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Optimization of solvent-free enzymatic esterification in eutectic substrate reaction mixture

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ABSTRACT

The *Candida rugosa* lipase catalyzed esterification of (-)-menthol and lauric acid (LA) was studied in a eutectic mixture formed by both substrates((-)-menthol:LA 3:1, mol/mol). No additional reaction solvent was necessary, since the (-)-menthol:LA deep eutectic solvent (DES) acts as combined reaction medium and substrate pool. Therefore, the esterification is conducted under solvent-free conditions. The thermodynamic water activity (a_w) was identified as a key parameter affecting the esterification performance in the (-)-menthol:LA DES. A response surface methodology was applied to optimize the esterification conditions in terms a_w, amount of *C. rugosa* lipase (m_{CRL}) and reaction temperature. Under the optimized reaction conditions (a_w = 0.55; m_{CRL} =60 mg; T =45 °C), a conversion of 95 ± 1% LA was achieved (one day), the final (-)-menthyl lauric acid ester concentration reached 1.36 ± 0.04 M (2.25 days). The experimental product formation rate agreed very well with the model prediction.

1. Introduction

Biocatalysis is often associated with aqueous reaction media, because water is accepted as the natural environment of enzymes. However, the high polarity of water limits its application as reaction solvent due to the low water solubility of many organic reactants of interest. Enzymatic processes are not only restricted by low solubilities of the substrates of interest, but also by a lack of enzyme activity/stability in the chosen solvent. Therefore, reaction medium engineering is an essential task in biocatalytic process development to compromise enzyme activity/stability with high substrate loads. To overcome the drawback of using water as solvent, enzymatic reactions have been performed in non-conventional reaction media (e.g. organic solvents, ionic liquids, supercritical fluids). A relatively new class of non-conventional solvents are deep eutectic solvents (DESs), which have been first described by Abbott and co-workers in 2003 [1]. DESs are eutectic mixtures of at least two components, which can form hydrogen bond interactions leading to a deep "freezing point depression of the mixture in contrast to the initial constituents [1]. Ideally, the mixtures are liquid around room temperature to be used as solvents. DESs have gained attention as alternative reaction media for biocatalysis, since Gorke et al. first performed a lipase catalyzed reaction in these kind of solvents [2]. The fact that DESs are mixtures of two components promoted the idea of incorporating substrates in a DES matrix to provide high substrate loads with the reaction solvent. Using substrate-based DESs as reaction media was exploited for a number of different enzymatic reactions [3–7]. For these reactions, one of the substrates was incorporated in the DES matrix, whereas it was also possible to form DESs with both substrates for solvent-free lipase catalyzed esterification reactions. For the esterification reactions of (-)menthol with fatty acids of different chain length (octanoic acid, decanoic acid or lauric acid), the terpene was mixed with the fatty acid in a certain molar ratio to form a eutectic and the reaction was started by adding a lipase after liquefaction of the substrates [8]. However, under the selected biphasic esterification conditions (water addition of 10 wt% to the DES) the conversion reached only 71% after a quite long reaction time of 7 days [8]. While the addition of water had a positive effect on the esterification for a similar reaction system [9], it is also known that enzymes only require trace amounts of water to be active in non-aqueous media [10]. The thermodynamic activity of water (a_w) is a measure for the amount of water present in non-aqueous reaction media and influences the reaction rate, enantioselectivity and equilibrium conversion of lipase catalyzed reactions in non-conventional media [11]. In non-aqueous reaction solvents, lipase catalyzed reactions were performed under a_w controlled conditions to supplement and maintain the reaction medium with the optimal amount of water [12–14]. Therefore, a_w is an important parameter for lipase catalyzed reactions in non-conventional media. Since there is only limited information on the effect of a_w for enzymatic reactions

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in DES reaction media available [15,16], the present study aims at exploiting the impact of a_w on an enzyme catalyzed reaction in a DES in more detail. The lipase catalyzed esterification in a (-)-menthol: lauric acid (3:1, mol/mol) eutectic mixture was selected as a model reaction, because the DES acts as solvent and substrate at the same time to enzymatically synthesise (-)menthyl laurate ester (cf. Scheme 1). The use of a substrate-based (-)-menhtol:lauric acid DES as 2-in-1 reaction medium is very attractive, as the approach enables solvent-free reaction conditions as well as extremely high substrate loads. The modification of (-)-menthol for cosmetic applications has been described for the esterification of (-)menthol with long-chain fatty acids [9]. In this context, the enzymatic synthesis of (-)-menthyl laurate can be regarded as an example of how (-)-menthol can be modified using a DES. Moreover, a response surface methodology (RSM) was used to optimize the reaction conditions in terms of a_w, applied enzyme amount and reaction temperature.

2. Experimental

2.1. Materials

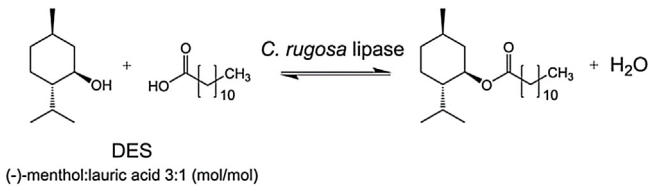
The DES components, (-)-menthol (purity ≥ 98.5%) and lauric acid (LA) (purity ≥ 97.5%) as well as the enzyme Candida rugosa lipase type VII ≥ 700 U/mg (CRL) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The salts used in this study were: lithium chloride (LiCl ≥ 99%, p.a., ACS), potassium acetate (KAc ≥ 99%), sodium chloride (NaCl > 99.8%) (all from Carl Roth GmbH + Co. KG, Karlsruhe, Germany); magnesium chloride hexahydrate ($MgCl_2 \bullet 6H_2O$ ≥ 99%), potassium iodide (KI > 99.5%), sodium bromide (NaBr ≥ 99.5%), sodium iodide (NaI ≥ 99%, ACS), potassium carbonate (K_2CO_3 ≥ 99%, anhydrous, Fluka) (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany), lithium bromide (LiBr > 99%, anhydrous, Alfa Aesar, Thermo Fisher GmbH, Kandel, Germany), and potassium nitrate (KNO_3 extra pure, Merck KGaA, Darmstadt, Germany). The ester standard ((-)-menthyl laurate) was kindly 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.

2.2. Preparation of DES

The substrate containing (-)-menthol:LA DES was prepared by mixing (-)-menthol and LA in a 3:1 M ratio ((-)-menthol:LA 3:1 mol/mol). The mixture was placed in an incubator at 37 °C with 180 rpm orbital shaking (Infors ecotron HT) until a homogeneous liquid was obtained.

2.3. DES water absorption

In order to determine the absorption of water, the DES was equilibrated over saturated salt solutions of defined a_w (cf. Table 1). Deionised water was used to obtain fully water saturated conditions, corresponding to an a_w of 1. The DES (6 g) was incubated at 35 °C for 24 h. Temperature control was accomplished with a thermostat (VWR) feeding the heating jacket of the reactor and by an additional incubator hood (Sartorius Certomat HK) for further temperature control in the head space. The water content of the DES before and after the incubation was determined by volumetric Karl-Fischer titration (KF titrator DL38, Mettler Toledo) using a one-component reagent with a titer of 1 mg/mL (Hydranal®-Composite 1, Fluka). The a_w of the saturated salt solutions was correlated with the DES water content after incubation to obtain a DES water absorption isotherm.



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

Table 1
Water activity of saturated salt solutions at 35 °C [17].

Sat. salt solution	a_w (35 °C)
LiBr	0.06
LiCl	0.11
KAc*	0.20
NaI	0.35
K_2CO_3*	0.43
NaBr	0.55
KI	0.67
NaCl	0.75
KNO_3	0.91

* water activity extrapolated to 35 °C.

2.4. Screening reactions with different saturated salt solutions

Prior to starting the reaction, the DES (2 g) and the enzyme (20 mg CRL) were incubated separately for 3 d at 35 °C. 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 by diluting 10 μL of the DES in 990 μL EtOH. The esterification reaction was started by the addition of 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 (Eppendorf MiniSpin Plus, 14100xg, 3 min) to separate the enzyme and 10 μL of the DES phase was diluted in 990 μL EtOH and analyzed by HPLC.

2.5. HPLC analysis

Quantitative analysis of (-)-menthol, LA and the ester was performed on a HPLC (Shimadzu Prominence) equipped with a C8 column (Phenomenex C8(2) Luna 5 μm, 100 Å, 150 x 4.6 mm), which was operated at 40 °C. Acetonitrile and water containing 0.05% formic acid served as mobile phase with a total flow rate of 0.75 mL/min. 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 sample injection volume was 10 μL. 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 laurate), whereas the substrates ((-)-menthol and LA) were analyzed with the refractive index detector (RID). An exemplified chromatogram is provided in the supplementary section (cf. Figure S2).

2.6. Response surface optimization of the esterification reaction

A central composite design (CCD) of the response surface methodology (RSM) was applied to optimize 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 optimize 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 S1. 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. Based on a regression analysis a model was obtained, which was analyzed by an ANOVA (analysis of variance) implemented in the Design-Expert® software. The experimental runs were performed with 2 g DES, which was incubated with different saturated salt solutions for 3 d at 35 °C and at 900 rpm (Velp multistirrer), whereas the enzyme was used without any pre-treatment due to a loss of activity after incubation at an increased a_w (cf. Figure S4). Temperature control was ensured by a thermostat (VWR) and an additional incubator hood (Sartorius Certomat HK) (cf. Figure S1). The esterification reaction was started by addition of CRL to the pre-incubated DES. Sampling and HPLC analysis were performed as described in the previous chapters.

2.7. Calculation of conversion and product formation rate

As a measure for the reaction velocity, the product formation rate was calculated by plotting the ester concentration over time and by approximating the data points with a linear function. At least three time points were used to fit a linear function (0, 2 and 6 h).

3. Results and discussion

3.1. Physicochemical characterisation of DES water absorption

Adjusting and controlling the water content in the (-)-menthol:LA solvent requires to determine the water absorption characteristics of the DES surrounded by different saturated salt solutions. Since saturated salt solutions have a specific a_w [17], they can be used to create an atmosphere of defined humidity in a closed system. Therefore, the water content of another fluid, surrounded by a separated saturated salt solution, can be adjusted in a closed system, as the water vapour partial pressures of the two phases will be in equilibrium. In case of the (-)-menthol:LA DES, the a_w of different saturated salt solutions was correlated with the water content in the DES after incubation. The experimental data could be approximated with a linear fit, showing that higher a_w values induced higher water contents in the DES in a proportional manner (cf. Fig. 1). The initial water content of the freshly prepared (-)-menthol:LA DES was relatively low (0.1 wt%), whereas the water content increased up to 1.4 wt% after the incubation with pure water ($a_w = 1$). Therefore, the use of different salt solutions varies the water content of (-)menthol:LA in a range of 0.1–1.4 wt% at a given temperature of 35 °C. These values are in the same order of magnitude as the water contents determined for dried (0.276 wt%) and water saturated (1.237 wt%) (±)-menthol:LA (2:1, mol/mol) DES [18].

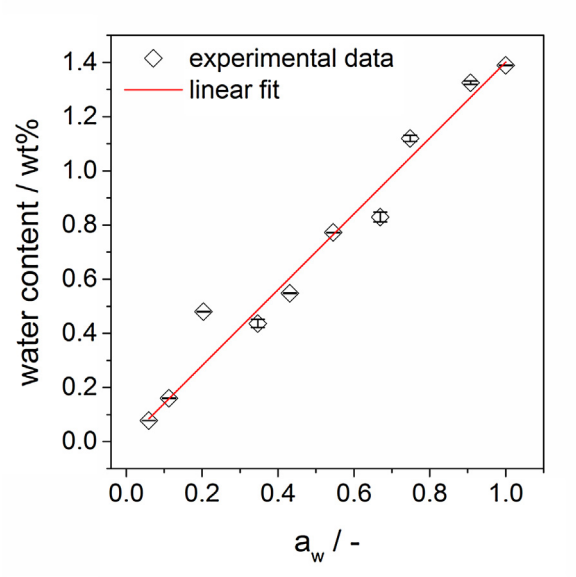


Fig. 1. Correlation between a_w and water content of (-)-menthol:LA DES at 35 °C. Line represents the linear fit of the experiment data.

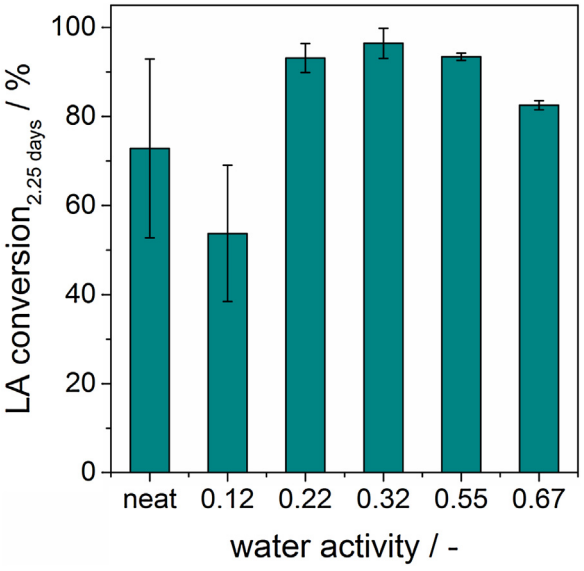


Fig. 2. Lauric acid (LA) conversion after 2.25 days of the CRL catalyzed esterification in neat (-)-menthol:LA in comparison to different water activity controlled reaction systems.

3.2. Screening esterification reactions with different a_w

Different methods have been developed to control the a_w during enzyme catalyzed reactions in non-aqueous media [19,20]. Most commonly saturated salt solutions are used to adjust a_w via the gas phase [11,12,21], which has been applied for the a_w controlled esterification reactions in (-)-menthol:LA. In a preliminary experiment the effect of different a_w values on the CRL catalyzed esterification in (-)menthol:LA was screened to characterise the water demand of the enzyme. As illustrated by Fig. 2, a_w values from 0.22 to 0.55 are beneficial for the esterification reaction. If the reaction was performed under dry conditions, no conversion was observed (cf. Figure S3). Full conversion was obtained with an a_w of 0.22 and 0.32 after 3 d. For a published CRL catalyzed reaction in an organic solvent with hexanol and ethyl decanoate as substrates, the highest transesterification rate occurred at $a_w = 0.33$, whereas the rate decreased at lower or higher a_w (at a reaction temperature of 25 °C) [13]. Generally, the results indicated that there is an optimal a_w for the CRL catalyzed esterification in (-)-menthol:LA. This was further investigated within the scope of an experimental RSM design.

3.3. CCD optimization of temperature, enzyme amount and a_w

The reaction optimization by a statistical design of experiments is a powerful method and has also been applied to CRL catalyzed esterification of (-)menthol [22,23]. For the optimization of the CRL

Table 2
Coded and actual levels of factors used for the central composite design.

		Coded levels				
Factor	Symbol	$-\alpha$	−1	0	+1	$+\alpha$
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

catalyzed esterification in the (-)menthol:LA DES, experimental data from 20 runs was collected according to a CCD plan with coded and actual factor levels shown by Table 2. The product formation rate was selected as response function to build the model and two runs were excluded, as the DES became solid at a low experimental temperature (run 18) and as one run was assigned with an unusually high influence on the model (supplementary information Section 4.2). The experimental data of 18 runs was fitted by stepwise regression (significance level of $\alpha_{in/out} = 0.1$) and the following quadratic model Eq. (1) was obtained, where *Y* is the product formation rate, *A* is the a_w, *B* is the enzyme amount (m_{CRL}) and *C* is the reaction temperature:

$$Y = 60.49 + 469.37\,A + 1.54\,B - 9.53\,C + 0.04\,BC - 526.29\,A^2 - 0.02\,B^2 + 0.14\,C^2 \quad (1)$$

The quadratic model is significant to describe the experimental data, since the adjusted R^2 (adj. $R^2 = 0.9564$) and the predicted R^2 (pred. $R^2 = 0.8883$) are in reasonable agreement and close to 1. The lack of fit is insignificant ($p = 0.2529$), indicating that the model can accurately represent the experimental data. The three parameters (a_w, m_{CRL}, T) were identified as significant influential factors with p-values of less than 0.05 ($p_{aw} = 0.0004$; $p_{mCRL} < 0.0001$; $p_T = 0.0015$). Moreover, the interaction of the enzyme amount and temperature ($p = 0.006$) has a significant impact on the synthesis rate. Generally, the esterification reaction is enhanced at elevated temperatures and with a high enzyme amount (cf. Fig. 3). Moreover, the esterification performance depends also on the a_w. If the reaction is conducted at 45 °C and with 60 mg CRL at low a_w, the product formation is decreased. After a certain a_w, a maximum is reached and the product formation is reduced upon further increase of the a_w. Since the product formation is maximized at the boundaries of the design space, it would be necessary to augment the experimental design towards higher temperatures and CRL amounts to further optimize the esterification reaction in (-)-menthol:LA. As the process costs rise with increasing temperatures and with using increased amounts of biocatalyst, a further optimisation of the process would also require an economic evaluation, which was out of scope of this study.

3.4. CRL catalyzed esterification under optimized conditions

In order to verify the model and to compromise the factors, an experiment was conducted with the predicted optimal conditions. The desirability function of DesginExpert8 was used to maximize the product formation rate (range: 80 up to 600 mM/h). The factors were allowed to vary within the design space, since extrapolation is not permitted ($0.11 \leq a_w \leq 0.67$; $20 \leq T \leq 45$ °C; $12 \leq m_{CRL} \leq 60$ mg). The factor combination with the highest desirability calculated by the model is: $a_w = 0.55$, m_{CRL} =60 mg, T =45 °C (cf. Figure S7). When this factor combination is applied up to 161.5 mM/h product formation is predicted by the model. This was confirmed by an experiment using sodium bromide (NaBr) as saturated salt solution ($a_w = 0.52$ at 45 °C [17]). The results are depicted Fig. 4A as an average of three individual reactions. The relative humidity (RH) was monitored in one reactor by a humidity sensor to check the a_w. The relative humidity is around 50% in the reaction vessel, corresponding to $a_w = 0.5$, but initially higher values were measured (cf. Fig. 4B). This is due to a temperature variation upon opening the incubator hood to withdraw samples and due to water formation as side product of the esterification reaction. Generally, the experimental data agrees well with the predicted values and a product formation of 174 mM/h was determined under optimized conditions. This is slightly higher than predicted by the model, but the product formation is within the 95% confidence interval (134.2–188.8 mM/h). Performing the reaction under the optimized conditions ($a_w = 0.55$, 45 °C, 60 mg CRL), resulted in 95% LA conversion after one day as well as in a final (-)-menthyl laurate ester concentration of up to 1.36 ± 0.04 M at the end of the reaction (2.25 days). In comparison to the preliminary screening results (96% LA conversion after 2.25 d, $a_w = 0.32$, 35 °C, 20 mg) the reaction time was reduced by one half. In contrast to the published esterification (71% LA conversion, 7

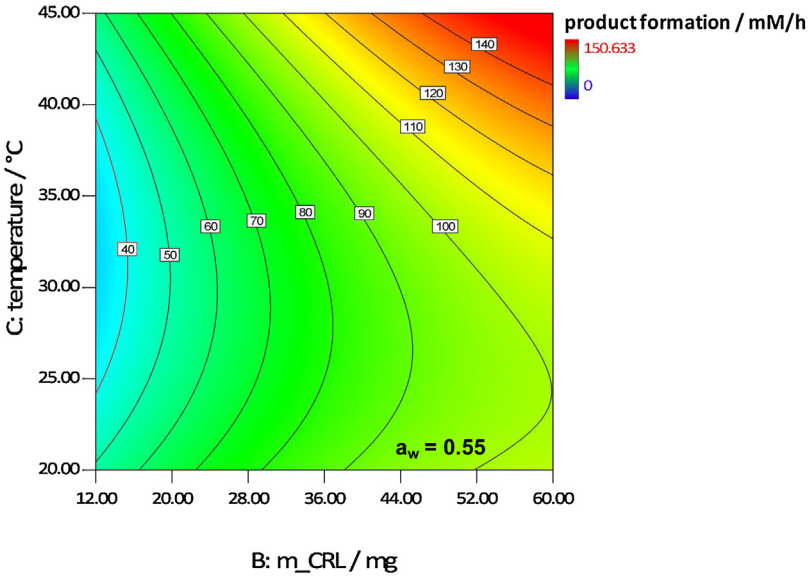


Fig. 3. Interaction of enzyme amount (m_{CRL}) and temperature affecting the product formation of the CRL catalyzed esterification in (-)-menthol:LA at a water activity of 0.55. The colour gradient of the heat map shows areas of low ester formation in blue and areas of high product formation are depicted in red. Auxiliary lines with numbers show the course of the product formation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

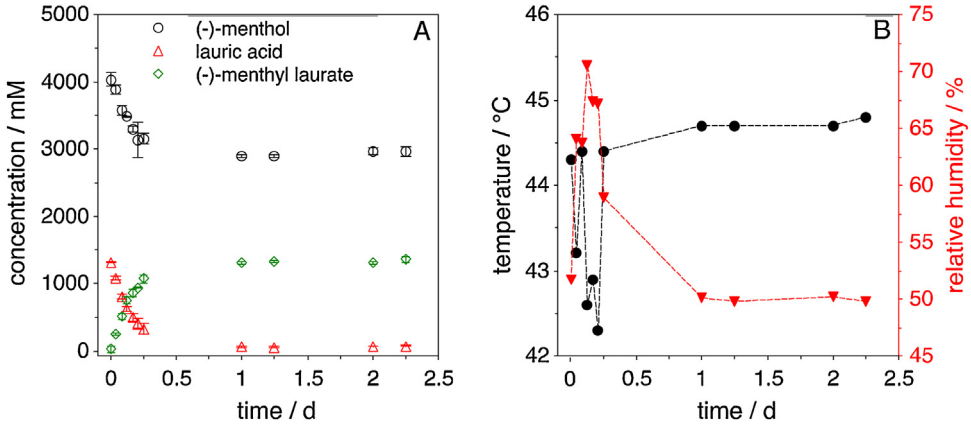


Fig. 4. CRL catalyzed esterification in (-)-menthol:LA using NaBr as saturated salt solution. A: Product formation and substrate depletion kinetics. B: Relative humidity and temperature during the reaction. Conditions: $a_w = 0.55$, m_{CRL} =60 mg, T =45 °C, 900 rpm.

days, 10 wt% water addition) [8], the reaction was significantly accelerated under the optimized conditions, reaching a higher LA conversion and reducing the reaction time by 7-fold. The improved ester formation under the optimized conditions can be explained on the one hand by a suitable water supply for the enzyme to be active at the DES-water interface and by a sufficient control of the water content in the reaction medium due to the presence of a saturated salt solution. 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.

4. Conclusion

In biocatalysis DESs, or more generally eutectic mixtures, are an extremely interesting solvent class, since they allow for melting solid substrates to form liquid reaction media with high substrate concentrations. In this study, the potential of DESs acting as solvent and substrate was further exploited for a CRL catalyzed esterification reaction. The a_w has an important influence on the esterification performance. A RSM optimization approach involving three factors (a_w, applied enzyme amount and reaction temperature) resulted in optimized reaction conditions that enhanced the enzymatic (-)-menthyl laurate synthesis in the DES composed of both substrates ((-)-menthol: LA 3:1, mol/mol). This example demonstrates that it is possible to establish a high performance and environmentally friendly reaction in a non-conventional reaction medium. The use of a 2-in-1 substrate and solvent eutectic mixture (1) enables high substrate concentrations, (2) contributes to the reduction of waste, (3) avoids the use of organic solvents and (4) facilitates product recovery, since no additive/solvents have to be separated. Product purification and recovery of excess substrates is currently under investigation in our laboratory to provide more insight into whether DESs can be valuable alternative reaction media for enzymatic processes.

Conflict of interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00333.

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