Process Biochemistry 102 (2021) 1–9

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Development of environmentally friendly lipase-catalysed kinetic resolution of (*R*,*S*)-1-phenylethyl acetate using aqueous natural deep eutectic solvents

Manuela Panić, Mia Radović, Izabela Maros, Ana Jurinjak Tušek, Marina Cvjetko Bubalo *, Ivana Radojčić Redovniković

Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000, Zagreb, Croatia

ARTICLE INFO

Keywords:
Green chemistry
Kinetic resolution
Natural deep eutectic solvents
Novozym 435
(*R*)-1-phenylethanol
Stereoselective biocatalysis

ABSTRACT

Over the past decade, natural deep eutectic solvents (NADES) have become promising green solvent from both environmental and technological perspectives. The number of structural combinations encompassed by NADES is tremendous; thus, NADES with unique physicochemical properties can be designed for a particular purpose, such as for efficient biocatalytic processes. The aim of this work was to prepare several NADES based on renewable sources (biomass energy) and to apply these solvents in the industrially interesting process of lipase-catalysed (*R*)-1-phenylethanol synthesis. Based on experimental data on lipase behaviour in the tested NADES, choline chloride:glycerol (ChGly) was selected as the most promising solvent for the given reaction. The resulting (*R*)-1-phenylethanol was also successfully isolated and purified from the reaction mixture using liquid-liquid extraction with ethyl acetate, whilst ChGly was recycled and reused. (*R*)-1-phenylethanol and unreacted (*S*)-1-phenylethyl acetate were purified on silica gel columns with yields of 81.91 %. The biocatalyst was also successfully recycled and reused in 4 cycles. Based on laboratory-scale optimum conditions, (*R*)-1-phenylethanol synthesis performed in a 500 mL batch reactor achieved reaction conversions comparable to those obtained with millilitre-scale biocatalytic reactions. The downstream process was also performed on a preparative scale with a yield of 49.48 %.

1. Introduction

Single-enantiomer drugs have become the standard in pharmaceutical companies when working with compounds featuring stereogenic centres, and they have mobilised the chemical and biotechnological industries to develop versatile methods for the production of biologically active chiral compounds of high enantiomeric purity. Enantiomers of a single chiral drug may differ significantly in their biological effect (sometimes even having an opposite effect), as well as in their bioavailability; rate of metabolism; metabolites; excretion; potency and selectivity for receptors, transporters and/or enzymes; and toxicity [1]. Among chiral compounds, (*R*)- or (*S*)- 1-phenylethanol is of special interest because it is a high-value intermediate for drug and fine chemical production [2,3]. Enantiomerically pure 1-phenylethanol can be synthesised by traditional synthesis methods, such as oxidation of ethylbenzene or reduction of acetophenone using organometallic compounds as catalysts; however, the poor enantioselectivity and residual metal catalysts in the final products makes these approaches rather undesirable [4]. Enzymes, by contrast, display much higher enantioselectivity and better regioselectivity than the organometallic catalysts used in traditional organic synthesis. In the case of 1-phenylethanol production, lipase-catalysed hydrolysis is a convenient method, as a dehydrogenase-catalysed reduction must also be designed to regenerate coenzymes.

Apart from the necessity for highly efficient chiral drug preparation, the pharma industry typically generates significant amounts of waste, by-products and pollutants (e.g. contaminated solvents, depleted reagents, air pollutants, etc.). Therefore, the incorporation of green

Abbreviations: CALB, *Candida antarctica* lipase B; ChGlc, Choline chloride:glucose; ChGly, Choline chloride:glycerol; ChEG, Choline chloride:ethylene glycol; EGGlcFru, Ethylene glycol:glucose:fructose; GlcGly, Glucose:glycerol; GlcEG, Glucose:ethylene glycol; NADES, Natural deep eutectic solvents; SD, Standard deviation; SorEG, Sorbose:ethylene glycol.

* Corresponding author.
E-mail address: mcvjetko@pbf.unizg.hr (M. Cvjetko Bubalo).

https://doi.org/10.1016/j.procbio.2020.12.001
Received 10 September 2020; Received in revised form 9 November 2020; Accepted 1 December 2020
Available online 3 December 2020

chemistry approaches for environmentally sustainable preparations have also become an imperative [5]. The basics of green chemistry suggest that chemical products and processes should be designed to avoid the application and creation of harmful and dangerous reagents, catalyst and products, with the unique goal of protecting the environment by inventing new chemical processes that do not pollute, rather than depending on clean-up. Numerous tools have been studied for green chemistry, such as the use of alternative solvents and environmentally benign raw (or renewable) materials, the use of alternative energy sources and performing reactions by biocatalysis [6].

An overview of the current literature on chiral drug production and formulation reveals exciting, new, efficient and green approaches, particularly involving the use of natural deep eutectic solvents (NADES) as media for the biochemical preparation of chiral biologically active compounds and as auxiliaries in the development of chiral drug delivery systems [7,8]. NADES have become promising green solvent from both environmental and technological perspectives. These solvents are mixtures of cheap, natural, non-toxic and readily available components prepared by mixing quaternary ammonium salts (e.g. choline chloride) and hydrogen bond donors based on natural products (e.g. alcohols, organic acids, sugars, vitamins and amines), as well as water in some cases, in a specific molar ratio. The end results is a homogenous solution based on hydrogen bonds between the NADES components [9,10].

NADES fully represent green chemistry principles owing to their specific properties, which include non-volatility, non-flammability and stability, together with a low ecological footprint. For these reasons, NADES are almost ideal solvents for a wide range of fields ranging from biocatalysis, extraction and electrochemistry to carbon dioxide capture and biomedical applications. NADES may be considered 'designer solvents' due to their numerous structural variations and the possibility to design their physicochemical properties for optimal performance in a specific process [11]. NADES are produced from naturally occurring molecules; therefore, they have an inherently low toxicity while also providing a natural environment for proteins, enzymes and other biologically active molecules [12].

The synergistic use of NADES and biotechnological methods (i.e. the use of enzymes as catalysts) fits logically with the efficient and sustainable production of enantiomerically pure compounds. Biotechnological approaches (e.g. biocatalysis) ensure the catalysis of otherwise difficult transformations with high regioselectivity, chemoselectivity and enantioselectivity under mild and cost-effective conditions, while the use of NADES can provide strong green support to modulate/direct the reaction route to obtain the desired product. Previous papers dealing with the use of NADES as media for enantiopure compound preparation/recovery have implied that the unique properties of these solvents make them promising candidates for reactions catalysed either by isolated enzymes or by whole cells. NADES use can improve substrate/product solubility, enhance enzyme activity and stability, increase reaction yields, provide the possibility of tailoring the reaction enantioselectivity and regioselectivity and allow NADES recycling and reuse [13–18].

The present study presents the development of green lipase-catalysed process for the production of an optically pure secondary alcohol (*R*)-1-phenylethanol, through screening of the NADES, optimization of the process, followed by isolation of the product and scale-up. The results were also used to formulate postulates for the development and scale-up of eco-friendly biocatalytic technology using NADES.

2. Material and methods

2.1. General

Novozym 435 (lipase B from *Candida antarctica*; immobilised on macroporous polyacrylic resin beads) was obtained from Novozym (Bagsvard, Denmark). (*R*)-1-phenylethyl acetate, (*S*)-1-phenylethyl acetate, (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, *n*-heptane, ethyl acetate and all chemicals for NADES preparation were purchased from Sigma–Aldrich, Germany (purity of ≥99 %) and used without further purification.

Reaction mixtures were analysed by gas chromatography on a Shimadzu QP2010PLUS instrument equipped with Varian CHIRASIL-DEX CB capillary chiral column (25 m ×0.25 mm ×0.25 μm) and a mass spectrum (MS) detector.

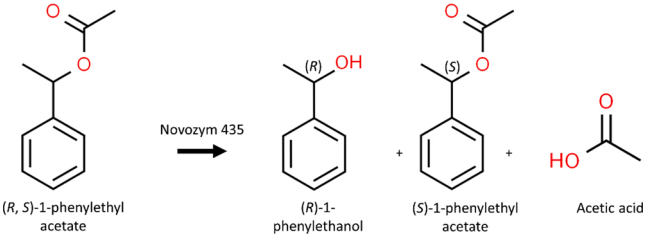
2.2. Preparation and characterisation of NADES

Choline chloride was dried at 60 °C for 24 h in a vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo Scientific, USA) prior to use. The following components, in specific ratios, together with 10, 30 or 50 % (v/v) water, were placed in a round-bottomed glass flask: choline chloride:glucose (ChGlc, 1:1), choline chloride:glycerol (ChGly, 1:2), choline chloride:ethylene glycol (ChEG, 1:2), glucose: glycerol (GlcGly, 1:2), glucose: ethylene glycol (GlcEG, 1:2), sorbose: ethylene glycol (SorEG, 1:2) or ethylene glycol:glucose:fructose (EGGlcFru, 2:1:1). The mixture of HBA (hydrogen bond acceptor), HBD (hydrogen bonf donor) and water was stirred in a flask at 50 °C for 2 h. The pH value and polarity of the prepared solvents were measured according to Panić et al. [19].

2.3. Lipase catalysed hydrolysis of (R,S)-1-phenylethyl acetate

For initial screening of NADES, the reaction was started by adding 5 mg Novozym 435 to 1.0 mL solvent (buffer or NADES) containing 0.05 mol L^{-1} (8.2 mg) (*R, S*)-1-phenylethyl acetate. Reactions without the enzyme were also performed. A separate reaction was conducted for each measurement point.

The lipase-catalysed hydrolysis is represented as Eq. (1):



(1)

At specified time intervals, the reaction was stopped, 1 mL water was added and the reaction mixture was extracted with 9 mL *n*-heptane on vortex shaker (3 min). The organic phase was analysed by gas chromatography under the following conditions: injector (220 °C); detector (200 °C); column temperature 80 °C for 2 min, then 80 °C – 140 °C (5 °C min^{-1}); and helium as the carrier gas at a flow rate of 96.9 mL min^{-1}. Substrates and products were identified with external standards and the quantified using calibration curves of (*R*)-1-phenylethanol (0.409–35 mmol L^{-1}).

The efficiency of the proposed biocatalytic reaction was monitored to determine the initial reaction rate (k_0), conversion (X), enantiomeric excess (*ee*) and volumetric productivity (V_p), (Eq. 2–5). The initial reaction rate (k_o, mmoL L^{-1} min^{-1}) was calculated from the linear parts of the plots of product concentration vs reaction time, according to Eq. (2):

$$k_0 = \frac{a_p}{t} \tag{2}$$

where a_p is the slope of the line (mmol L^{-1}) and t is time.

The reaction conversion (X, %) was calculated according to Eq. (3):

$$X = \frac{c_A}{c_{AT}} \times 100 \tag{3}$$

where c_A is the concentration of (*R*)-1-phenylethanol (mol L^{-1}) and c_{AT} is the maximal theoretical concentration of (*R*)-1-phenylethanol (mol L^{-1}).

Enantiomeric excess (ee, %) was calculated according to Eq. (4):

$$ee = \frac{(R_{OH} - S_{OH})}{(R_{OH} + S_{OH})} \times 100 \quad (4)$$

where R_{OH} is the area under the curve of (*R*)-1-phenylethanol (mol L^{-1}) and S_{OH} is the area under the curve of (*S*)-1-phenylethanol (mol L^{-1}).

Productivity (V_P, mmol L^{-1} min^{-1}) was calculated according to Eq. (5):

$$V_P = \frac{c_{p2} - c_{p1}}{t} \quad (5)$$

where c_{P1} is the concentration of (*R*)-1-phenylethanol (mol L^{-1}) at 0 h, c_{P2} is the concentration of (*R*)-1-phenylethanol (mol L^{-1}) at the end of the reaction, and *t* is the time of the reaction (min).

All experimental measurements were performed in triplicate, and the average values were reported (at 95 % confidence intervals, the results have no statistically significant differences).

2.4. Storage stability of the enzyme

The storage stability of Novozym 435 in the prepared solvents was measured according to the procedure reported by Cvjetko Bubalo et al. [15]. Briefly, the enzyme was incubated in ChGly and ChEG with 10, 30 and 50 % water or in phosphate buffer at room temperature. At the specified time intervals, the enzyme reaction was initiated by adding the butyl acetate substrate and the reaction was monitored over time. The results were presented as residual enzyme activity.

The residual activity (*A*, %) was calculated according to Eq. (6):

$$A = \frac{k_1}{k_0} \quad (6)$$

where k_1 is the initial reaction rate obtained by the enzyme after incubation in a given solvent and k_0 is the initial reaction rate obtained without prior incubation.

Inactivation rate constants (K_d) were calculated according to first order kinetics using StatSoft Statistica version 8.0 software.

2.5. Optimisation of (R)-1-phenylethanol synthesis in NADES

The kinetic resolution of (*R,S*)-1-phenylethyl acetate in ChGly was optimised using the Box-Behnken design. The influence of the independent variables of time (X_1, 1–6 h), water content in ChGly (X_2, 10–50 %, v/v) and temperature (X_3, 20–60 °C) was studied on the dependent variables of the reaction conversion (Y, *X*, %).

The extraction process was optimised by performing 15 experiments with 3 centre points per block, and the responses were analysed using the numerical tools provided by Design Expert (Version 7.0.0., Suite 480, Minneapolis, MN 55,413). Each experiment was performed with 5 mg Novozym 435 and 1 mL ChGly containing 0.05 mol L^{-1} (8.2 mg) (*R, S*)-1-phenylethyl acetate. The responses were fitted with second-order polynomial equation (response surface methodology [RSM] model), as described by Sontakke [20],

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \; i < j \quad (7)$$

where X_1, X_2,.. ., X_k are the independent variables, Y is the dependent variable, β_0, βj (i = 1, 2,.. ., k), β_{ii} (i = 1, 2,.. ., k), and β_{ij} (i = 1, 2,.. ., k; j = 1, 2,.. ., k) are the regression coefficients for the intercept, linear, quadratic and interaction terms, respectively, and k is the number of variables.

The second-order polynomial coefficients were calculated by applying analysis of variance (ANOVA) using Design Expert (Version 7.0.0., Suite 480, Minneapolis, MN 55,413) software. The value of the determination (R^2) was used to predict the model capability.

In all 15 experiments, the reaction was stopped at specified time intervals and the products were analysed by GCMS, as described in section 2.3.

2.6. Downstream process for enzyme and NADES recovery at the laboratory scale

Upon completion of the kinetic resolution of (*R,S*)-1-phenylethyl acetate under optimal reaction conditions (50.4 °C, 47.75 % of water in ChGly and 5 h 52 min), the reaction medium containing the Novozym 435 was filtered under vacuum. The recovered enzyme was washed with water (5 × 10 mL) and ethyl acetate (5 × 10 mL), dried in an oven at 40 °C overnight and stored in a fridge until the next run. A 5 mg sample of the recovered Novozym 435 was weighed and used again for hydrolysis of (*R,S*)-1-phenylethyl acetate in fresh ChGly with 50 % water. The reaction was monitored and analysed as described in section 2.3. This recycling and reuse of the enzyme was repeated for 5 cycles.

Enzyme reusability ($\eta_{relative}$, %) was calculated according to Eq. (8):

$$\eta_{relative\ (\%)} = \frac{\eta_{run\ (1+n)}}{\eta_{run\ (1)}} \cdot 100, \; n = 1, 2, 3.... \quad (8)$$

where $\eta_{run(1)}$ is the reaction yield with fresh enzyme and $\eta_{run(1+n)}$ is the reaction yield with the enzyme after recycling once, twice...,five times.

NADES recycling and recovery was performed as a separate experiment. The kinetic resolution of (*R,S*)-1-phenylethyl acetate in optimal conditions was stopped after 5 h 52 min, the enzyme was filtered from the reaction medium, and the reaction medium was placed in a separatory funnel and extracted with ethyl acetate (5 × 10 mL). The organic phases were collected and evaporated under reduced pressure to yield crude products. The regenerated NADES were tested for all four rounds of assays.

NADES reusability ($V_{relative}$, %) was calculated according to Eq. (9):

$$V_{relative\ (\%)} = \frac{V_{run\ (1+n)}}{V_{run\ (n)}} \cdot 100, \; n = 1, 2, 3... \quad (9)$$

where, $V_{run(1)}$ is the volume of NADES in the n-cycle and $V_{run(1+n)}$ is the volume of the recycled NADES after each n-cycle.

The reaction conversion in the recycled NADES was calculated according to Eq. (3).

The (*R*)-1-phenylethanol and unreacted (*S*)-1- phenylethyl acetate from the organic fraction were separated on a silica gel column (1.2 cm × 5 cm) using *n*-heptane:ethyl acetate (4:1, v/v) as the mobile phase.

Separation efficiency was calculated according to Eq. (10):

$$\eta = \frac{m_k}{m_0} \quad (10)$$

where m_k is the mass of the (*R*)-1-phenylethanol after the silica gel column separation and m_0 is the mass of (*R*)-1-phenylethanol achieved by hydrolysis.

2.7. Preparative scale of kinetic resolution of (R,S)-1-phenylethyl acetate in ChGly and isolation of (R)-1-phenylethanol

A 250 mg sample of Novozym 435 was added to 300 mL choline chloride:glycerol in 47.75 % water containing 0.05 mol L^{-1} (2.5 g) (*R, S*)-1-phenylethyl acetate. The reaction was run at 50.4 °C for 5 h 52 min on an ES-20/60 Orbital Shaker-Incubator. The Novozym 435 was then filtered from the reaction medium under vacuum. The corresponding (*R*)-1-phenylethanol was isolated by extraction using ethyl acetate as the renewable solvent. The extraction was performed in 5 cycles, and the ethyl acetate fraction was collected each time. The (*R*)-1-phenylethanol and unreacted (*S*)-1- phenylethyl acetate from the organic fraction were separated on a silica gel column, as described in section 2.6.

The reaction yield was calculated according to Eq. 11:

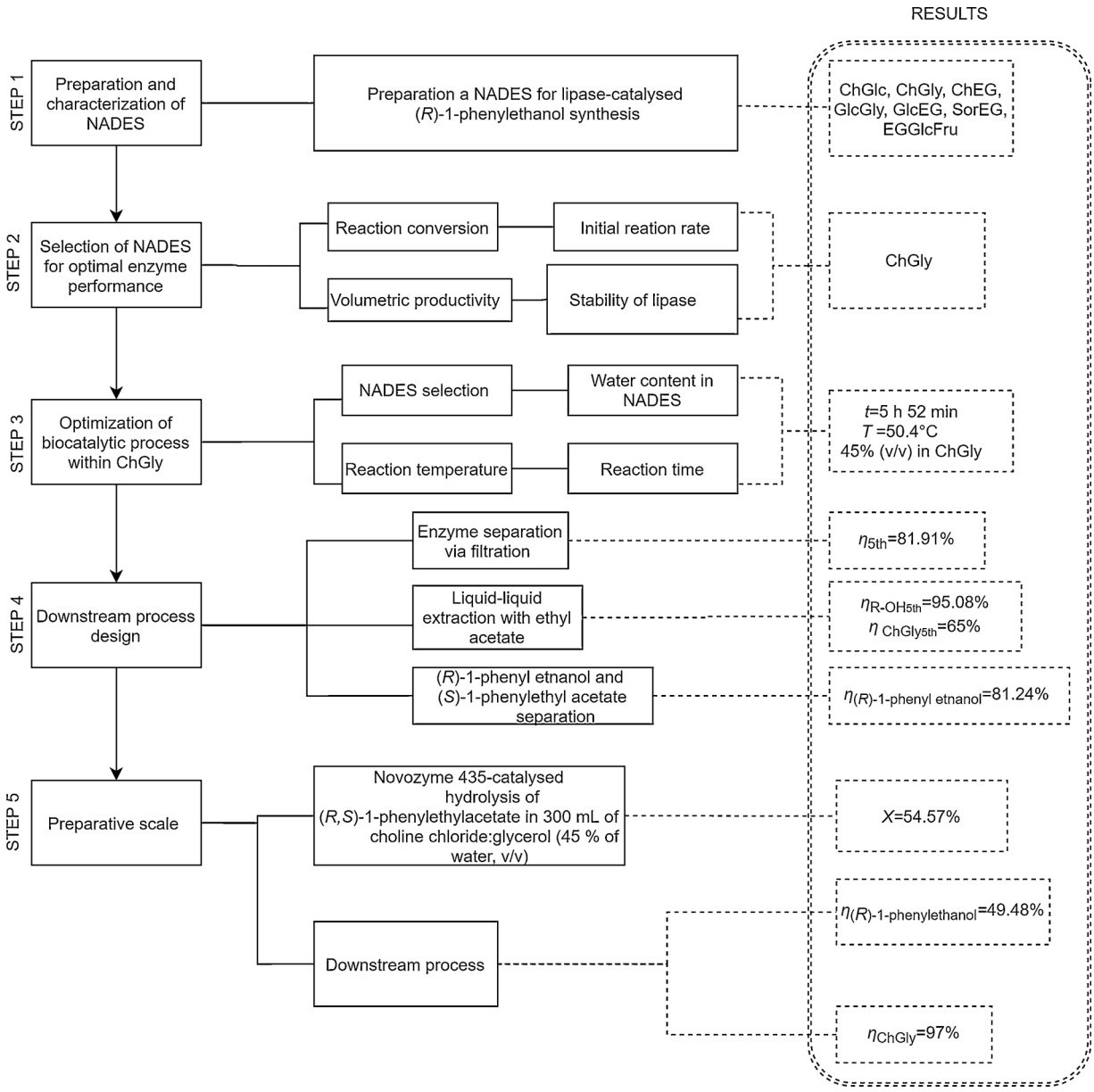


Fig. 1. Flowchart of experimental design and results of development of an environmentally friendly method for lipase-catalyzed (*R*)-1-phenylethanol production in natural deep eutectic solvents (NADES).

$$\eta = \frac{m1}{mo} \tag{11}$$

where m_1 is the mass of the (*R*)-1-phenylethanol after reaction and m_0 is the mass of (*R*)-1-phenylethyl acetate before the biocatalytic reaction.

2.8. Data analysis

All experimental results were statistically analysed using Statistica 8 software. Data in the text and tables are expressed as the mean ± standard deviation (±SD), and error bars in the figures indicate the SD. The differences between the means were analysed by ANOVA, followed by a post-hoc Tukey's test. A p value < 0.05 was considered statistically significant.

3. Results and discussion

The aim of this study was to develop a NADES-assisted protocol for the synthesis of (*R*)-1-phenylethanol that would be of interest from both economic and ecological points of view. For that purpose, the kinetic resolution of lipase catalysis of (*R,S*)-1-phenylethyl acetate within NADES was performed. For this research, the following major points governed the design of the biocatalytic reaction involving NADES: (*i*) screening of NADES for optimal enzyme performance; (*ii*) optimisation of the biocatalytic protocol; and (*iii*) preparative-scale upstream and downstream (*R*)-1-phenylethanol synthesis (Fig. 1).

3.1. NADES selection for optimal lipase-catalysed performance for (R)-1-phenylethanol synthesis

This work examined the enantioselective hydrolysis of (*R, S*)-1-phenylethyl acetate to (*R*)-1-phenylethanol by immobilised *Candida antarctica* lipase B (Novozym 435) in 8 different cholinium-based or cholinium-free NADES (Table 1S). Enantioselective hydrolysis was also performed in 0.025 M potassium phosphate buffer as a reference solvent. Blank experiments without the enzyme were also performed for all tested NADES, and no conversion was observed within the reaction

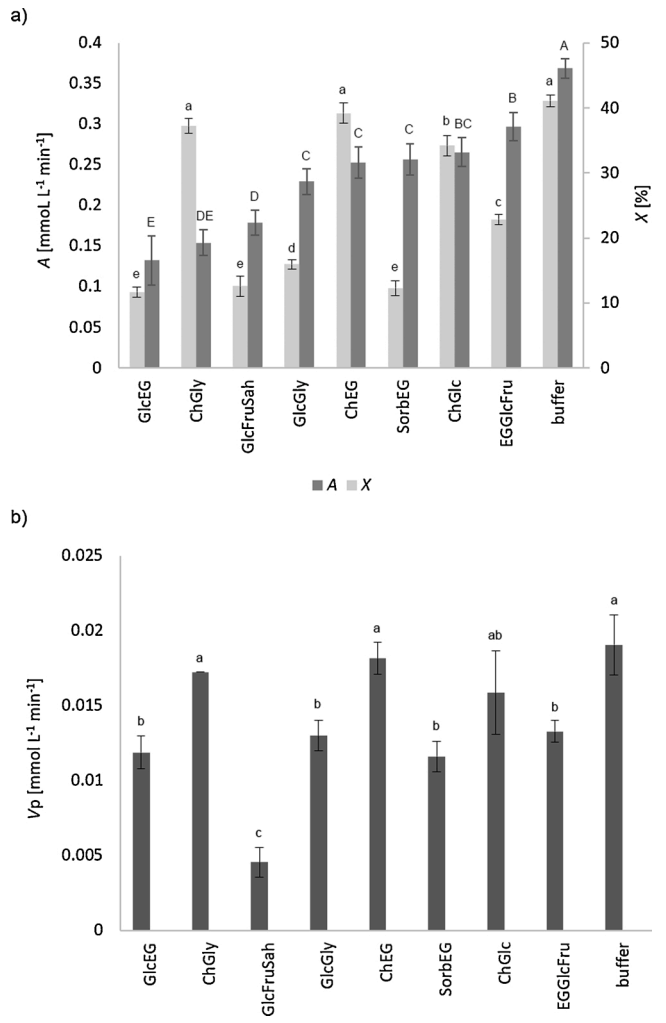


Fig. 2. Initial reaction rates (*A*), conversion (*X*) (a) and productivity (*Vp*) (b) of Novozym 435—mediated hydrolysis of (*R,S*)-1-phenylethyl acetate within different natural deep eutectic solvents (NADES) (50 %, v/v) and buffer. Reaction conditions: 0.05 M (*R,S*)-1-phenylethyl acetate; 5 mg Novozym 435; 25 °C; 1 mL. Each data point represents the mean of triplicate experiments; error bars represent the standard deviation (SD). Every group of results followed by nonidentical letters (a-e and A—E) indicate signifcant diff ;erence ($p < 0.05$) determined by Tukey's HSD test.

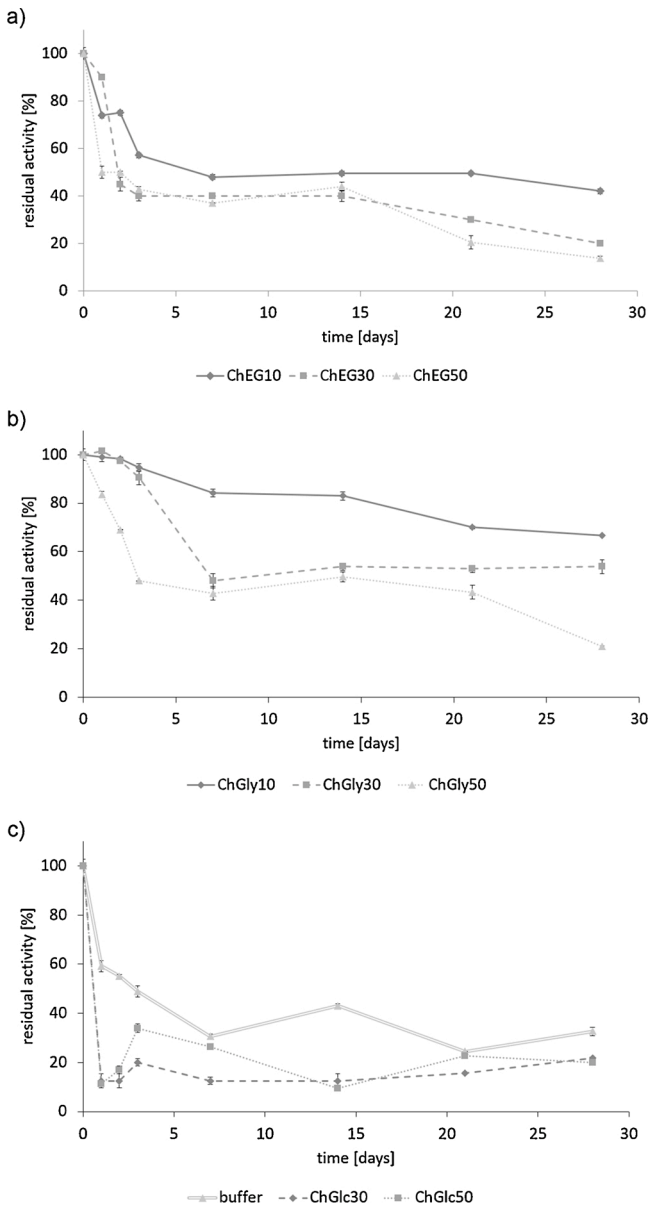


Fig. 3. Residual activity of Novozym 435 after incubation in choline chloride: ethylene glycol (ChEG) (a) choline chloride:glycerol (ChGly) (b), choline chloride:glucose (ChGlc), and buffer (c) at 25 °C. Reaction conditions: 0.1 mol L^{-1} butyl acetate; 5 mg Novozym 435; 25 °C; 1 mL of solvent. Each data point represents the mean of triplicate experiments; error bars represent the standard deviation (SD).

times used in this study (data not shown).

The selected NADES were weakly acidic to neutral, since lipase is well known to perform reactions under mild conditions (pH and temperature), with remarkable chemoselectivity, regioselectivity and stereoselectivity [6,21]. The price of using NADES was estimated based on the cost of raw materials and varied from approximately 5.57 € kg^{-1} for GlcGly up to 656.89 € kg^{-1} for SorEG, in agreement with the cost of conventional solvents (Table 1S).

The results indicated that lipase exhibits a strong *R*-stereopreference, regardless of the solvent choice (ee~99 %). This stereopreference is advantageous, as it allows the direct production of the desired *R*-alcohol without further chemical manipulation. Rios et al. [2] also noted enantioselectivity of a lipase to hydrolyse only (*R*)-phenylethyl acetate in a racemic mixture of this ester. Conversely, the measured initial reaction rates (k_o), conversion (*X*) and productivity (V_p) showed a dependence on the NADES used. The values for k_o, *X* and V_p ranged from 0.1323–0.369 mmoL L^{-1} min^{-1}, 11.69–41.14 % and 0.00456–0.01905 mmoL L^{-1} min^{-1}, respectively (Fig. 2). The highest k_o was observed in the conventional solvent, potassium-phosphate buffer.

No improvement was observed in the *X* and V_p values in the optimal NADES (ChGly, ChEG and ChGlc) compared to the reference solvent (buffer); however, the stability of the enzyme was significantly enhanced in certain NADES (Fig. 2 and 3). The influence of NADES on lipase-catalysed reaction mechanism was previously proposed by Cvjetko Bubalo et. al. [15]. Authors suggested that lipase activity in cholinium-based NADES containing polyols as HBD could be explained by increase in nucleophilicity of the hydroxyl group of the serine residue in active lipase site and by the fact that this solvent supports stabilization of tetrahedral inter mediates through H-bonding, leading to decrease in the activity energy barrier and therefore increase the reactivity. The storage stability of Novozym 435 was monitored at 25 °C for 28 days in ChGly, ChEG, ChGlc and buffer (Fig. 3). ChGlc with 10 % of water was too viscous for manipulation, so no experiments were performed in that solvent. In summary, no differences were observed in Novozym 435 stability in the various solvents at room temperature.

Table 1
Deactivation constants (K_d) of Novozym 435 under different inactivation solvents on 25 °C during 30 days.

Solvent	Abbreviation	water content in solvent [%, v/v]	K_d [day^{-1}]
Choline chloride: ethylene glycol	ChEG	10	0.0244 ± 0.00699
		30	0.0428 ± 0.00113
		50	0.0519 ± 0.0103
Choline chloride: glycerol	ChGly	10	0.015 ± 0.00125
		30	0.0249 ± 0.00840
		50	0.0401 ± 0.00959
Choline chloride: glucose	ChGlc	30	0.0215 ± 0.0303
		50	0.0388 ± 0.0422
buffer			0.0317 ± 0.0116

The only exception was ChGly, where immobilised *Candida antarctica* lipase B showed a much higher stability (−80 % of initial activity, 28 days) compared to buffer (−40 % of initial activity, 28 days) (Fig. 3). This finding implied that NADES could serve as a stabilising medium for this lipase. Similarly, when monitoring the stability of *Candida antarctica* lipase B immobilisation with cross-linking aggregates, Guajardo et al. also noted a higher stability of the enzyme in ChGly than in other NADES (ChEG and ChU) [23]. These results regarding storage stability of Novozym 435 in ChEG are in agreement with the results of Cvjetko Bubalo et al. [15], who found an approximately 50 % decrease in the initial lipase activity after a 3 day incubation of the enzyme in ChEG. Nevertheless, extending the incubation time up to 15 days did not result in any significant enzyme activity loss [15]. In the present study, NADES stabilised the enzyme. Zhou et al. used circular dichroism spectroscopy to examine the effect of DES (deep eutectic solvents) on the structural integrity of *Candida antarctica* B for a deeper understanding of the stability of the enzyme [24]. They concluded that the increased stability of the enzyme was due to the preservation of its structural integrity in DES.

The inactivation rate constants (K_d) were also calculated (Table 1). The rate of enzyme inactivation was the highest in ChEG with 50 % water (0.0519 day^{-1}) and the lowest in ChGly with 10 % water (0.0150 day^{-1}). The measured K_d values decreased with a higher content of NADES. The dependence of Novozym 435 deactivation on the water content in NADES was also reported in a previous study of the influence of water content in ChGly for the *Candida antarctica* lipase B-catalysed reaction of acetic anhydride with 1-butanol to give the butyl acetate ester [15].

Based on the experimental data on lipase behaviour in the tested NADES and the enzyme stability in NADES, ChGly was selected as the most promising solvent for the given reaction; therefore, it was used for further optimisation.

3.2. Laboratory scale process optimisation

The biocatalytic process has two parts which should be optimised: the upstream (product development) and downstream (product purification) parts. After choosing the optimal NADES and optimisation of the pH, polarity and viscosity of the medium, the next step was optimisation of the biocatalytic protocol. A search of the literature revealed that the amount of water in the NADES, the temperature, the enzyme-to-substrate ratio, the pH and the reaction time all influenced the enzyme activity [6]. The presence of water in the NADES (>60 %, w/w) may damage the strong hydrogen-bonding network of the NADES [24, 25], whereas lower amounts of water (approx. 30 %) could negatively influence hydrolysis. Apart from water, other well-known process parameters that need optimisation are the reaction temperature and the substrate(s) concentration [26–28]. It is well known that raising temperature up to the temperature optimum speeds up enzyme-catalyzed reactions, whereas high temperatures (depending of enzyme origin and type) cause an enzyme to lose its active shape. Adjusting reaction temperature in DES-mediated biocatalytic reactions is especially important as elevation of temperature reduces viscosity of DES, enabling better mass transfer in such viscous environment and consequently higher reaction rate [22,29]. Based on our preliminary data and the current literature, the following independent variables for RSM analysis were selected in this study: reaction time (1–6 h), water content in ChGly (10–50 %) and temperature (20–60 °C). The enzyme-to-substrate ratio was fixed at 1.3 according to our preliminary results (data not shown).

Table 2
Experimental matrix and values of observed response. The numbers in bracket are level factorials of experimental matrix.

Run	t [h]	$w_{(H2O,NADES)}$ [%, w/w]	T [°C]	X [%]
1	3.5(0)	10(-1)	20(-1)	20.02
2	3.5(0)	50(+1)	60(+1)	50.52
3	3.5(0)	50(+1)	20(-1)	24.90
4	6(+1)	50(+1)	40(0)	42.03
5	3.5(0)	30(0)	40(0)	35.99
6	1(-1)	30(0)	60(+1)	36.65
7	3.5(0)	10(-1)	60(+1)	41.06
8	6(+1)	30(0)	20(-1)	27.11
9	3.5(0)	30(0)	40(0)	35.71
10	1(-1)	10(-1)	40(0)	21.44
11	1(-1)	50(+1)	40(0)	24.20
12	3.5(0)	30(0)	40(0)	35.75
13	6(+1)	10(-1)	40(0)	31.58
14	6(+1)	30(0)	60(+1)	55.28
15	1(-1)	30(0)	20(-1)	19.74

*t -time, $w_{(H2O,NADES)}$- water content in NADES, T- temperature.

Table 3
Analysis of variance (ANOVA) of the modelled responses.

Source	*Conversion (Quadratic)*			
	Sum of squares	*Mean square*	*F-value*	*p-value*
Model	1640.42	182.27	906.56	< 0.0001
X_1	364.08	364.08	1810.83	< 0.0001
X_2	94.82	94.82	471.63	< 0.0001
X_3	1052.04	1052.04	5232.61	< 0.0001
$X_1 X_2$	14.79	14.79	73.57	0.0004
$X_1 X_3$	31.72	31.72	157.76	< 0.0001
$X_2 X_3$	5.25	5.25	26.13	0.0037
X_1^2	27.20	27.20	135.29	< 0.0001
X_2^2	39.90	39.90	198.47	< 0.0001
X_3^2	9.41	9.41	46.81	0.0010
Residual	1.01	0.20		
Lack of fit	0.96	0.32	13.81	0.0683
Pure error	0.046	0.02		
Total	1641.42			
R^2	0.9994			

$p < 0.01$ highly significant; $0.01 \leq p < 0.05$ significant; $p \geq 0.05$ not significan. X_1:time; X_2: water content in ChGly; X_3: temperature.

The RSM model was evaluated and analysis of variance (ANOVA)

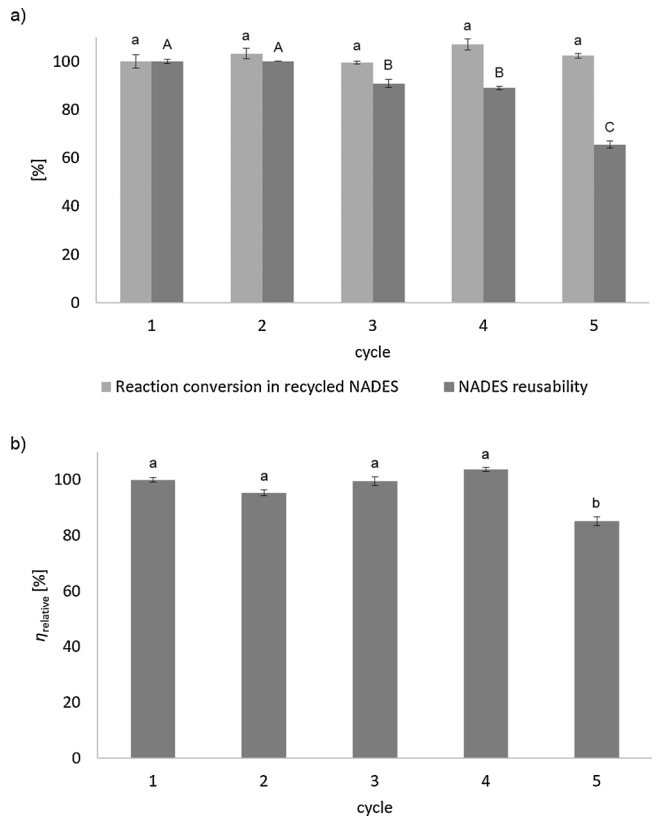


Fig. 4. Natural deep eutectic solvents (NADES) reusability and reaction conversion in recycled NADES (a) and enzyme reusability (%) (b), of (*R*)-1-phenylethanol synthesis in choline chloride:glycerol (ChGly) with 47.75 % of water. Reaction conditions: 0.05 M (*R,S*)-1-phenylethyl acetate; 5 mg Novozym 435; 40 °C; 1 mL. Each data point represents the mean of triplicate experiments; error bars represent the SD. Every group of results followed by nonidentical letters (a-b and A—C) indicate signifcant diff ;erence ($P < 0.05$) determined by Tukey's HSD test.

was used to calculate the statistical significance of the model. The model for the independent variable was selected according to this determination and the adjusted coefficient (R^2) and the model *p* value (Table 3).

The second-order polynomial equations for conversion were as follows:

$$Conversion\ (\%) = \\ = 35.81 + 6.75 \cdot X_1 + 3.44 \cdot X_2 + 11.47 \cdot X_3 + 1.92 \cdot X_1X_2 \\ + 2.82 \cdot X_1X_3 + 1.15 \cdot X_2X_3 - 2.71 \cdot {X_1}^2 - 3.29 \cdot {X_2}^2 \\ + 1.60 \cdot {X_3}^2$$

where X_1 is time, X_2 is the water content in ChGly and X_3 is the temperature.

The results show that the independent variables had a significant effect ($p < 0.001$). All four variables also had a positive coefficient, whereby an increase in a specific variable led to an increased conversion. One important point to mention is that ANOVA also showed statistical significance of both the quadratic and interaction factors.

ANOVA also showed good agreement between the data predicted by the model and the experimental data, with a determination coefficient of $R^2 = 0.9994$, $R^2_{adjusted} = 0.9983$ and with no significant lack of fit at $p > 0.05$. The determination coefficient indicated that the proposed model was able to explain 99.94 % of the results. The results also showed that the model used to fit the response variable was significant (< 0.0001) and suitable for description of the relationship between the independent variables and the response. The F-test suggested that the model had a very high model *F*-value ($F = 906.56$), indicating that this model was highly significant.

The experimental data acquired were used for the creation of supplementary Fig. 1S and 2S, which showed that the hydrolytic conversion of (*R,S*)-1-phenylethyl acetate to (*R*)-1-phenylethanol increased with the reaction temperature and reaction time. Conversely, the water content in the ChGly had a double effect on each response. An increase in water content from 10 % to 45 % in the ChGly resulted in an increased conversion, whereas a further increase led to a decreased conversion. This once again indicates the importance of optimising the water content when applying NADES as reaction solvents. The developed RSM model showed that the optimum conditions for conversion were a time of 5 h 52 min, a water content of 47.74 % and a temperature of 50.4 °C. The model predicted a conversion of 50.14 % as optimum. After performing the experiment under these optimal conditions, the value of *X* was 52.30 ± 1.11 %, which closely agreed with the predicted value.

The major cost in a biocatalytic process in industry lies in the downstream purification [30]; therefore, the last steps in designing an efficient biocatalytic process involving NADES are product isolation and purification and enzyme and NADES recycling and reuse. For this reason, downstream separation of (*R*)-1-phenylethanol from ChGly was performed. When performing biocatalysis in an organic solvent, the solvent is usually evaporated and the final product is further purified. However, the low vapour pressure of NADES makes these solvents very difficult to evaporate, which may pose a problem for industrial applications [31]. Only a few research groups have reported a downstream separation of a product after a biocatalytic process in NADES [14,32,33]. In the present research, the obtained *R*-alcohol and unreacted (*S*)-1-phenylethyl acetate were isolated by extraction using ethyl acetate as the renewable solvent. Novozym 435 was removed by filtration, and the (*R*)-1-phenylethanol and unreacted (*S*)-1-phenylethyl acetate from the organic fraction were separated on a silica gel column using *n*-heptane:ethyl acetate (4:1) as the mobile phase (Fig. 4). The yield of the separated product was 81.24 %.

After removing the product and unreacted substrate, the NADES was recycled. The ChGly with 47.74 % water was also recycled and reused for another round of (*R*)-1-phenylethanol production (Fig. 4). The yield of the NADES ChGly after recycling five times decreased by 35 % compared to the freshly synthesised solvent due to the downstream procedures. Hydrolysis was also performed in the recycled NADES, and the conversion and enantioselectivity in the recycled ChGly with 47.75 % water was similar to that achieved with freshly synthesised NADES.

After the isolation and purification of (*R*)-1-phenylethanol under the RSM-optimised conditions (at a millilitre scale), Novozym 435 was also recycled and reused. Novozym 435 was washed with ethyl acetate and water, dried, and used again for the kinetic resolution of (*R,S*)-1-phenylethyl acetate in (*R*)-1-phenylethanol in fresh ChGly. The procedure was repeated four times (Fig. 4). At the end of the fifth cycle, the relative reaction yield of Novozym 435 was 81.91 %, while the *ee* (>99 %) did not decrease in any of the five repeated batches. Liang et al. also noticed a similar effect when studying recycling of Novozym 435 in a buffer-based media, as the *ee* value did not change through 11 cycles and the relative activity was 70 % after the eleventh cycle [34]. By contrast, a slightly larger decline in Novozym 435-activity was reported, at 47 % after just five cycles of recycling in 92 % (v/v) ChGly [35].

3.3. Preparative scaling (up and down)

After laboratory scale optimisation, the biocatalytic reaction was performed in a 500 mL batch reactor under the optimum conditions determined at the laboratory scale, followed by the downstream process, again following the conditions determined at the laboratory scale.

The preparative-scale upstream experiment proceeded as follows. Novozym 435 was used for hydrolysis of 0.05 mol L^{-1} (*R,S*)-1-phenylethyl acetate in an overall reaction volume of 300 mL ChGly with 47.75 % water at 50.4 °C for 5 h 52 min. The reaction conversion was 54.57 %, similar to that achieved at the laboratory scale. The enantioselectivity

Table 4
Summary of up- and down-stream results of biocatalytic process on laboratory and preparative scale.

	Upstream	Downstream	
	X [%]	η_{ChGly} [%]	$\eta_{(R)\text{-1-phenyethanol purification}}$ [%]
Laboratory scale	50.14	100−65.5	81.24
Preparative scale	54.57	97	49.48

was the same as for the laboratory scale experiment (Table 4). The available literature indicates that the scalability of NADES-assisted biocatalysis has been performed only for a preparative scale (up to 500 mL). Xu et al. (2015) carried out the reduction of 3-chloropropiophenone catalysed by immobilised *Acetobacter* sp. CCTCC M209061 cells in a ChU- containing system at a 500 mL scale under the optimal reaction conditions determined at the laboratory scale [36]. The yield (82.3 %) was lower than that obtained at the 5.0 mL scale (93.3 %), but the product *ee* was still high as at the laboratory scale. Panić et al. scaled up the reduction of 1-(3,4-dimethylphenyl)ethanone (DMPA) by *S. cerevisiae* in ChGly to 300 mL from the laboratory scale [14]. The conversion of hydrolysis at the larger scale was 54.57 %, similar to the millilitre scale (Table 4).

The yield of purified (*R*)-1-phenylethanol was 49.48 %, or approximately 30 % lower than the laboratory scale. The NADES recycling was 97 %. Panić et al. also performed a downstream process after preparative scale-up and their reaction yield was lower or similar to the millilitre scale, as in our case [14].

4. Conclusions

A highly efficient and eco-friendly laboratory method was developed for the preparation of (*R*)-1-phenylethanol with lipase in NADES. Based on the experimental data on lipase behaviour in the tested NADES, the NADES characteristics and the enzyme stability in NADES, choline chloride:glycerol (ChGly) was selected as the most promising solvent for the given reaction. Response surface methodology used to optimise the biocatalytic process parameters gave the following optimum conditions for the highest conversion of (*R*)-1-phenyethyl acetate in (*R*)-1-phenylethanol: a time of 5 h 52 min, a water content of 47.74 % and a temperature of 50.4 °C. The produced (*R*)-1-phenylethanol was successfully isolated and purified from the reaction mixture using liquid-liquid extraction with ethyl acetate, and ChGly was recycled and reused. The (*R*)-1-phenylethanol and unreacted (*S*)-1-phenylethyl acetate were also purified on a silica gel column. The biocatalyst was also successfully recycled and reused in four cycles. After laboratory-scale optimisation, the production of (*R*)-1-phenylethanol was also successfully performed with the similar efficiency at a preparative scale, making this process very interesting for future industrial applications. ChGly was successfully recycled and reused after biocatalysis. Special emphasis should be given to the observed enhanced stability of Novozym 435 in ChGly. This contribution can be valuable from an economic point of view for recycling and reuse of enzymes, as well as performing integrated processes.

Credit Author Statement

MCB and IRR designed the main experimental work. IM, MR and MP carried the experimental work. MP drafted the manuscript while MCB reviewed the manuscript. AJT performed statistical calculations. All authors together finalized the manuscript and approved it.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The work was supported by the Croatian Science Foundation (Grant No. 9550and7712).

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.procbio.2020.12.001.

References

[1] L.A. Nguyen, H. He, C. Pham-Huy, Chiral drugs: an overview, Int. J. Biomed. Sci. 2 (2006) 85–100. http://www.ncbi.nlm.nih.gov/pubmed/23674971%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3614593.
[2] N.S. Rios, M.P. Pinheiro, J. Cleiton, S. Santos, T.D.S. Fonseca, L.D. Lima, M.C. De Mattos, D.M.G. Freire, J. Ivanildo, S. Júnior, E. Rodríguez-aguado, L.R.B. Gonc, Strategies of covalent immobilization of a recombinant Candida antarctica lipase B on pore-expanded SBA-15 and its application in the kinetic resolution of (R, S) -Phenylethyl acetate, J. Mol. Catal., B Enzym. 133 (2016) 246–258, https://doi.org/10.1016/j.molcatb.2016.08.009.
[3] L. Ma, X. Liu, D. Li, Z. Zhang, (R) -1-Phenylethanol Production from Racemic 1-Phenylethanol By Double Strains Redox-Coupling, 238, 2011, pp. 981–985, https://doi.org/10.4028/www.scientific.net/AMR.236-238.981.
[4] G.A. Burdock, Fenaroli's Handbook of Flavor Ingredients, sixth edition, 2016.
[5] J.L. Tucker, M.M. Faul, Industrial research: drug companies must adopt green chemistry, Nature 534 (2016) 27–29, https://doi.org/10.1038/534027a.
[6] M. Panić, M. Cvjetko Bubalo, I. Radojčić Redovniković, Designing a biocatalytic process involving deep eutectic solvents, J. Chem. Technol. Biotechnol. (2020), https://doi.org/10.1002/jctb.6545.
[7] A. Paiva, A.A. Matias, A.R.C. Duarte, How do we drive deep eutectic systems towards an industrial reality? Curr. Opin. Green Sustain. Chem. 11 (2018) 81–85, https://doi.org/10.1016/j.cogsc.2018.05.010.
[8] H. Vanda, Y. Dai, E.G. Wilson, R. Verpoorte, Y. Hae, Comptes Rendus Chimie Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents, Comptes Rendus - Chim. (2018) 1–11, https://doi.org/10.1016/j.crci.2018.04.002.
[9] Y.H. Choi, J. van Spronsen, Y. Dai, M. Verberne, F. Hollmann, I.W.C.E. Arends, G.-J. Witkamp, R. Verpoorte, Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology? Plant Physiol. 156 (2011) 1701–1705, https://doi.org/10.1104/pp.111.178426.
[10] B. Tang, K.H. Row, Recent developments in deep eutectic solvents in chemical sciences, Monatshefte Fur Chemie 144 (2013) 1427–1454, https://doi.org/10.1007/s00706-013-1050-3.
[11] A. Paiva, R. Craveiro, I. Aroso, M. Martins, R.L. Reis, A.R.C. Duarte, Natural deep eutectic solvents - solvents for the 21st century, ACS Sustain. Chem. Eng. 2 (2014) 1063–1071, https://doi.org/10.1021/sc500096j.
[12] Y. Dai, J. van Spronsen, G.J. Witkamp, R. Verpoorte, Y.H. Choi, Natural deep eutectic solvents as new potential media for green technology, Anal. Chim. Acta 766 (2013) 61–68, https://doi.org/10.1016/j.aca.2012.12.019.
[13] E. Durand, J. Lecomte, P. Villeneuve, Deep eutectic solvents: synthesis, application, and focus on lipase-catalyzed reactions, Eur. J. Lipid Sci. Technol. 115 (2013) 379–385, https://doi.org/10.1002/ejlt.201200416.
[14] M. Panić, D. Delač, M. Roje, I. Radojčić Redovniković, M. Cvjetko Bubalo, Green asymmetric reduction of acetophenone derivatives: saccharomyces cerevisiae and aqueous natural deep eutectic solvent, Biotechnol. Lett. 41 (2019) 253–262, https://doi.org/10.1007/s10529-018-2631-3.
[15] M. Cvjetko Bubalo, A. Jurinjak Tušek, M. Vinković, K. Radošević, V. Gaurina Srček, I. Radojčić Redovniković, Cholinium-based deep eutectic solvents and ionic liquids for lipase-catalyzed synthesis of butyl acetate, J. Mol. Catal., B Enzym. 122 (2015) 188–198, https://doi.org/10.1016/j.molcatb.2015.09.005.
[16] M. Panić, M. Majerić Elenkov, M. Roje, M. Cvjetko Bubalo, I. Radojčić Redovniković, Plant-mediated stereoselective biotransformations in natural deep eutectic solvents, Process Biochem. 66 (2018) 33–139, https://doi.org/10.1016/j.procbio.2017.12.010.
[17] I. Juneidi, M. Hayyan, M.A. Hashim, Intensification of biotransformations using deep eutectic solvents: overview and outlook, Process Biochem. 66 (2017) 33–60, https://doi.org/10.1016/j.procbio.2017.12.003.
[18] M. Cvjetko Bubalo, M. Mazur, K. Radošević, I. Radojčić Redovniković, Baker's yeast-mediated asymmetric reduction of ethyl 3-oxobutanoate in deep eutectic solvents, Process Biochem. 50 (2015) 1788–1792, https://doi.org/10.1016/j.procbio.2015.07.015.
[19] M. Panić, V. Gunjević, G. Cravotto, I. Radojčić Redovniković, Enabling technologies for the extraction of grape-pomace anthocyanins using natural deep eutectic solvents in up-to-half-litre batches extraction of grape-pomace anthocyanins using NADES, Food Chem. 300 (2019) 125185, https://doi.org/10.1016/j.foodchem.2019.125185.
[20] J.B. Sontakke, G.D. Yadav, Optimization and kinetic modeling of lipase catalyzed enantioselective N-acetylation of (? ?)-1-phenylethylamine under microwave irradiation, J. Chem. Technol. Biotechnol. 86 (2011) 739–748, https://doi.org/10.1002/jctb.2582.

[21] M. Pätzold, S. Siebenhaller, S. Kara, A. Liese, C. Syldatk, D. Holtmann, Deep eutectic solvents as efficient solvents in Biocatalysis, Trends Biotechnol. 37 (2019) 943–959, https://doi.org/10.1016/j.tibtech.2019.03.007.
[22] E. Durand, J. Lecomte, B. Baréa, G. Piombo, E. Dubreucq, P. Villeneuve, Evaluation of deep eutectic solvents as new media for Candida antarctica B lipase catalyzed reactions, Process Biochem. 47 (2012) 2081–2089, https://doi.org/10.1016/j.procbio.2012.07.027.
[23] N. Guajardo, K. Ahumada, P. Domínguez de María, R.A. Schrebler, Remarkable stability of Candida antarctica lipase B immobilized via cross-linking aggregates (CLEA) in deep eutectic solvents, Biocatal. Biotransformation 37 (2019) 106–114, https://doi.org/10.1080/10242422.2018.1492567.
[24] P. Zhou, X. Wang, B. Yang, F. Hollmann, Y. Wang, Chemoenzymatic epoxidation of alkenes with Candida antarctica lipase B and hydrogen peroxide in deep eutectic solvents, RSC Adv. 7 (2017) 12518–12523, https://doi.org/10.1039/c7ra00805h.
[25] Q.-B. Cheng, L.-W. Zhang, Highly efficient enzymatic preparation of Daidzein in deep eutectic solvents, Molecules 22 (2017) 186, https://doi.org/10.3390/molecules22010186.
[26] S. Khandelwal, Y.K. Tailor, M. Kumar, Deep eutectic solvents (DESs) as eco-friendly and sustainable solvent/catalyst systems in organic transformations, J. Mol. Liq. 215 (2016) 345–386, https://doi.org/10.1016/j.molliq.2015.12.015. Review.
[27] M. Pätzold, A. Weimer, A. Liese, D. Holtmann, Optimization of solvent-free enzymatic esterification in eutectic substrate reaction mixture, Biotechnol. Reports. 22 (2019) e00333, https://doi.org/10.1016/j.btre.2019.e00333.
[28] R. Craveiro, L. Meneses, L. Durazzo, Â. Rocha, J.M. Silva, R.L. Reis, S. Barreiros, A. R.C. Duarte, A. Paiva, Deep eutectic solvents for enzymatic esterification of racemic menthol, ACS Sustain. Chem. Eng. 7 (2019) 19943–19950, https://doi.org/10.1021/acssuschemeng.9b05434.
[29] A.P. Abbott, D. Boothby, G. Capper, D.L. Davies, R.K. Rasheed, Deep Eutectic Solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic liquids, J. Am. Chem. Soc. 126 (2004) 9142–9147, https://doi.org/10.1021/ja048266j.
[30] J.A. Asenjo, B.A. Andrews, Mini-review challenges and trends in bioseparations, Chem. Eng. 120 (2008) 117–120, https://doi.org/10.1002/jctb.
[31] M. Cvjetko Bubalo, S. Vidović, I. Radojčić Redovniković, S. Jokić, Green solvents for green technologies, J. Chem. Technol. Biotechnol. 90 (2015) 1631–1639, https://doi.org/10.1002/jctb.4668.
[32] Z. Maugeri, W. Leitner, P. Domínguez De María, Practical separation of alcohol-ester mixtures using deep-eutectic-solvents, Tetrahedron Lett. 53 (2012) 6968–6971, https://doi.org/10.1016/j.tetlet.2012.10.044.
[33] H. Jiang, B. Diao, D. Xu, L. Zhang, Y. Ma, J. Gao, Y. Wang, Deep eutectic solvents effect on vapor-liquid phase equilibrium for separation of allyl alcohol from its aqueous solution, J. Mol. Liq. 279 (2019) 524–529, https://doi.org/10.1016/j.molliq.2019.01.163.
[34] F. Liang, J. Huang, J. He, P. Wang, Improved Enantioselective Hydrolysis of Racemic Ethyl-2, 2- dimethylcyclopropanecarboxylate Catalyzed by Modified Novozyme, 435, 2012, pp. 952–958, https://doi.org/10.1007/s12257-012-0135-x, 958.
[35] N. Guajardo, H.P. Domínguez de María, K. Ahumada, R.A. Schrebler, R. Ramírez-Tagle, F.A. Crespo, C. Carlesi, Water as cosolvent: nonviscous deep eutectic solvents for efficient lipase-catalyzed esterifications, ChemCatChem. 9 (2017) 1393–1396, https://doi.org/10.1002/cctc.201601575.
[36] P. Xu, Y. Xu, X.F. Li, B.Y. Zhao, M.H. Zong, W.Y. Lou, Enhancing asymmetric reduction of 3-chloropropiophenone with immobilized acetobacter sp. CCTCC M209061 cells by using deep eutectic solvents as cosolvents, ACS Sustain. Chem. Eng. 3 (2015) 718–724, https://doi.org/10.1021/acssuschemeng.5b00025.