl fatty acid esters in (–)-menthol:caprylic acid, (–)-menthol:capric acid and (–)-menthol:lauric acid DESs without the need to add buffers or another solvent.

It was observed that controlling the a_w of the (–)-menthol:lauric acid (3:1 mol/mol) DES reaction system was more efficient than performing the esterification in binary DES-water reaction systems or in neat DES. With the continuous control of the a_w, the batch productivity for (–)-menthyl laurate reached $287\,\mathrm{g\,L^{-1}\,d^{-1}}$ and a fatty acid conversion of 65 % was achieved. Moreover, the esterification of (–)-menthol:lauric acid DES was optimised in terms of a_w, temperature and enzyme amount by a central composite statistical design of experiments. The ester synthesis rate was maximised at $a_w = 0.55$, $m_{CRL} = 60\,\mathrm{mg}$ and $T = 45\,^{\circ}\mathrm{C}$ to reach $174\,\mathrm{mM\,h^{-1}}$. An ester concentration of $1.36 \pm 0.04\,\mathrm{M}$ (2.25 d) was achieved and the ester productivity reached $443\,\mathrm{g\,L^{-1}\,d^{-1}}$ at 95 % (24 h) conversion. The eutectic (–)-menthol:lauric acid (3:1 mol/mol) composition was altered for the esterification reaction to reduce the excess of (–)-menthol, which also increased the ester formation velocity (e.g. $236\,\mathrm{mM\,h^{-1}}$ at 3:1 mol/mol vs. $368\,\mathrm{mM\,h^{-1}}$ at 1.5:1 mol/mol). CRL did not catalyse the esterification in (+)-menthol:lauric acid 3:1 mol/mol DES, and the resolution of (±)-menthol could be an interesting future application.

In order to demonstrate the separation of the ester product and to increase the overall process efficiency, a recycling step was developed to recover the excess substrate (–)-menthol from a reaction mixture starting with the (–)-menthol:lauric acid (3:1 mol/mol) DES mixture. First, the CRL catalysed esterification reaction was optimised to achieve full conversion in terms of lauric acid (3 d, 35 °C, a_w of 0.16). Second, surplus (–)-menthol was recovered by a vacuum distillation step to separate (–)-menthol (overhead product) from the ester (bottom product). The ester product (735 mg, 94 % purity) and pure (–)-menthol (622.5 mg, 4 mmol) were obtained. (–)-Menthol was reused to prepare new (–)-menthol:lauric acid (3:1 mol/mol) DES and a second esterification step with fresh CRL was successfully performed yielding more than 98.7 % lauric acid conversion.

A Supplementary information

A.1 Rheological characterisation of ChCl:U

A rheometer (MCR 302 Anton Paar) was equipped with a plate-plate (PP50, $D = 50\,\text{mm}$) or cone-plate (CP50, $D = 50\,\text{mm}$) measuring system and a temperature control device (P-PTD200/AIR). The gap width was adjusted to 0.25 mm, if not stated differently. The measuring system was covered with a mountable hood (H-PTD200) to enable measurements at controlled temperature and dry atmosphere. This was important as the studied ChCl:U 1:2 mol/mol DES (ChCl:U) was extremely hygroscopic and rapidly absorbed water through air moisture. Initially, an amplitude sweep of ChCl:U was performed at 18 °C to determine the linear viscoelastic deformation range. The angular frequency was kept constant at 10 $\text{rad}\,\text{s}^{-1}$ with a logarithmic increasing deformation from 0.01 to 100 %. The storage modulus (G'), which describes the viscous (liquid-like) properties, and the loss modulus (G''), which defines the elastic (solid-like) properties of a material were measured with an oscillatory amplitude sweep. As expected, the viscous (i.e. liquid) properties were predominant in ChCl:U and the DES was fully liquid at the amplitude test temperature of 18 °C. This was reflected by the G'' dominating over the storage modulus G' in the entire deformation range (see Figure A.1).

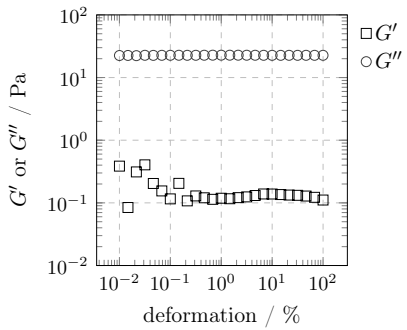
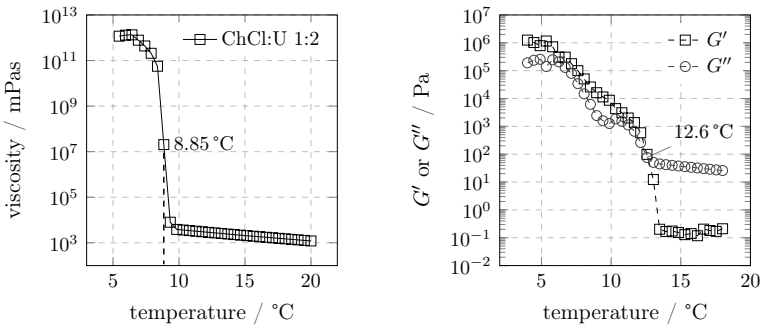


Figure A.1: Amplitude sweep of ChCl:U 1:2 mol/mol at 18 °C showing the storage (G') and loss modulus (G'') of the DES (CP50, 0.1 mm gap width, $\omega = 10\,\text{rad}\,\text{s}^{-1}$).

It was furthermore observed that $G'' \gg G'$, which is typical for ideal viscous fluids.

Although ChCl:U is a composite solvent synthesized from two solids, the liquid DES can be regarded as an ideal fluid. The linear viscoelastic region of ChCl:U stretches across the entire deformation range (linearity of G' and G''). This means that any deformation from 0.01 to 100 % can be selected for further oscillatory experiments without any risk of irreversibly changing the molecular structure of the DES.

Crystallisation experiments were performed both in rotational and oscillatory mode to investigate the freezing/melting behaviour of the ChCl:U. A cooling rate of 0.1 °C min^{-1} from 20 to 5 °C was applied. Upon cooling, the DES starts to from solid crystals (i.e. crystallisation of ChCl and U) until the liquid becomes completely solid. Since the DES volume changes during cooling, the gap width was controlled automatically by the rheometer through setting the normal force to 0 ($F_N = 0$ N). For rotational experiments, the shear rate ($\dot{\gamma}$) was kept constant at 10 s^{-1}. For oscillatory experiments, the deformation (γ) was kept constant at 0.1 % at a constant frequency of 1 Hz.



(a) rotational experiment (b) oscillatory experiment

Figure A.2: Determination of freezing temperature of ChCl:U 1:2 mol/mol at a cooling rate of 0.1 °C min^{-1} using a PP50 measuring system.

The reported freezing point of the ChCl:U 1:2 mol/mol occurs at 12 °C [31]. This was approximated by the rotational experiment, where a strong increase of the viscosity was observed at about 9 °C (see Figure A.2a). With the oscillatory exper-

iment, the change in the DES structure from liquid ($G'' > G'$) to solid ($G' > G''$) was marked by the intersection of G' and G'' at about 12.6 °C (see Figure A.2b). This comes very close to the reported freezing point of the ChCl:U DES mixture.

The time- and temperature-dependent behaviour of ChCl:U was further characterised to simulate cooling and remelting of the DES (e.g. storage in the fridge and remelting at higher temperature). The DES was first cooled in the rheometer from 18 to 4 °C at a cooling rate of 0.1 °C min^{-1}. Then the DES was solidified completely at 4 °C. After 120 min at 4 °C, ChCl:U was eventually heated to 80 °C at a rate of 0.5 °C min^{-1}. During the initial cooling phase G'' (i.e. liquid behaviour) dominated up to 10 °C (see Figure A.3). When the temperature was held at 4 °C, the DES became completely solid after 180 min, which was indicated by a dominating G' in this phase (see Figure A.3). Upon heating, ChCl:U became liquid again ($G'' > G'$) after 310 min at a temperature of approximately 27 °C (see Figure A.3). The results confirm that the crystallisation of ChCl:U is reversible and that the DES can be remelted by appropriate heating within a relatively short period of time.

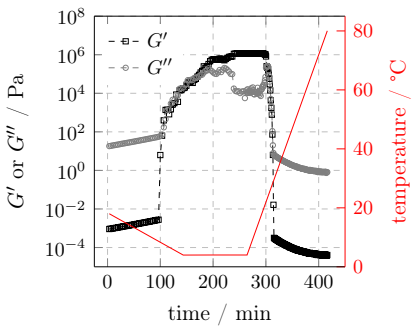


Figure A.3: Temperature- and time-dependent behaviour of ChCl:U 1:2 mol/mol (PP50, 0.5 mm gap width, $F_N = 0$ N, $\gamma = 0.1$ %, $\omega = 10$ rad s^{-1}).

A.2 Lipase characterisation

The lipase from *Candida rugosa* was obtained from Sigma-Aldrich (Schnelldorf, Germany) as the commercially available product '*Candida rugosa* lipase Type

VII' with a unit specification of $\geq$700 U mg^{-1}_{solid}, which refers to the hydrolytic acitivity (1 U releases one microequivalent of fatty acid from a trigylceride at pH 7.2, 37 °C within 1 h)[8]. For this study, the hydrolytic activity of CRL was assessed by the cleavage of a *p*-nitrophenyl ester in 50 mM Tris-HCl buffer at pH 8 and 35 °C (see chapter B.3.6). Using this assay, CRL had an activity of 2 U mg^{-1}_{solid} (1 U = 1 µmol$_{pNP}$ min^{-1}). Since only little was known about the composition of the lipase powder, except that it was free of protease and α-amylase[8], the protein content of the powder and the molecular weight of the enzyme was measured. In order to characterise the commercial CRL powder, the protein content was determined by a BCA assay (see chapter B.3.7). The protein content of the CRL powder was 13 wt%. The CRL solutions were also spiked with a known amount of BSA to exclude that any of the unknown compounds of the powder interfere with the assay. The recovery of the BSA spiked CRL sample was 102 %, which might be explained by a varying assay response towards different proteins (different amino acid sequence, pI, structure etc.). Thus, it is unlikely that unknown powder components would interfere with measuring the protein content. This was also confirmed for other lipase powders (i.e. BCL, PCL and PFL), for which recoveries of more than 97 % were observed (see Table A.1).

Table A.1: Recovery of protein in lipase samples spiked with a known amount of BSA.

Lipase	Recovery %
CRL	102
BCL	97
PFL	97
PCL	99

The information on the protein content of the lipases powders allowed for the calculation of the specific hydrolytic activity related to the amount of protein. CRL exhibited the lowest specific activity in terms of U $g^{-1}_{protein}$ compared to the

[8]CRL specification sheet
Available on: `www.sigmaaldrich.com/catalog/DataSheetPage.do?brandKey=SIGMA&symbol=L1754`

other lipase powders used for enzyme screening experiments (see Figure A.4).

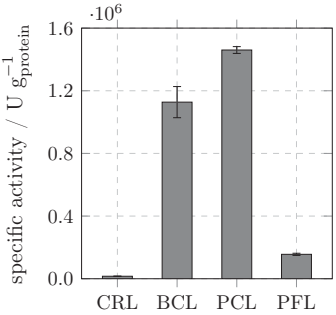


Figure A.4: Specific activity per amount of protein in different lipases powders. ($1\,\mathrm{U} = 1\,\mu\mathrm{mol_{pNP}}\,\mathrm{min^{-1}}$ in 50 mM Tris-HCl buffer at pH 8 and 35 °C)

Moreover, SDS-PAGE was performed to determine the molecular weight of CRL. One band with a molecular weight just below 70 kDa was obtained (see Figure A.5). This coincided with a reported molecular weight of 60 kDa determined by SDS-PAGE for a commercial CRL consisting of two active Lip3 isoforms [186].

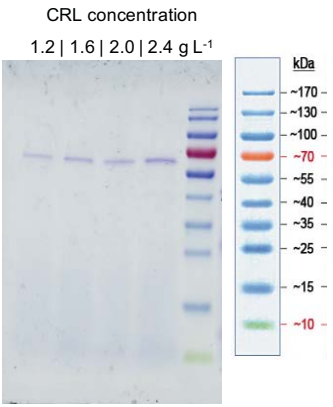


Figure A.5: SDS-PAGE of CRL at various concentrations.

A.3 Identification of menthyl esters by LC-MS

The product peaks of the lipase catalysed and DES-based (–)-menthyl fatty acid ester formation (i.e. (–)-menthyl caprylic acid ester, (–)-menthyl capric acid ester and (–)-menthyl lauric acid ester) were identified by APCI-LC-MS (APCI, atmospheric pressure chemical ionisation). The stationary phase was a C8 column (Phenomenex C8(2) Luna 5 µm, 100 Å, 150×4.6 mm). Acetonitrile and water containing 0.05 % formic acid were used as mobile phases (MS grade solvents). The elution conditions were as follows: hold 65 % acetonitrile for 14 min, increase to 95 % acetonitrile within 0.5 min and hold for 10.5 min, decrease to 65 % acetonitrile within 0.5 min and hold for 9.5 min. The total flow rate was 0.75 mL min^{-1} and the column oven temperature was set to 30 °C. The APCI ion source was operated in positive mode, where the analytes are protonated to produce $[M + H]^+$ molecules. Mass-to-charge (m/z) ratios from 250 to 380 were scanned to detect the esters with expected m/z ratios of 283, 311 and 339 for (–)-menthyl caprylate, caprate and laurate, respectively. Table A.2 shows the retention times and corresponding m/z ratios of peaks that were found to increase for reaction samples, containing the esters, in comparison to blank samples without any esters present. The protonated ester ions were detected in all samples, but also other m/z ratios were found, which could be caused by thermal degradation of the esters in the ionisation process (see Table A.2). The ester ions occurred in all three DES reaction systems with or without the addition of water, which confirmed the CRL catalysed ester formation in the DESs.

Table A.2: Detected m/z ratios and possible ions from CRL catalysed esterification in (–)-menthol:caprylic acid, (–)-menthol:capric acid and (–)-menthol:lauric acid DESs.

	Exact mass[a] *u*	Retention time *min*	Detected m/z values	Possible corresponding ion
(–)-Menthyl caprylate	282.25588	16.9	299	–
		19.4	nd[b]	–
		19.9	281	–

Continued on next page

Table A.2 – *(Continued)*

	Exact mass[a]	Retention time	Detected m/z values	Possible corresponding ion
	u	*min*		
		20.6	338	–
		20.8 - 21.3	283	$[M+H]^+$
(–)-Menthyl caprate	310.28718	18.1	327	–
		21.6	309	–
		22.9 - 23.6	311	–
		24.6	nd[b], 311, 325	$[M+H]^+$
(–)-Menthyl laurate	338.31848	19.0	nd[b], 353	–
		20.7	338	–
		22.9	337	–
		23.9 - 24.4	337	–
		25.9 - 26.8	339	$[M+H]^+$

[a] Data from `https://www.sisweb.com/referenc/tools/exactmass.htm` (accessed on 01/12/2017).

[b] No distinct m/z was readable from the mass spectrum.

A.4 Esterification at controlled water activity

The water content in the DES was regulated by gas phase pre-equilibration of the DES and CRL powder with different saturated salt solutions. Due to the presence of the saturated salt solutions, kept in a separated compartment, the water content of the DES was also controlled during the esterification reactions. A reference reaction was performed in neat (–)-menthol:lauric acid DES without controlling the water content. Therefore, the water content in the neat DES gradually increased with ongoing esterification, as water is formed as a byproduct. Lauric acid was depleted and ester formation was observed in both the a_w controlled reactions and in the neat DES, whereas no conversion or product formation was observed under dry reaction conditions, where the DES was surrounded by a mixture of molecular sieve and silica gel in a separate compartment (see Figure A.6).

Additionally, the effect of equilibrating the enzyme powder over different saturated

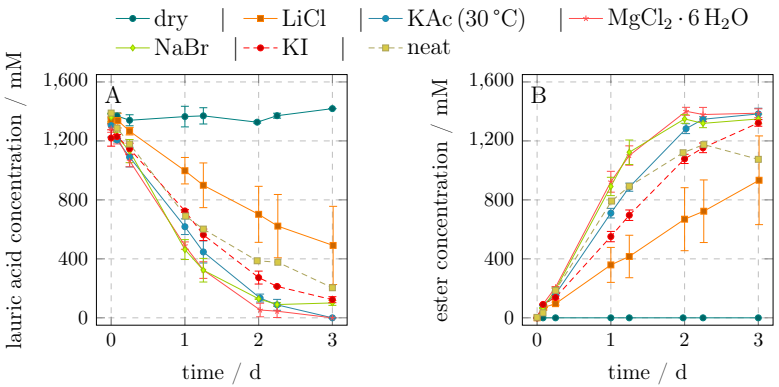


Figure A.6: Time course of lauric acid (A) and ester (B) concentrations in the presence of different saturated salt solutions, in neat DES or under dry conditions in the presence of molecular sieves and silica. Reaction conditions: $m_{DES} = 2\,\mathrm{g}$, 20 mg CRL, 300 rpm, $T = 35\,^{\circ}\mathrm{C}$, $n = 3$.

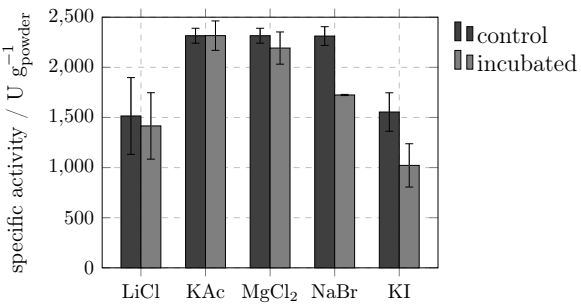


Figure A.7: CRL activity after equilibration (35 °C, 3 d) over different saturated salt solutions in comparison to non-treated controls (1 U = 1 µmol$_{\mathrm{pNP}}$ min^{-1} at 35 °C and pH 8).

salt solutions was investigated to make sure that the water content of the reaction mixture (DES+CRL) is constant at the beginning of the esterification. A hydrolysis lipase activity assay was performed before and after gas phase equilibration of the CRL powder for three days at 35 °C. The CRL activity after the incubation was compared to a control sample, which was not treated. A loss of activity was observed with an increasing a_w, especially for NaBr (a_w = 0.55, 35 °C) and KI (a_w = 0.67, 35 °C) saturated salt solutions (see Figure A.7). In contrast to that, low a_w values, adjusted with LiCl (a_w = 0.11, 35 °C), KAc (a_w = 0.2, 35 °C) or $MgCl_2 \cdot 6\,H_2O$ (a_w = 0.32, 35 °C) saturated salt solutions, did not impair the enzyme activity.

A.5 Reaction optimisation - CCD experimental plan

A three factorial central composite design (CCD) was created with Design-Expert8 software (Stat-Ease, Inc.). The a_w was adjusted by different saturated salt solutions with known a_w values [146]. Since the a_w depends on the type of salt and temperature, it cannot varied gradually. In some cases the a_w calculated by Design-Expert could not be matched with the a_w of the selected salt at a given temperature. If it was not possible to adjust the a_w to the value required by the experimental plan, the published a_w values were plotted over temperature and a curve was fitted using the software Origin 2015G (instrumental weighing). The a_w was then calculated based on the obtained function or if necessary, by extrapolation. An example for the a_w calculation method is given for NaI at 32.5 °C. With the equation obtained by fitting the literature data (see Figure A.8), a a_w (y) of 0.36 was calculated at a temperature (x) of 32.5 °C. Fit functions, which were obtained for all salts, are listed in Table A.3.

The a_w levels output by Design-Expert are listed in Table A.4, column 'a_w by CCD plan'. If the a_w was not available from the literature at the temperature given by the experimental plan, the a_w values were replaced by the calculated values to match the temperature levels (see Table A.4, column: 'a_w for modelling'). These values were used for building the model in terms of optimising the product formation rate (see chapter 5.4.4, 5.1). In total, the design comprises 20 experimental runs

including six center points, which are repetitions of the same experiment under identical conditions (runs 1, 4, 8, 9, 11 and 13). The design boundaries were chosen based on the experiences from previous experiments.

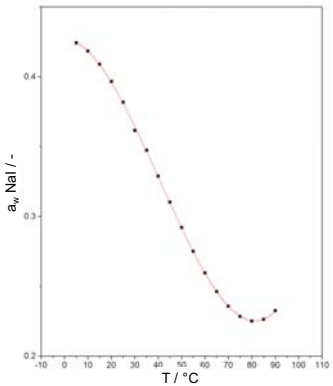


Figure A.8: Literature data for temperature and a_w retrieved from [146] and plotted to enable calculation of a_w at 32.5 °C by cubic fit function (—).

Table A.3: Equations obtained by plotting and fitting temperatur-a_w data from [146]. x and y denote temperature in °C and a_w, respectively.

Salt	Fit	Equation
LiBr	quadratic	$y = 0.078 - 6.7 \cdot 10^{-4}x + 4.36 \cdot 10^{-6}x^2$
NaI	cubic	$y = 0.43 - 2.57 \cdot 10^{-5}x - 9.1 \cdot 10^{-5}x^2 + 7.4 \cdot 10^{-7}x^3$
K_2CO_3	linear	$y = 0.43 + 1.43 \cdot 10^{-5}x$
NaCl	cubic	$y = 0.76 + 3.96 \cdot 10^{-4}x - 2.65 \cdot 10^{-5}x^2 + 2.84 \cdot 10^{-7}x^3$

Table A.4: CCD experimental plan for optimisation of the esterification in (–)-menthol:lauric acid 3:1 mol/mol DES by factors (FAC) water activity (a_w), temperatue (T) and enzyme amount (m_{CRL}). Model responses (RESP) were conversion after 1 d and product formation rate.

Run	**Salt**	**FAC 1**		**FAC 2**	**FAC 3**	**RESP 1**	**RESP 2**
		a_w by CCD plan	a_w for modelling	m_{CRL}	T	$Conversion_{1d}$	Product formation rate
				mg	*°C*	*%*	mMh^{-1}
1	NaI	0.39	0.36[a]	36	32.5	94.3	78.18
2	NaCl	0.76	0.75[a]	36	32.5	38.3	50.91
3	KI	0.67	0.65[b]	12	45	65.6	35.28
4	NaI	0.39	0.36[a]	36	32.5	91.5	78.15
5	KI	0.67	0.70[b]	60	20	45.7	77.55
6	LiCl	0.11	0.11[b]	12	45	0	10.05
7	LiBr	0.02	0.06[a]	36	32.5	0	0
8	NaI	0.39	0.36[a]	36	32.5	95.7	86.27
9	NaI	0.39	0.36[a]	36	32.5	95.3	95.90
10	KI	0.67	0.65[b]	60	45	91.6	138.92
11	NaI	0.39	0.36[a]	36	32.5	95.1	85.70
12	LiCl	0.11	0.11[b]	60	20	84.7	44.80
13	NaI	0.39	0.36[a]	36	32.5	95.2	73.77
14	KI	0.67	0.70[b]	12	20	23.7	16.07
15	LiCl	0.11	0.11[b]	12	20	14.7	9.80
16	NaI	0.39	0.36[a]	4.41	32.5	17.3	11.05
17	K_2CO_3	0.39	0.43[a]	36	49	94.6	150.63
18	NaI	0.39	0.41[a]	36	16	59.6	19.46
19	LiCl	0.11	0.11[b]	60	45	53.8	24.64
20	NaI	0.39	0.36[a]	67.6	32.5	95.7	122.14

[a] a_w was calculated based on a fit function or extrapolation of a fit function
[b] a_w was taken from literature [146]

B Materials and methods

B.1 Materials

Chemicals

The following tables provide an overview on the chemicals used for the preparation of DESs (see Table B.1), the experimental reagents (see Table B.2), solvents (see Table B.3) and lipases (see Table B.4).

Table B.1: Chemicals used for the preparation of DESs.

Compound	Quality/purity	Manufacturer/supplier
Choline chloride	$\geq$ 98 %	AppliChem GmbH, Darmstadt (Germany)
Urea	98 %, reagent grade	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
Glycerol	$\geq$ 99.5 %, p.a., anhydrous	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Ethylene glycol	99.7 %	Fluka Chemie AG, Buchs (Switzerland)
1,5-Pentanediol	96 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
1-Hexanol	99 %	Alfa Aesar/Thermo Fisher, Kandel (Germany)
1-Octanol	$\geq$ 99 %	Merck KGaA, Darmstadt (Germany)
1-Decanol	$\geq$ 99 %	Merck KGaA, Darmstadt (Germany)
Tetra-n-butylammonium chloride (TbACl)	$\geq$ 95 %	Merck KGaA, Darmstadt (Germany)
Tetra-n-octylammonium bromide (ToABr)	$\geq$ 95 %	Merck KGaA, Darmstadt (Germany)

Table B.2: Reagents.

Compound	Quality/purity	Manufacturer/supplier
n-Butylamine	≥ 99 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
Capric acid	≥ 98 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
Caprylic acid	≥ 99 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
(±)-β-Citronellol	90-95 %	Fluka/Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
Ethyl valerate	≥ 98 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
Geraniol	96 %	Fluka/Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
4-Hydrazino-7-nitro-2,1,3-benzoxadiazole (NBD-H)	suited for fluorescence	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
KF titrating agent	Hydranal™ Composite 1	Honeywell Specialty Chemicals GmbH, Sleeze (Germany)
Lauric acid	≥ 97.5 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
(–)-Menthol	≥ 98.5 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
(±)-Menthol	≥ 99 %	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Methanol Rapid	Hydranal™	Honeywell Specialty Chemicals GmbH, Sleeze (Germany)
4-Nitrobenz-aldehyde (4-NBA)	99 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
p-Nitrophenol palmitate (*p*NPP)	≥ 98 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)

Continued on next page

Table B.2 – *(Continued)*

Compound	Quality/purity	Manufacturer/supplier
Phenylmethylsulfonyl fluoride (PMSF)	$\geq$ 98.5 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)
Vinyl laurate	$\geq$ 99 %	Sigma-Aldrich Chemie GmbH, Steinheim (Germany)

Table B.3: Solvents used for analyses/reaction media.

Solvent	Quality/purity	Manufacturer/supplier
Acetone	$\geq$ 99.9 %, Rotisolv® HPLC	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Acetonitrile	$\geq$ 99.9 %, gradient grade for HPLC	VWR International GmbH, Darmstadt (Germany)
Ethanol	$\geq$ 99.9 %, HPLC gradient grade	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Ethyl acetate	$\geq$ 99.5 %, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
1-Propanol	$\geq$ 99.5 %, Rotisolv® HPLC	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
i-Propanol	$\geq$ 99.8 %, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Toluene	$\geq$ 99.5 %, for synthesis	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)

Table B.4: Immobilised lipases and lipase powders.

Lipase	Abbreviation	Type	Supplier units
Candida antarctica lipase B	CALB	immobilised	$8100\,\mathrm{LU\,g^{-1}}$
Candida antarctica lipase B	N435	immobilised, lipase arcrylic resin	$\geq 5000\,\mathrm{U\,g^{-1}}$
Candida rugosa lipase	CRL	type VII	$\geq 700\,\mathrm{U\,mg^{-1}}$
Porcine pancreas lipase	PPL	type II	$100-500\,\mathrm{U\,mg^{-1}}$
Burkholderia cepacia lipase	BCL	Amano lipase PS	$\geq 30\,000\,\mathrm{U\,g^{-1}}$
Pseudomonas cepacia[a] lipase	PCL	–	$\geq 30\,\mathrm{U\,mg^{-1}}$
Pseudomonas fluorescence lipase	PFL	Amano	$\geq 20\,000\,\mathrm{U\,g^{-1}}$

[a] Strain is today renamed as *Burkholderia cepacia*

All lipases were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), except for CALB, which was obtained from c-LEcta GmbH (Leipzig, Germany).

Salts, which were used for a_w experiments (see Table B.7), were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Alfa Aesar/Thermo Fisher (Kandel) GmbH (Kandel, Germany) or Merck KGaA (Darmstadt, Germany).

Some reference materials were custom synthesized for analytical purposes. (–)-Menthyl laurate was gratifyingly synthesized by the Institute of Applied Synthetic Chemistry (research group of Prof. Marko D. Mihovilovic, Vienna University of Technology, Austria). The aldol product [4-hydroxy-4-(4-nitrophenyl)butan-2-on] and its olefinic condensation product [4-(4-nitrophenyl)butan-3-en-2-on] were grat-

ifyingly synthesized by the Deska Synthetic Biocatalysis Research Group (research group of Prof. Jan Deska, Aalto University, Espoo, Finland).

Laboratory equipment
The laboratory equipment, which was used for this study, is listed in the following table (see Table B.5).

Table B.5: Laboratory equipement.

Device	Type	Manufacturer/Supplier
Analytical balance	Extend ED224S	Sartorius AG, Göttingen (Germany)
Centrifuge	MiniSpin® Plus	Eppendorf AG, Hamburg (Germany)
Centrifuge	5415R	Eppendorf AG, Hamburg (Germany)
Gas chromatograph	GC-17A	Shimadzu Deutschland GmbH, Duisburg (Germany)
Gel electrophoresis	Mini-PROTEAN® 3 electrophoresis system	Bio-Rad Laboratories GmbH, München (Germany)
HPLC [a]	Prominence	Shimadzu Deutschland GmbH, Duisburg (Germany)
Incubator	Certomat HK®	Sartorius Stedim Biotech GmbH, Göttingen (Germany)
Incubator	Ecotron	Infors AG, Bottmingen (Switzerland)
Magnetic stirrer	Multistirrer Digital 6	Velp Scientifica, Usmate (Italy)
Magnetic stirrer	C-MAG HS 7	IKA®-Werke GmbH & Co. KG, Staufen (Germany)

Continued on next page

Table B.5 – *(Continued)*

Device	Type	Manufacturer/Supplier
pH-metre	FiveEasy™ Plus	Mettler Toledo, Gießen (Germany)
Pipettes	5 ml, 1000, 200, 100, 20, 10 and 2.5 µl	Eppendorf AG, Hamburg (Germany)
Plate reader	infinite® 200	Tecan Group Ltd., Männedorf (Switzerland)
Rheometer	MCR 302	Anton Paar GmbH, Graz (Austria)
Thermal mixer	BioShake iQ	Quantfoil Instruments GmbH, Jena (Germany)
Thermostat	AD07R-20	VWR International GmbH, Darmstadt (Germany)
Vacuum drying oven	Heraeus® VT6025	Thermo Electron LED GmbH, Langenselbold (Germany)
Vacuum pump	Laboport® mini laboratory pumps	KNF Neuberger GmbH, Freiburg (Germany)

[a] HPLC components were: degasser unit (DGU-20A3), two pumps (LC-20AT), autosampler (SIL-20AC), column oven (CTO-20AC), system controller (CBM-20A), PDA detector (SPD-M20A), RID detector (RID-10A) and ELSD detector (ELSD-LTII).

B.2 Enzymatic syntheses and product purification

B.2.1 Aminolysis

The lipase catalysed aminolysis of ethyl valerate with 1-butylamine was studied in different ChCl-based DESs (HBDs: glycerol, urea or acetamide; ChCl:HBD 1:2 mol/mol). The DESs were compared to toluene as a reference organic solvent for the aminolysis reaction. The reactions were performed in an incubator at 60 °C and 200 rpm orbital shaking (Ecotron, Infors HT). To 1 g of DES 111 µmol 1-butylamine and 100 µmol ethyl valerate were added corresponding to 11 and 15 µl, respectively. The reactions were started by the addition of 10 mg of immobilised

CALB (N435) and proceeded for 6 h. An individual reaction sample was prepared for each time point. After the respective reaction time, the DES mixtures were extracted by the addition of 5 ml toluene, briefly vortexed, centrifuged and 1 mL of the toluene phase was used for GC analysis.

B.2.2 Aldol reaction

The aldol reaction was performed according to a modified procedure described in [58]. A typical reaction mixture comprised 30 ml DES containing a substrate mixture of 1 mol 4-NBA and 5 mol acetone. The amount of 4-NBA varied with its maximum solubility in each DES. The substrate 4-NBA was dissolved in the DES at 60 °C. Subsequently, 1.5 ml of water and porcine pancreas lipase (PPL) were added to the reaction mixture. The same amounts of 4-NBA and PPL were used to catalyse the reaction (PPL:4-NBA 1:1 (w/w)). The reaction was started with the addition of acetone and proceeded at 60 °C with constant stirring (400 rpm). The reaction conditions are summarised in Table B.6. Samples (aliquots of approximately 1 ml) were withdrawn in regular time intervals and extracted with water and ethyl acetate. For the first extraction step, 6 ml of water were added and the sample was vigorously mixed to dilute and dissolve the water-soluble DES components. Then, 6 ml of ethyl acetate were added to extract the reactants, the sample was vigorously mixed and centrifuged (3100×g, 3 min) for phase separation. Three aliquots (1 ml) of the organic phase were used to prepare the HPLC samples. Ethyl acetate was evaporated overnight and the sample was re-dissolved with 5 ml of an acetonitrile:water (53:47 (v/v)) mixture. 1 ml of the solution was filtered through a Nylon syringe filter (0.2 µm pore diameter) and analysed by HPLC.

Experiments with inactivated PPL were performed in small scale, involving just 1 ml of the DESs. The substrate ratio (4-NBA:acetone 1:5 mol/mol) and the amount of inactivated enzyme (PPL:4-NBA 1:1 (w/w)) as well as the reaction conditions (T, rpm) were the same as previously described. 50 µl of water were added to the reacion mixtures. PPL was inactivated thermally by autoclaving or heating (95 °C) in 12 M urea. Additionally, the enzyme was inactivated chemically by an the inhibitor phenylmethylsulfonyl fluoride (PMSF). The PPL activity/inactivity was

monitored photometrically by a hydrolysis assay based on *p*-nitrophenyl palmitate.

Table B.6: Reaction parameters of PPL catalysed aldol reaction in 30 mL DES.

DES	PPL	4-NBA	Acetone	Water
	g	*mmol*	*mmol*	*ml*
ChCl:Gly 1:2 mol/mol	1.35	9	45	1.5
ToABr:ethylene glycol 1:3 mol/mol	6	40	200	1.5
ToABr:1,5-pentanediol 1:3 mol/mol	3	20	100	1.5
TbACl:4-NBA 2.2:1.5 mol/mol	7.5	50	250	1.5

B.2.3 Transesterifications

Transesterification of vinyl laurate

The transesterification of vinyl laurate and 1-propanol was studied in different hydrophobic TbACl:alcohol based DESs (HBDs: 1-hexanol, 1-octanol or 1-decanol; TbACl:HBD 1:2 mol/mol). The progress of the reaction was measured by coupling a secondary reaction step involving the transformation of the fluorescence dye 4-hydrazino-7-nitro-2,1,3-benzoxadiazole (NBD-H). The dye reacts with acetaldehyde, released by the transesterification of the vinyl fatty acid, to become fluorescence active. A substrate mix was prepared by mixing the DES with 150 mM vinyl laurate. NBD-H was dissolved in 1-propanol (220 µM). The immobilised lipase (CALB, c-LEcta) was weighed into the reaction vessel and the DES-substrate mixture as well as the NBD-H solution was added in a 2:1 ratio (v/v). The reaction was conducted at 40 °C either in 96-well microtiter plates with 432 rpm orbital shaking or in glass vials with 1300 rpm (magnetic stirrer and waterbath). The fluorescence intensity was measured in a plate reader (infinite 200, Tecan) with an excitation wavelength of 480 nm and an emission wavelength of 545 nm. The plates were sealed with a foil to prevent evaporation.

Transesterification of terpenes

In order to convert geraniol and citronellol to the corresponding geranyl and citronellyl acetate, the compounds were mixed with TbACl in a 1:1 molar ratio and

heated to form 5 g of the respective DES. Different lipases (immobilised CALB c-LEcta or N435, and PPL powder) were investigated as potential catalysts for the transesterification of the terpene alcohols with vinyl acetate. To the DES mixtures 50 mg of the lipase and 500 mg vinyl acetate were added and the transesterification was performed at 50 °C under continuous stirring (400 rpm). The reactions were stopped after 20 h and the mixture was extracted twice with 5 ml ethyl acetate. The organic phases were combined and washed with 40 ml deionised water to remove the residual TbACl salt. After centrifugation (1200×g, 1 min, 20 °C) the organic layer was transferred into another centrifugation tube and washed again with 15 ml water. This procedure was repeated twice to obtain ammonium salt-free extracts.a The extracts were filtered (Nylon syringe filters, 0.2 µm pore diameter) and diluted 1:20 with ethyl acetate prior to GC analysis.

B.2.4 Esterification of menthol

Screenings

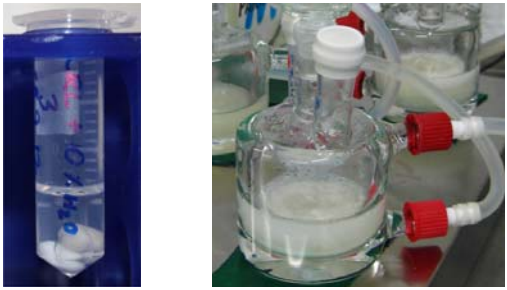
Six different lipases were screened for the synthesis of (–)-menthyl esters, when the DES acts as substrate and solvent simultaneously. Two immobilised lipases, N435 and c-LEcta CALB, and four powdered lipases, CRL, BCL, PCL and PFL, were used to produce (–)-menthyl caprylate, (–)-menthyl caprate, (–)-menthyl laurate, (–)-menthyl L-lactate, (–)-menthyl DL-lactate and (–)-menthyl acetate in the respective 2-in-1 DESs. 0.5 g of each DES was weighed into a 2 ml reaction tube containing a stirring rod and the reaction was started by the addition of 5 mg immobilised beads or powder of the respective enzyme (i.e. for screening reasons the lipases were simply used in equal amounts). A negative control was prepared for each DES containing no enzyme. The reactions in (–)-menthol:organic acid DESs were allowed to proceed for 24 h, whereas esterifications in (–)-menthol:fatty acid DESs proceeded for 120 h. The reactions were performed at 35 °C under constant stirring (400 rpm). Before the addition of the lipases and after the respective reaction time, 10 µl of the reaction mixtures were diluted with 990 µl ethanol for analysis. If necessary, the samples were centrifuged (14 100×g, 2 min) to separate the enzyme from the DES phase. Samples from (–)-menthol:organic acid DESs were analysed by GC and samples from (–)-menthol:fatty acid DESs were analysed

by HPLC.

For the lipase catalysed esterification of (–)-menthol and benzoic acid, a 2-in-1 DES was prepared by mixing and heating (55 °C, 30 min, 1400 rpm) a 2:1 molar mixture of both constituents in a thermal shaker (BioShake iQ, QInstruments). The reaction was then started by the addition of 5 mg immobilised CALB (c-LEcta). The reaction was allowed to proceed in the thermal shaker at 55 °C and 1000 rpm for 15 h. Before the reaction was started and after 15 h, 10 µl of the reaction mixture were combined with 990 µl EtOH (dilution factor 100) and analysed by means of GC.

Esterification with water addition
The effect of water addition to the (–)-menthol:fatty acid DES reaction systems was studied in a small scale set-up (0.5 g DES) and in large scale glass reactors (25 g DES), as depicted by Figure B.1. For the small scale reactions, 5 mg CRL powder was weighed into 2 ml reaction tubes and either no (+ 0 wt% H_2O, i.e. neat DES) or different amounts of water were added (+ 1, 5 or 10 wt% H_2O, percentage referring to DES weight). Negative controls containing no enzyme were prepared for each DES and water amount. The reaction was started by the addition of the 0.5 g DES. For temperature control the samples were placed in an incubator with temperature set to 35 °C (Certomat® HK, Sartorius). The esterifications were allowed to proceed for a certain time under constant agitation (700 rpm). An increased stirrer speed was selected to mix the DES and aqueous phase well. The samples were centrifuged (16 100×g, 2 min, 22 °C) to withdraw a 10 µl aliquot of the DES phase, which was then diluted in 990 µl EtOH for HPLC analysis.

The esterification of (–)-menthol with lauric acid was studied in a larger scale (25 g DES), using double-walled glass reactors, which were coupled to a heating circuit with a set temperature of 35 °C. The esterifications were performed in neat (–)-menthol:lauric acid DES (+ 0 wt% H_2O) and in biphasic reaction mixtures with 20 or 50 wt% of water added (percentage referring to the DES weight). The reaction mixtures were incubated for 30 min at 35 °C to reach the reaction temperature. The reactions were started by the addition of 250 mg CRL powder.



(a) Small scale, 0.5 g DES (b) Large scale, 25 g DES

Figure B.1: Experimental set-up for small scale screenings (a) and large scale esterifications (b) in biphasic DES-water reaction mixtures.

Samples (0.5 ml) were taken in regular intervals. The enzyme powder and water were separated from the DES by centrifugation (16 100×g, 2 min, 22 °C) and 10 µl of the DES was diluted in EtOH (factor 100) for HPLC analysis.

Esterification at controlled a_w
In order to adjust the water content of the (–)-menthol:lauric acid DES to a defined water content, the DES was incubated in the presence of saturated salt solutions via gas phase equilibration. Table B.7 gives an overview on the a_w of selected salt solutions at defined temperatures.

Table B.7: Water activity of selected saturated salt solutions at defined temperatures. [146]

Salt	a_w [–]	T °C
LiBr	0.06	35
LiCl	0.11	35
KAc	0.22	30
$MgCl_2 \cdot 6\,H_2O$	0.32	35
NaI	0.35	35

Continued on next page

Table B.7 – *(Continued)*

Salt	a_w [–]	T °C
K_2CO_3	0.43	30
NaBr	0.55	35
KI	0.67	35
NaCl	0.75	35
KCl	0.83	35
KNO_3	0.91	35
K_2SO_4	0.97	35

Since the a_w is a temperature-dependent parameter, temperature control is important for a suitable experimental set-up. This was achieved by putting the reaction vessels, which had an outer jacket connected to a heating circuit, additionally into an incubating hood to control the gas phase temperature. The a_w screening experiments were conducted without an additional incubating hood (see chapter 5.4.2, Figure 5.9), whereas the CCD experiments were performed with a double temperature control (see chapter 5.4.4, Figure 5.12). Prior to starting the reaction, the DES (2 g) was incubated for 3 d at 35 °C in the presence of different saturated salt solutions. The DES was separated from the saturated salt solutions by an insert glass tube (see Figure 5.12) to achieve gas phase equilibration. The stirring rate was 200 rpm during the DES incubation. Just before the enzyme was added, an initial sample of the DES phase was prepared for HPLC analysis by diluting 10 µl of the DES in 990 µl EtOH. The esterification reactions were started by the addition of 20 mg CRL to the pre-incubated DES, for which the reactor was briefly opened. The mixing rate was increased to 300 rpm to evenly distribute the enzyme powder. A small sample volume (typically 50 µl) was withdrawn in regular intervals via a septum and syringe. The samples were centrifuged (14 100×g, 2 min) to separate the enzyme and the DES phase was diluted in EtOH as previously described.

B.2.5 Distillative product purification

A vacuum distillation step using a micro distillation apparatus was performed to separate unreacted (–)-menthol after the esterification reaction. The cooling temperature of the condenser circuit was set to 40 °C to avoid crystallisation of (–)-menthol, since its melting point at atmospheric pressure is about 41 to 43 °C. Before the distillation was started, a HPLC sample was prepared by diluting the DES in EtOH (dilution factor 100). At a pressure of approximately 3 mbar the temperature of the oil bath was slowly increased up to 210 °C to evaporate (–)-menthol, with a vapor temperature of approximately 72 °C. The overhead product was collected in a round bottom flask, which was kept on ice to crystallise (–)-menthol and therefore minimise further evaporation. The amount of bottom and overhead product was calculated by differential weighing and a sample of both fractions was prepared for HPLC analysis (liquid fraction: 1:100 dilution in EtOH; solid fraction: $14.4\,\mathrm{g\,l^{-1}}$ of the solid in EtOH).

B.3 Analytical Methods

B.3.1 Density and pH measurement

The density of (–)-menthol and (±)-menthol-based fatty acid DESs was determined by a differential weighing method. An empty pipette tip was placed into a weighing boat on an analytical balance and tared to 0. Then a defined DES volume (typically 1 ml) was taken up with the same pipette tip. The tip containing the DES was again placed in the weighing boat on the tared balance and the mass was read. The densities were calculated as the average of at least four measurements according to the following equation:

$$\bar{\rho} = \frac{\bar{m}}{V} \tag{B.1}$$

The pH of ChCl-based DESs mixed with water was measured using a pH meter (FiveEasy Plus™, Mettler Toledo). DES aqueous mixtures ($m_{total} = 5\,\mathrm{g}$) containing 10, 20, 50, 75 or 99 wt% of water were prepared and their pH was measured. Additionally, the same aqueous solutions were prepared with the single DES components and their pH was measured also to determine the influence of the HBA/HBD on

the overall DES pH. The pH of neat DESs was also measured, but was associated with high uncertainty as the electrode could not reach a constant pH value.

B.3.2 Karl-Fischer titration

The water content of the DESs was determined by volumetric Karl-Fischer titration (KF titrator DL38, Mettler Toledo) using a one-component reagent with a titer of 1 mg ml^{-1} (Hydranal®-Composite 1, Fluka). Di-sodium tartrate ($Na_2C_4H_4O_6 \cdot 2\,H_2O$) was used for calibration. An average water content of 15.51 wt% ($n = 4$) of $Na_2C_4H_4O_6 \cdot 2\,H_2O$ was determined by the weight loss of the salt after drying at 150 °C for 4 h.

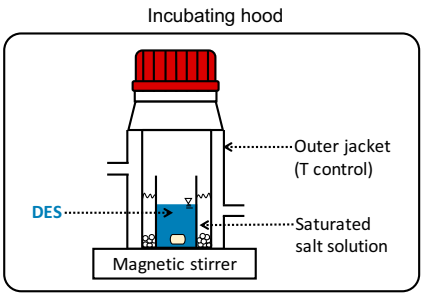


Figure B.2: Experimental set-up for the determination of the water absorption isotherm of (–)-menthol:lauric acid DES.

The a_w of different saturated salt solutions (see Table B.7) was correlated with the water content of (–)-menthol:lauric acid 3:1 mol/mol DES ($m_{total} = 6$ g) after incubation at 300 rpm and 35 °C for 24 h to obtain a water absorption isotherm. In order to dry the DES to achieve $a_w = 0$, a mixture of molecular sieves (0.4 nm) and silica gel was used to surround the DES containing glass insert. The experimental data (average of three water content measurements) was approximated by linear regression with instrumental weighing (QtiPlot software version Qt: 5.9.7). The experimental set-up is shown by Figure B.2. The temperature was controlled by an external heating circuit and by an incubating hood (Certomat® HK, Sartorius) for liquid and gas phase temperature control.

B.3.3 Rheological methods

The rheological characterisation of DESs was performed with a modular rheometer (MCR 302, Anton Paar), which can be equipped with different measuring systems and the corresponding peltier temperature devices (PTD, temperature range from −5 to 200 °C). The configurations are listed in Table B.8. In the plate-plate (PP) and cone-plate configuration (CP), a hood (H-PTD200) can be used to cover the sample enabling measurements at controlled temperature and dry atmosphere. This was especially important to measure hygroscopic ChCl-based DESs.

Table B.8: Configurations of the modular rheometer.

Abbrevation	Measuring system, diamter	Peltier temperature device (PTD)
PP25	Plate-plate, 25 mm	P-PTD200/AIR (+ H-PTD200)
PP50	Plate-plate, 50 mm	P-PTD200/AIR (+ H-PTD200)
CP25	Cone-plate, 25 mm	P-PTD200/AIR (+ H-PTD200)
CP50	Cone-plate, 50 mm	P-PTD200/AIR (+ H-PTD200)
DG26.7	Double-gap, 26.7 mm	C-PTD200
CC27	Concentric cylinders, 27 mm	C-PTD200

Flow curves of the DESs were recorded by rotational experiments at 20 °C. For ChCl:U and ChCl:Gly 1:2 mol/mol DESs, the flow curves were measured using the PP50 measuring system with the temperature and atmosphere control devices (P-PTD200/AIR + H-PTD200). Since ChCl:EG and TbACl-based DESs had a much lower viscosity than the other ChCl-based DESs, the double-gap measuring system was used to measure these DESs in order to prevent the sample from pouring out of the measuring gap. The flow curves of all DESs were measured with a logarithmic decreasing shear rate profile from 1000 to $1\,s^{-1}$ and a corresponding logarithmic data point duration from 3 to 25 s.

The temperature-dependent flow behaviour was measured in controlled shear rate mode at a constant shear rate of $50\,s^{-1}$ with a linearly increasing temperature gradient from 20 to 80 °C wit a heating rate of $1\,°C\,min^{-1}$ and constant data point duration of 1 min. For temperature and atmospheric control, the DESs were mea-

sured using the PP50 and the P-PTD200/AIR and H-PTD200 configuration.

In order to characterise the viscosity as a function of the DES water content, the water-miscible DES (ChCl:U 1:2, ChCl:Gly 1:2, ChCl:EG 1:3 mol/mol) were diluted with a certain amount of water. The DG26.7 measuring system was used for these experiments. The viscosity of each DES-water mixture was retrieved by the slope of a linear fit of the flow curves, which were recorded with a logarithmic decreasing shear rate profile from 1000 to 1 s^{-1} and at 20 °C.

B.3.4 GC analysis

GC analysis for aminolysis

The aminolysis reactants were analysed by GC-FID using a fused silica capillary column (Supelco Equity-5, 30 m; 0.25 mm ID; 0.25 µm thickness). The injector temperature was set to 250 °C and the split ratio was 1:100. The temperature was held for 6 min at 76 °C, then increased to 165 °C with 25 °C min^{-1}. The temperature was further increased to 200 °C with 35 °C min^{-1} and held at 200 °C for 5 min. The FID temperature was set to 275 °C. The retention time for the product butyl valeraic acid amide was typically 10.8 min.

GC analysis for menthol screenings

The gas chromatograph was equipped with a flame ionisation detector (FID) and a high polarity polyethylene glycol capillary column (Agilent, DB-WAXetr, 30 m; 0.25 mm ID; 0.25 µm thickness). The injector temperature was set to 220 °C and the split ratio was 1:20. The temperature was held for 4 min at 60 °C, then increased to 200 °C with 10 °C min^{-1} and held at 200 °C for 12 min. The total program time was 30 min. The injection volume was 1 µl. Substrate peaks were identified by retention times of standard compounds ($t_{R,\ (-)\text{-menthol}}$ = 13.4 min, $t_{R,\ caprylic}$ = 18 min, $t_{R,\ capric\ acid}$ = 20.7 min, $t_{R,\ lauric\ acid}$ = 25.1 min), while potential esters peaks were identified putatively (occurrence of a third peak after 24 h reaction time).

B.3.5 HPLC methods

HPLC-UV/Vis for aldol reactants

The HPLC (Prominence, Shimadzu) was coupled with a photodiode array detector (PDA) for the UV/visible detection of 4-NBA, the aldol product and the condensation product. Acetonitrile was used as mobile phase A and mobile phase B was water. The compounds were separated in an isocratic elution mode with a 53:47 (v/v) mixture of acetonitrile:water. A C18 column (Phenomenex Luna C18(2), 5 µm, 100 Å, 125×4 mm) was used and the total flow rate was 0.5 ml min^{-1}. The detection wavelengths were set to 263 nm, 273 nm and 306 nm corresponding to the absorption maxima of 4-NBA, the aldol product and the olefinic condensation compound, respectively. The oven temperature was set to 40 °C and the injection volume was 10 µl. The total run time was 10 min. Typical retention times were 3.2 min, 4.4 min and 5.2 min for the aldol product, 4-NBA and the condensation product, respectively.

HPLC-RID-ELSD for detection of menthol and fatty acids

Quantitative analysis of fatty acids, (–)-menthol and (–)-menthyl fatty acid esters was carried out by means of HPLC analysis. The HPLC (Prominence, Shimadzu) was equipped with a refractive index detector and an evaporative light scattering detector (RID-ELSD). A gradient elution mode was used with acetonitrile and water containing 0.05 % formic acid serving as mobile phases. A C8 column (Phenomenex C8(2) Luna 5 µm, 100 Å, 150×4.6 mm) was selected as stationary phase for the separation of the target compounds. The column oven temperature was set to 40 °C. The elution conditions were as follows: hold 68 % acetonitrile for 9 min, increase to 100 % acetonitrile within 0.25 min and hold for 9.75 min, decrease to 68 % acetonitrile within 0.25 min and hold for 5.75 min. The total flow rate was 0.75 ml min^{-1} and the injected sample volume was 10 µl. (–)-Menthol and the fatty acids were detected and quantified via the RID signal. The ELSD detector was coupled to the RID for the detection of (–)-menthyl fatty acid esters and was operated with 3.5 bar N_2 pressure at 30 °C. The gain of the ELSD was set to 5 for the detection of (–)-menthyl caprylate and caprate and to 4 for the detection of (–)-menthyl laurate. Typical retention times are listed in Table B.9.

Table B.9: Typical retention times of (–)-menthol esterification reactants.

Compound	Retention time *min*
(–)-Menthol	5.6
Caprylic acid	4.2
Capric acid	6
Lauric acid	9.3
(–)-Menthyl caprylate	16
(–)-Menthyl caprate	17
(–)-Menthyl laurate	18.5

B.3.6 Lipase activity assay

The lipase activity assay was based on the hydrolysis of *p*-nitrophenol palmitate (*p*NPP) to release *p*-nitrophenol, which can be detected photometrically at 405 nm. The lipase powder (typically 4 mg) was dissolved in 10 ml Tris-HCl buffer (50 mM, pH 8, 0.01 wt% triton X-100, 0.01 wt% gum arabic). A *p*NPP stock solution ($1.33\,\mathrm{g\,l^{-1}}$) was prepared using *i*-propanol. The stock solution was stable for one week at ambient temperature. For the assay, the *p*NPP stock solution was diluted in buffer (dilution factor 12.5). Aliquots of this substrate solution (1 ml) were pre-incubated at 35 °C and 750 rpm orbital shaking in a thermal mixer (BioShake iQ, QInstruments) for at least 45 min. 180 µl of the pre-warmed substrate were transferred into a microtiter plate and 20 µl of the CRL solution was injected automatically. A plate reader (infinite 200, Tecan) was used to follow the reaction at 405 nm and 35 °C for 4 min. The sampling rate was one measurement every 2 s with a short mixing interval in between (orbital shaking for 1 s at 2.5 mm amplitude). One activity unit U was defined as the amount of enzyme that catalyses the hydrolysis of *p*NPP to form $1\,\mu\mathrm{mol_{pNP}\,min^{-1}}$ in 50 mM Tris-HCl buffer at pH 8 and 35 °C. The absolute activity was obtained by approximating the initial rate within the linear range and the relative activity was calculated based on the average activity of the control.

B.3.7 Characterisation of enzyme powders

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a 2D electrophoresis cell (mini-PROTEAN™ 3 electrophoresis system, Bio-Rad) and precast gels to determine the molecular weight of CRL. A prestained protein ladder from 10 to 180 kDa (PageRuler™ Prestained Protein Ladder, Thermo Fisher Scientific) was used as reference. 5 µl of denaturing buffer were mixed with 20 µl CRL solution (2.4, 2.0, 1.6 or 1.2 $g\,l^{-1}$) and boiled at 95 °C for 5 min. The samples were loaded on the gel and a Tris-base buffer containing glycine and SDS was used as running buffer. The voltage was set to 200 V. The gel was stained with Coomassie blue for 15 min and washed with a destaining solution (20 % EtOH, 10 % acetic acid). The gel was maintained in the washing solution overnight to decolourise.

Protein concentration

The protein content of the enzyme powders (CRL, BCL, PCL and PFL) was determined by a BCA assay (Pierce™ BCA protein assay kit, Thermo Fisher Scientific). Enzyme solutions (2.4, 1.6, 1.2 or 0.6 $g_{powder}\,l^{-1}$) were prepared with ultrapure water. Pre-diluted BSA solutions (Pierce™ Bovine Serum Albumin Standard, Thermo Fisher Scientific) were used as protein standard. 25 µl of each standard and of the enzyme samples were pipetted into a 96-well microtitre plate. 200 µl of working solution (prepared as described in the kit protocol) were added to each standard and sample. Standards and enzyme samples were prepared in triplicates. The plate was briefly mixed and incubated at 37 °C for 30 min. After the plate was cooled down to ambient temperature, the absorption at 562 nm was read using a plate reader (infinite 200, Tecan). The BSA calibration curve was fitted with a quadratic function and the protein concentration of the enzyme samples was calculated from the obtained calibration equation.

B.3.8 Response surface optimisation of menthol esterification

A central composite design (CCD) of the response surface methodology (RSM) was applied to optimise the esterification reaction. A three-factorial central CCD was set up using Design-Expert® (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) software to optimise the reaction in terms of water activity (a_w), enzyme amount (m_{CRL}) and reaction temperature (T). Each factor was varied within certain boundaries that were determined based on experiences from previous experiments ($0.06 \leq a_w \leq 0.75$; $4.41 \leq m_{CRL} \leq 67.59$ mg; $16 \leq T \leq 49$ °C). The experimental plan is shown by Table A.4. The design comprised six centre points, which are repetitions of the same experiment under identical conditions. In order to conduct different runs in parallel, experiments with the same temperature were run in groups. The reaction velocity was selected as a model response. As a measure for the reaction velocity, the product formation rate was calculated by plotting the ester concentration over time and approximating the data points with linear function using Orign2015G (OriginLab). At least three time points (0, 2 and 6 h) were used to fit a linear curve and the slope was read as the product formation rate. Based on a regression analysis a model was obtained, which was analysed by an ANOVA (analysis of variance) implemented in the Design-Expert software. The experimental runs were performed with 2 g (–)-menthol:lauric acid 3:1 mol/mol DES, which was incubated with different saturated salt solutions for 3 d at the targeted reaction temperature and at 900 rpm (Velp multistirrer). An exeperimental set-up with double temperature control was used, which is depicted by Figure 5.12. The esterification reaction was started by addition of CRL to the pre-equilibrated DES. Sampling and HPLC analysis were performed as described in the previous chapters.

C Abbreviations

4-NBA	4-Nitrobenzaldehyde
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
API	Active pharmaceutical ingredient
BCA	Bicinchoninic acid assay
BCL	*Burkholderia cepacia* lipase
BSA	Bovine serum albumin
C8:0	Capric acid, octanoic acid
C10:0	Caprylic acid, decanoic acid
C12:0	Lauric acid, dodecanoic acid
CALB	*Candida antarctica* lipase B
CCD	Central composite design
ChCl	Choline chloride
CRL	*Candida rugasa* lipase
DES	Deep eutectic solvent
DoE	Design of experiments
***E* factor**	Environmental factor
EG	Ethylene glycol
ELSD	Evaporative light scattering detector
EtOH	Ethanol

Gly	Glycerol
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
IL	Ionic liquid
N435	Novozymes *Candida antartica* lipase B
NADES	Natural deep eutectic solvent
NBD-H	4-Hydrazino-7-nitro-2,1,3-benzoxadiazole
n.d.	not detected
PCL	*Pseudomonas cepacia* lipase
PFL	*Pseudomonas fluorescens* lipase
PMSF	Phenylmethylsulfonyl fluoride
pNP	*p*-Nitrophenol
pNPP	*p*-Nitrophenyl palmitate
PPL	Porcine pancreas lipase
RH	Relative humidity
RID	Refractive index detector
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TbACl	Tetra-*n*-butylammonium chloride
THEDES	Therapeutic deep eutectic solvent
ToABr	Tetra-*n*-octylammonium bromide
U	Urea

D Symbols

A	Model variable for water activity
a_w	Water activity
B	Model variable for enzyme amount
c	Concentration
C	Model variable for temperature
F_N	Normal force
g	Earth's gravitational acceleration
G'	Storage modulus
G''	Loss modulus
H_{sorp}	Henry's constant for water absorption by (–)-menthol:lauric acid DES
m	Mass
m/z	Mass-to-charge ratio
n	Number of experiments
p-value	Probability value
R^2	Coefficient of determination
t	Time
T	Temperature
T^E	Eutectic temperature
T_m	Melting temperature
V	Volume

x	Mole fraction
x^E	Mole fraction at eutectic composition
Y	Model response variable for product formation velocity
ρ	Density
γ	Deformation
$\dot{\gamma}$	Shear rate
η	Viscosity
τ	Shear stress

E List of Figures

F List of Schemes

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H References

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