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RAPID COMMUNICATION

Product recovery of an enzymatically synthesized (−)-menthol ester in a deep eutectic solvent

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Abstract
Deep eutectic solvents (DESs) have gained increased attention as alternative reaction media for biocatalysis in recent years. There are many investigations on biotransformations in a variety of DESs, but the purification of bioproducts from DES reaction mixtures has not yet been sufficiently addressed. The present study demonstrates a product recovery strategy from a DES reaction medium composed of (−)-menthol and dodecanoic acid. Since the DES is not formed by equimolar amounts of the substrates, but the eutectic point occurs at a 3:1 molar ratio, product isolation is an important task for effective biocatalytic process development, even if the limiting substrate is converted completely. Both DES compounds acted as substrates and reaction solvent in the lipase-catalyzed esterification to synthesize (−)-menthyl dodecanoate. The product (−)-menthyl dodecanoate ester was separated from the DES reaction mixture by a vacuum distillation step and a second esterification reaction can be performed with the recovered (−)-menthol.

Keywords Deep eutectic solvent · Downstream processing · Lipase · Menthol · Vacuum distillation

Introduction

In biocatalysis, water is certainly the most frequently used solvent [1]. However, water cannot always be used for biotransformations. For example, the high polarity and the resulting low solubility for hydrophobic substances or thermodynamic effects can prevent effective applications of water as solvent. Therefore, several non-conventional reaction media, e.g., organic solvents, ionic liquids and supercritical liquids were evaluated as reaction media in biotransformations. In recent years, deep eutectic solvents (DESs) gained more and more attention as novel reaction media. The term DES generically describes liquid eutectic mixtures of at least two compounds (solid:solid or solid:liquid) that form a hydrogen bond network. The hydrogen bond acceptor is mixed with a hydrogen bond donor in a certain molar ratio yielding a liquid after heating and stirring, which is characterized by a considerably lower freezing point compared to its initial constituents [2]. There are also so-called natural DES (NADES), which are prepared solely from 'natural' precursors [3]. While their constituents are still solid:solid or liquid:solid mixtures, NADESs only differ from DESs in the 'natural' quality of the starting materials. Kazlauskas et al. showed a first enzymatic reaction in this type of solvent in 2008 [4]. Since then, the number of studies evaluating DESs as environmentally and catalytically preferable alternatives to established reaction solvents in biotransformations is steadily increasing. One of the reasons for this continuous rise in the attention given to DESs is that these new solvents can be designed as non-toxic, non-volatile, non-flammable or even biodegradable liquids. Therefore, DESs are frequently considered as a novel solvent class contributing to "greener" process development [5]. It is already certain based on literature that the application of DESs can be beneficial in various steps of the entire biocatalytic process chain—from upstream across the reaction to downstream [6–8]. However, if reactions were performed in DES-based reaction media, studies on the purification of the products and the re-use of unconverted substrates are often neglected. There are only a few examples dealing with an entire process development for enzyme-catalyzed

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reactions in DESs [9, 10]. Therefore, the aim of this work was to develop a product purification strategy for a DES-based reaction mixture. The enzyme-catalyzed synthesis of a (−)-menthyl dodecanoic acid ester from a DES consisting solely of the substrates has been recently published [11] and served as a model reaction system (cf. Scheme 1) for the subsequent purification of the ester and recovery of unreacted menthol in this study. Menthol has eight stereoisomers, of which (−)-menthol is commercially most relevant due to its characteristic mint flavor and its refreshing cooling effect. The strong flavor of (−)-menthol can be extenuated by esterification with short-chain fatty acids [12], which can be desirable if a slow release of (−)-menthol is required (e.g., in ointments with a lasting cooling effect). In this context, the preparation of the (−)-menthyl dodecanoic acid ester can be regarded as an example for the modification of (−)-menthol. The synthesis of (−)-menthyl dodecanoate in a DES formed by the substrates is beneficial, since both substrates [dodecanoic acid and (−)-menthol] are hardly soluble in water and additionally water would shift the reaction equilibrium to an unfavorable side for ester synthesis. However, the DES for (−)-menthyl dodecanoic acid ester synthesis is not composed of equimolar amounts of the substrates, as the eutectic point occurs at a mole fraction of 0.29 of dodecanoic acid (DDA) at 290.97 K [13]. Therefore, separation of the ester product from the mixture of unreacted DES compounds is an important task, which was addressed in this study, next to the recovery and re-use of excess (−)-menthol for a subsequent esterification reaction.

Materials and methods

Materials

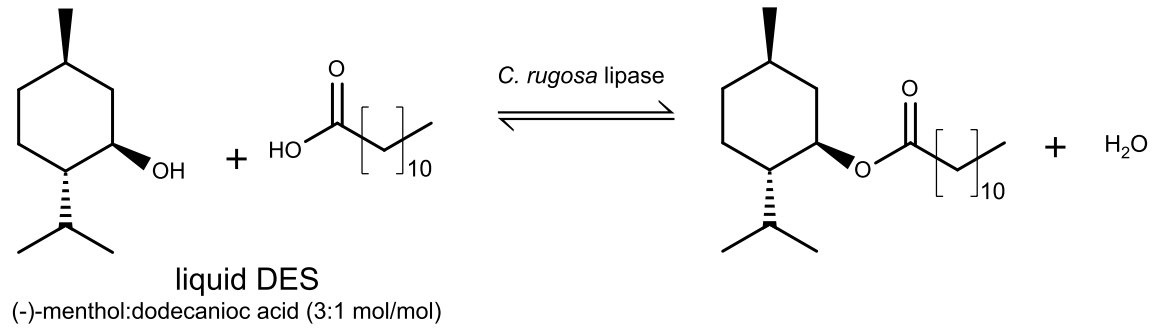
Candida rugosa lipase type VII ≥ 700 U/mg (CRL), (−)-menthol (purity ≥ 98.5%) and dodecanoic acid (DDA) (purity ≥ 97.5%) were obtained from Sigma-Aldrich (Steinheim, Germany). The product ester [(−)-menthyl dodecanoate] was gratifyingly synthesized by the Institute of Applied Synthetic Chemistry (Research Group of Prof. Marko D. Mihovilovic, Vienna University of Technology). All chemicals were used without any additional purification or dehydration step. Potassium acetate (KAc ≥ 99%) was purchased from Carl Roth (Karlsruhe, Germany) and was used to control the water activity (a_w) during the esterification reactions.

Preparation of DES

The substrate containing (−)-menthol:DDA DES was prepared by mixing (−)-menthol and DDA at molar ratio of 3:1 [(−)-menthol:DDA 3:1 mol/mol]. The mixture was placed in an incubator (Ecotron, Infors HT, Einsbach, Germany) at 37 °C with 180 rpm orbital shaking until a homogeneous liquid was obtained. The DES remained liquid after cooling down to room temperature.

General procedure for CRL-catalyzed esterification in DES

The (−)-menthol:DDA DES (2 g) was weighed into glass tubes ($V = 5$ mL) and a small magnetic stir bar was added. The glass tubes were used as inserts to be placed in a reaction vessel filled with a saturated KAc solution (approximately 3 mL) for a_w control during the esterification reaction. The saturated salt solution was not in direct contact with the DES and a_w adjustment took place via the gas phase. The double-walled reaction vessels were connected to an external thermostating circuit with the temperature set to 35 °C and the DES was pre-equilibrated at 200 rpm (Velp Scientifica Multistirrer 6 Digital). Additionally, the CRL powder was pre-incubated for 3 days with saturated KAc at 35 °C (Certomat HK, Sartorius, Göttingen, Germany) in rubber-sealed plastic boxes. After a_w adjustment of the DES and enzyme, the reaction was started by the addition of the pre-equilibrated enzyme (1 wt% m_{CRL}/m_{DES}; corresponding to



Scheme 1 *Candida rugosa* lipase-catalyzed esterification of (−)-menthol with dodecanoic acid in a DES formed by the substrates in a 3:1 molar ratio

≥ 700 U/g_{DES} according to supplier units) and the stirrer rate was increased (300 rpm) to suspend the insoluble enzyme powder within the DES phase. The esterification proceeded for 3 days under controlled a_w conditions. Samples were withdrawn in regular intervals and centrifuged (Eppendorf MiniSpin Plus, 2 min, 14,100 *g*) to separate the enzyme. The supernatant DES was diluted in ethanol (dilution factor 100) for HPLC analysis. After the esterification reaction, approximately 1 mL of the reaction mixture was transferred to 2 mL reaction tubes and the enzyme was separated by a centrifugation step (5 min, 14,100 *g*). The supernatant was stored at − 20 °C for the following product purification step.

HPLC analysis

Quantitative analysis of (−)-menthol, DDA and the ester was performed on an HPLC (Shimadzu Prominence) equipped with a C8 column (Phenomenex C8(2) Luna 5 µm, 100 Å, 150 × 4.6 mm), which was operated at 40 °C. An evaporative light-scattering detector (ELSD operated with 3.5 bar N_2 at 30 °C, gain set to 4) was used to detect the product ester [(−)-menthyl dodecanoate], whereas the substrates [(−)-menthol and DDA] were analyzed with a refractive index detector (RID). Typical retention times were 5.6 min for (−)-menthol, 9.3 min for DDA and 18.3 min for the ester. Details of the analytical method are described in [11].

Ester purification

A vacuum distillation step using a micro-distillation apparatus was performed to separate (−)-menthol after the esterification reaction. The cooling temperature of the condenser circuit was set to 40 °C to avoid crystallization of (−)-menthol, since its melting point at atmospheric pressure is about 41–43 °C [14]. Before the distillation was started, a HPLC sample was prepared by diluting the DES in ethanol (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 crystallize (−)-menthol and, therefore, minimize 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 ethanol; solid fraction: 14.4 g/L of the solid in ethanol).

Results and discussion

Lipase-catalyzed synthesis of (−)-menthyl dodecanoate

The thermodynamic water activity (a_w) is an important parameter for lipase-catalyzed reactions in non-aqueous media [15] and (trans-)esterifications at controlled a_w have been conducted in organic solvents [16] and ionic liquids [17]. Therefore, the CRL-catalyzed esterification reaction in (−)-menthol:DDA was performed at controlled a_w using saturated KAc solutions. Two individual esterifications were carried out in (−)-menthol:DDA and both reactions reached 100% fatty acid conversion after 3 days. As the esterification proceeds, the initial binary DES mixture [(−)-menthol, DDA] becomes a ternary mixture [(−)-menthol, DDA, ester] and eventually a binary mixture again [(−)-menthol, ester]. To separate the excess (−)-menthol from the product, a fraction of the reaction mixture was harvested for further purification by vacuum distillation.

Development of a downstream process for ester purification

The DES compounds can be separated by a thermal separation process due to a lower boiling point of (−)-menthol (T_b = 212 °C) compared to DDA (T_b = 298.9 °C) and the (−)-menthyl dodecanoate ester (T_b = 379.1 ± 10.0 °C) [14]. As the reaction mixture contained no residual DDA after the esterification reaction, the separation of DDA was not necessary. A DES mixture consisting of (−)-menthol and (−)-menthyl dodecanoate (m = 1.4957 g, from two esterification reactions) was used to separate (−)-menthol from the ester by vacuum distillation. Due to its lower boiling point, (−)-menthol was collected as overhead product, while the (−)-menthyl dodecanoate ester remained at the bottom. After distillation, 735 mg ester and 622.5 mg (−)-menthol were obtained. HPLC analysis revealed that pure (−)-menthol is recovered as overhead product, while the ester was accumulated as bottom product with 94% purity (cf. Fig. 1). The purification of the product was generally limited by the use of a small-scale distillation apparatus lacking refluxing trays or fixtures. Moreover, the (−)-menthol impurity in the ester fraction could only be reduced by increasing the temperature above the boiling point of the ester, which caused high product losses. A total loss of approximately 10% was calculated, which can be explained by the high volatility of (−)-menthol and by the evaporation of residual water from the reaction mixture.

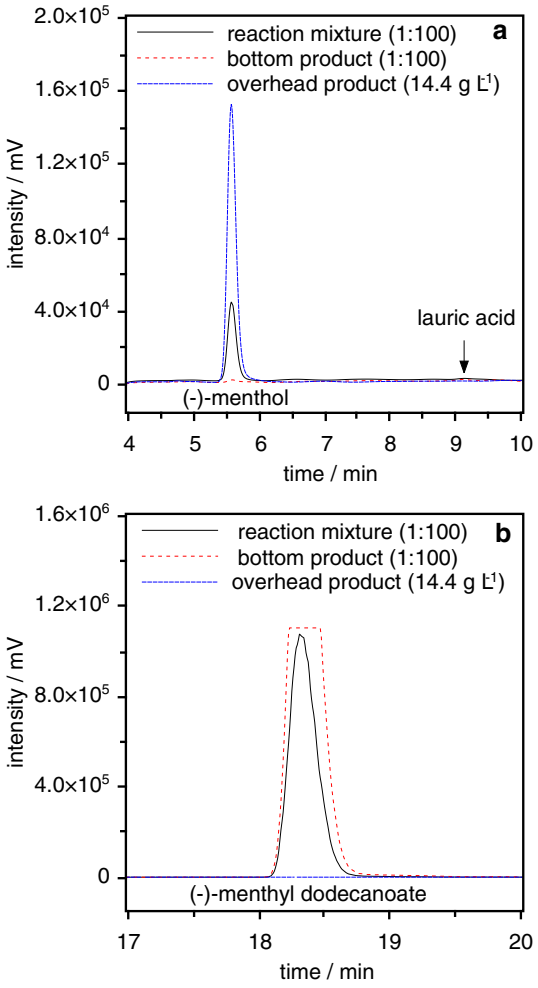


Fig. 1 RID chromatogram (**a**) of (−)-menthol ($t_R = 5.6$ min) and dodecanoic acid (DDA, $t_R = 9.3$ min) and ELSD chromatogram (**b**) of the (−)-menthyl dodecanoate ester ($t_R = 18.3$ min). Samples were taken from the reaction mixture before the distillation process (black), from the bottom (red) and overhead (blue) fractions after the separation process (colour figure online)

Re-use of unconverted (−)-menthol

The recovered 3.98 mmol (−)-menthol ($m = 622.5$ mg) from the vacuum distillation step was used to prepare a new (−)-menthol:DDA (3:1, mol/mol) DES by adding 1.33 mmol of the fatty acid ($m = 266.9$ mg). 836.3 mg of this new DES was used to perform another esterification reaction with fresh CRL powder and under the previously applied reaction conditions, including the pre-equilibration of the DES and the enzyme powder. The course of the reactant concentrations and conversion of the esterification reaction in the DES made of recycled (−)-menthol is shown by Fig. 2. Similar to the previous esterification reactions, DDA is almost fully converted (≥ 98.7%) after 3 days in the DES with re-used (−)-menthol. This example demonstrates that product purification from a DES reaction mixture and the recycling of excess DES compounds is possible.

Conclusion

DESs can be regarded as promising alternative reaction media for biotransformations, in particular if the starting materials represent the DES hydrogen bond donor and acceptor compounds itself. In this study, an enzymatic esterification reaction catalyzed by CRL was performed in a substrate DES [(−)-menthol:DDA 3:1 mol/mol] without the use of any additives. However, the substrate DES is not composed of equimolar amounts of the hydrogen bond donor and acceptor, which is also the case for most of the reported DESs. Due to the presence of an excess compound, product purification is necessary, even if the reaction reaches 100% conversion and the limiting substrate is completely consumed. The separation of products from DES reaction mixtures is often not addressed in studies dealing with biotransformations in DESs. With the present study, it was demonstrated that a DES reaction mixture can be purified by a thermal separation process due to the different boiling points of the substrates and the product. Subsequent to complete conversion of the limiting substrate DDA, (−)-menthol was separated from the (−)-menthyl dodecanoate ester by vacuum distillation. The ester was obtained with a purity of up to 94% and pure (−)-menthol was recovered to be re-used for a new (−)-menthol:DDA DES esterification. It was possible to perform a second esterification reaction in the recycled DES reaching almost full conversion (≥ 98.7%) after 3 days. Thus, the purification of a product [(−)-menthyl dodecanoate] and the re-use of an unconverted DES compound [(−)-menthol] were successfully accomplished, while the recycling of the biocatalyst was out of scope of this study. As a future perspective, the efficiency of the DES-based enzymatic synthesis of (−)-menthyl esters might be further improved, if a re-use of the lipase was possible. In summary, the enzymatic esterification of a substrate-based DES and the subsequent recycling and re-use of the excess DES compound represent an interesting approach to intensify the process economics of an enzymatic reaction in a DES reaction medium. For the model system, involving the separation of (−)-menthol from the synthesized ester, purification by a distillation step was especially well suited to compromise a high ester product purity with the recovery of pure (−)-menthol to enable its re-use.

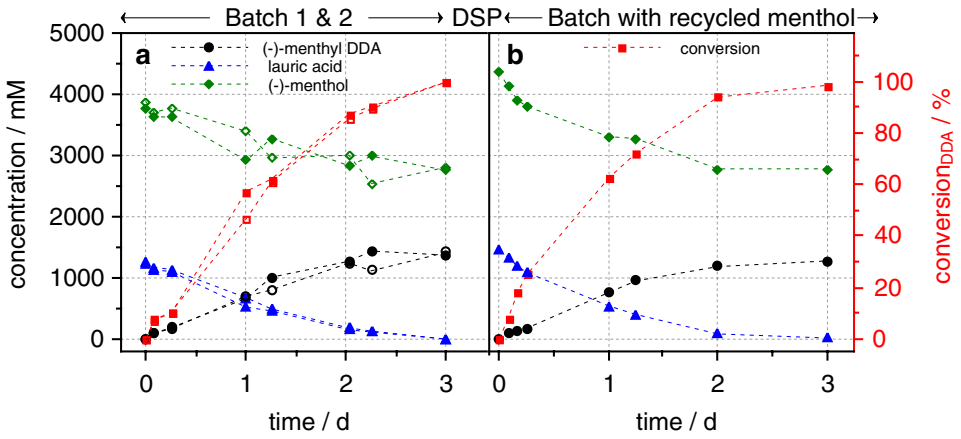


Fig. 2 Reactant concentrations (left *y*-axis) and fatty acid conversion (right *y*-axis, red) of the CRL catalyzed esterification in (−)-menthol:DDA of batch 1 (closed symbols) and batch 2 (open symbols) (**a**) and in the DES composed of the recycled (−)-menthol of combined fractions of batch 1 and 2 (**b**). The DES and CRL were equilibrated with saturated KAc at 35 °C for 3 days. Reaction conditions: 1 wt% CRL (m_{CRL}/m_{DES}), T = 35 °C, KAc saturated salt solution with measured $a_{w,initial}$ = 0.16, 300 rpm

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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