Scientific paper

Enzyme-catalyzed Acylation of (*R,S*)-1-phenylethanol in 1-butyl-3-methylimidazolium Based Ionic Liquids

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Abstract

Ionic liquids represent an exciting new class of reaction solvents for catalysis, which have been used successfully for enzyme-catalyzed reactions. In present research, three different ionic liquids, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium hexafluorophosphate, and 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl)]amide, were synthesized. They were used as a reaction medium for enzyme-catalyzed acylation of (R,S)-1-phenylethanol with vinyl acetate. Mentioned enzymatic reaction was performed in a batch stirred-tank reactor in order to optimise different reaction parameters (biocatalyst concentration, temperature, ...). The influence of three different immobilized lipases on reaction performance was studied as well.

The highest reaction rate and conversion of 49.7% after 5 h of reaction performance was achieved in the case when immobilized lipase Novozym 435 from *Candida antarctica* was used as a biocatalyst and hydrophilic ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate as a solvent. Therefore, the optimization of different reaction parameters on lipase-catalyzed acylation of (*R,S*)-1-phenylethanol was carried out in 1-butyl-3-methylimidazolium tetrafluoroborate.

Keywords: Immobilized lipase, acylation, (*R,S*)-1-phenylethanol, ionic liquids, 1-butyl-3-methylimidazolium tetrafluoroborate

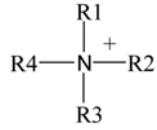
1. Introduction

Ionic liquids (ILs), a novel class of green solvents, have recently emerged as interesting non-aqueous reaction media for enzymatic reactions.[1] They are organic salts made of cations, which are generally bulky, organic with low symmetry, e.g. derivatives of imidazolium, pyridinium, pyrrolidinium, ammonium, sulfonium, phosphonium, ..., and anions, which are either organic or inorganic and can be classified in two classes: (i) those which give polynuclear anions, such as $Al_2Cl_7^-$, $Al_3Cl_{10}^-$, $Au_2Cl_7^-$, ..., and (ii) those that corresponds to mononuclear anions which lead to neutral, stoichiometric ILs, such as tetrafluoroborate, hexafluorophosphate, bis[(trifluoromethyl) sulfonyl]amide, nitrate, trifluoroacetate, methylsulfate ...[2] Common ions involved in ILs for biocatalysis are shown in Table 1.

The possible choices of cation and anion that will result in the formation of ILs with different physico-chemical properties are numerous.[4] Unlike conventional organic solvents, ILs exhibit very low vapor pressure under ambient conditions and thus are effectively non-volatile.[5] Moreover, ILs have good thermal stability, tunable polarity, hydrophobicity, viscosity, miscibility with water and organic solvents,[6,7] as well as good solubility for polar and non-polar organic, inorganic and polymeric compounds.[3] Furthermore, ILs are compounds that have a potential to be recycled and reused.[8] Due to unique properties, ILs are attractive alternative to conventional organic solvents. As functional materials, ILs are being used in a wide variety of applications. In biocatalysis, ILs may be used as reaction media for different types of enzyme-catalyzed reactions, such as hydrolysis,[9] esterification,[10] transesterification,[11] acylation,[12] acetylation,[13] ..., as co-solvents in aqueous phase, as two-phase systems together with other solvents and as pure solvents.[14]

In our case, ILs were used as reaction media for lipase-catalyzed acylation (kinetic resolution) of chiral substrate, (*R,S*)-1-phenylethanol, with vinyl acetate. Generally only one of the enantiomers of chiral compounds has

Table 1. Common ions involved in ILs for biocatalysis.[3]

Cation	Full Name	Cation	Full Name
	1,3-dialkylimidazolium		1,1-dialkylpyrrolidinium
	1-alkylpyridinium		1-alkylpyridiniumtetraalkylammonium

Anion	Full Name	Abbreviation
BF_4^-	tetrafluoroborate	$[BF_4]$
PF_6^-	hexafluorophosphate	$[PF_6]$
NO_3^-	nitrate	$[NO_3]$
$CH_3CO_2^-$	acetate	[Ac]
$CF_3CO_2^-$	trifluoroacetate	[TFA]
$CH_3SO_4^-$	methylsulfate	$[MeSO_4]$
$CF_3SO_3^-$	trifluoromethylsulfonate	[TfO]
$(CF_3SO_2)_2N^-$	bis[(trifluoromethyl)sulfonyl]amide	$[NTf_2]$

the desired biological activity. The biologically inactive enantiomer may merely be inactive and is at best just useless ballast, but it may also cause unwanted side-effects.[15] The optically active 1-phenylethanol, especially (*R*)-1-phenylethanol, is used as chiral building block and synthetic intermediate in fine chemical, pharmaceutical and agrochemical industries.[16] The synthesis of many pharmaceutical agents and complex natural products relies on the availability of chiral intermediates that can serve as building blocks for further structural and stereochemical elaboration.[17] In pharmaceutical industry, (*R*)-1-phenylethanol is used as ophthalmic preservative and may also inhibit cholesterol intestinal adsorption and thus decrease high cholesterol level.[16] The other application area of the enantiomers is in chemical analysis. Both the (*R*)- and (*S*)-enantiomer of 1-phenylethanol are used as chiral reagent for the determination of enantiomeric purity and for the asymmetric opening of cyclic anhydrides and epoxides.[18] Moreover, (*R*)-1-phenylethanol can be used in Solvatochromic dye.[16]

In present research, the main aim was to optimize different reaction parameters, such as biocatalyst concentration, temperature and substrate ratio, for lipase-catalyzed acylation of (*R,S*)-1-phenylethanol with vinyl acetate in ILs at atmospheric pressure. For that reason, three different ILs were synthesized and used as reaction media and three different immobilized lipases were used as biocatalysts for the acylation reaction. After determining the most suitable IL as reaction medium and immobilized lipase as a biocatalyst for the acylation of (*R,S*)-1-phenylethanol with vinyl acetate, reaction parameters were optimized.

2. Materials and Methods

2. 1. Enzymes and Chemicals

Immobilized lipases from *Candida antarctica* (Novozym 435, SP 382) and immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) were kindly donated from Novozymes (Bagsvaerd, Denmark). (*R*)-1-phenylethanol and (*S*)-1-phenylethanol were supplied from Sigma-Aldrich (Saint Louis, USA). (*R,S*)-1-phenylethanol (≥98%), vinyl acetate (≥99%), 1-ethyl-3-methyl-imidazolium bromide (≥97%), and hexafluorophosphoric acid 65% (gravimetric) in water were supplied from Fluka (Buchs, Switzerland). Decane – Reagent Plus® (≥99%), *N*-lithiotrifluoromethane-sulfonimide (97%) and sodium tetrafluoroborate (98%) were provided from Aldrich Chemical Co. (Diesenhofen, Germany). Acetone (≥99.8%), anhydrous magnesium sulfate (≥98%), 1-chlorobutane (≥99%), dichloromethane (≥99.8%), ethyl acetate (≥99.5%), *n*-heptane (≥99%) and 1-methylimidazole (≥99%) were purchased from Merck (Darmstadt, Germany). Helium 6.0 was supplied from Linde plin (Celje, Slovenia).

2. 2. Synthesis of Ionic Liquids

2. 2. 1. 1-Butyl-3-methylimidazolium Chloride [bmim][Cl]

The ionic liquid [bmim][Cl] was prepared by reaction of 1-methylimidazole (82.0 g, 1 mol) and 1-chlorobutane (92.5 g, 1 mol) in a round-bottomed flask fitted with a reflux condenser. The reaction mixture was stirred at 333.15 K for 70 h. The resulting yellow, viscous liquid mixture was cooled to room temperature, washed three times with ethyl acetate portions (100 mL) and the [bmim][Cl] crystals were dried under vacuum at 333.15 K for 24 h.[19] Using this ionic liquid, $[bmim][BF_4]$ and $[bmim][PF_6]$ were synthesized.

2 .2. 2. 1-Butyl-3-methylimidazolium Tetrafluoroborate $[bmim][BF_4]$

The sodium tetrafluoroborate $NaBF_4$ (29.15 g, 0.266 mol) was added to a solution of [bmim][Cl] (46.5 g, 0.266 mol) in acetone (250 mL). The reaction mixture was stir-

red at room temperature for 24 h. After 24 h under stirring, the reaction mixture was filtered through a plug of celite to remove the formed sodium chloride crystals. The solvent (acetone) was evaporated by rotary evaporation from hydrophilic ionic liquid [bmim][BF_4] at 313.15 K for 24 h.[20] Initial water content in the synthesized [bmim][BF_4] was 0.18% (w/w).

2. 2. 3. 1-Butyl-3-methylimidazolium Hexafluorophosphate [bmim][PF_6]

An aqueous solution of hexafluorophosphoric acid HPF_6 (50 mL) was slowly added to a solution of [bmim][Cl] (52 g, 0.297 mol) in water (300 mL) and stirred at room temperature for 36 h. The two-phase system was separated, and the lower phase (the ionic liquid [bmim][PF_6]) was washed with water portions (100 mL) until the neutral pH value. The light yellow [bmim][PF_6] was dried under vacuum at 353.15 K for 24 h.[19] Initial water content in the synthesized [bmim][PF_6] was 0.04% (w/w).

2. 2. 4. 1-Ethyl-3-methylimidazolium Bis[(trifluoromethyl)sulfonyl]amide [emim][NTf_2]

1-Ethyl-3-methylimidazolium bromide (13.16 g, 0.0689 mol) and *N*-lithiotrifluoromethanesulfonimide LiNTf_2 (20.5 g, 0.0689 mol) were mixed in hot water (50 mL, 343.15 K) for 1 h. The ionic liquid [emim][NTf_2] was extracted with dichloromethane (2 × 50 mL), and dried under vacuum at 373.15 K for 24 h.[20] Initial water content in the synthesized [emim][NTf_2] was 0.17% (w/w).

2. 3. Enzyme-catalyzed Acylation of (*R,S*)-1-phenylethanol Performed in Batch Stirred Tank Reactor at Atmospheric Pressure

Acylation of (*R,S*)-1-phenylethanol with vinyl acetate, catalyzed with chiral biocatalyst lipase B from *Candida antarctica* (CALB) (Figure 1), was performed in a batch stirred-tank reactor. (*R,S*)-1-Phenylethanol (0.61 g, 5.0 mmol) and vinyl acetate (0.43 g, 5.0 mmol) were dissolved in ionic liquid (5.0 mmol), which was used as a reaction medium. The reactor filled up with substrates, was immersed in a water bath, heated to the desired operating temperature and stirred with a magnetic stirrer. The reaction was started by addition of the immobilized lipase. Aliquots of the sample (0.010 g) were periodically withdrawn from the reaction mixture at fixed time intervals, suspended in 0.2% solution of decane (internal standard, IS) in *n*-heptane (2 mL) and 1 μL of the resulting solution was analyzed by gas chromatograph.

2. 4. Gas Chromatography Analysis

Enantiomers content during the reaction time course

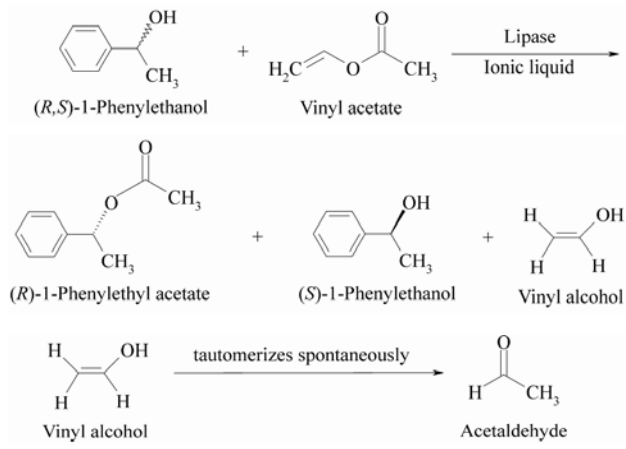


Figure 1. Schematic representation of immobilized lipase – catalyzed acylation of (*R,S*)-1-phenylethanol with vinyl acetate as acyl donor.

was monitored using an HP 5890 series A gas chromatograph equipped with a flame-ionisation detector (FID), using helium as carrier gas and a β-cyclodextrin capillary column (β-DEX 120) with the dimension length × I.D. 30 m × 0.25 mm with 0.25 μm film thickness (Supelco, Schnelldorf, Germany), at following temperature program: 373.15 K hold for 5 min, rise up to 393.15 K at rate of 5 K/min and hold for 11 min; the temperature of injector and detector were maintained at 493.15 K and 523.15 K, respectively.

The conversion (X) was calculated by applying the equation, which is valid for irreversible reactions:

$$X[\%] = \frac{ee_R}{ee_R + ee_P} \times 100 .$$

At least two replicates of experiments were carried out at each operative condition. All samples, which were withdrawn from the reaction mixture, were analyzed by gas chromatograph two times. The relative deviation was evaluated to be within ± 1%.

The initial reaction rate was calculated, as well. The rate of a chemical reaction is defined as the change in the concentration of one of the reactants or products in unit time. The initial reaction rate (v_i) was determined as the slope of the tangent on the curve, which presents the concentration of produced ester [$mmol_{(R)\text{-}1\text{-}PEA}/g_{RM}/g_E$] in time dependence [h] in the first 10 minutes of the reaction performance.

3. Results and Discussion

3. 1. Screening of three Different Ionic Liquids for Acylation of (*R,S*)-1-phenylethanol with Vinyl Acetate

Some potential advantages of enzymatic reactions in ILs, such as high activity, thermal and operational sta-

bility of biocatalysts, and good enantioselectivity of biotransformation in comparison with conventional media have been reported.[21] But not all ILs are suitable for biocatalysis. Enzymes are usually active in ILs containing tetrafluoroborate [BF_4^-], hexafluorophosphate [PF_6^-] and bis[(trifluoromethyl)sulfonyl]amide [NTf_2^-] anions, but not in ILs containing chloride [Cl^-], nitrate [NO_3^-], trifluoroacetate [$CF_3CO_2^-$] or acetate [$CH_3CO_2^-$] anions.[22] Enzyme activity in ILs is affected by other parameters, such as polarity and viscosity of ILs, as well, because they can affect both the enzyme activity and mass-transfer limitations, respectively.[20] The polarity of solvents can be measured by the log*P* value (partition coefficient of a given compound in the octanol and water two-phase system).[23] Organic solvents with a log*P* lower than 2 are considered hydrophilic in nature and often ineffective. The high hydrophobic solvents with log*P* higher than 4 are considered as the most suitable solvents for biocatalysis since they generally support enzymatic activity.[22,24] High viscosities of ILs can affect the diffusion of substrate and product molecules through the micropores in the biocatalist particles in the way which slows down the reaction.

The influence of three different ILs on acylation of (*R,S*)-1-phenylethanol with vinyl acetate was studied. ILs based on the *N,N'*-dialkylimidazolium cations associated with mononuclear anions, such as [BF_4^-], [PF_6^-] and [NTf_2^-], were used as reaction media. Reactions were performed in batch stirred-tank reactor at 313.15 K and atmospheric pressure, with an equimolar ratio of (*R,S*)-1-phenylethanol/vinyl acetate 1:1 and with 100 mg of immobilized CALB, magnetically stirred and thermostated in a water bath.

The influence of assayed ILs on conversion of (*R*)-1-phenylethanol to (*R*)-1-phenylethyl acetate is presented in Figure 2.

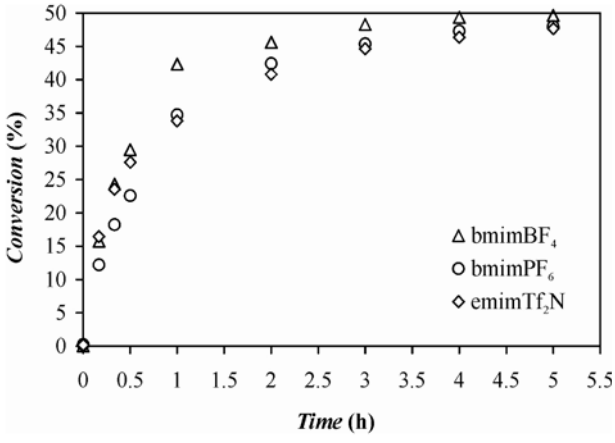


Figure 2. Influence of three different ILs on acylation of (*R,S*)-1-phenylethanol with vinyl acetate during the 5 h of reaction performance. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol ILs, 100 mg immobilized CALB, 313.15 K, 600 rpm.

The assayed ILs, [bmim][BF_4], [bmim][PF_6] and [emim][NTf_2] with log*P* values –2.44, –2.38 and –1.18,[22,25,26] respectively, proved to be adequate reaction media for lipase-catalyzed acylation of (*R,S*)-1-phenylethanol with vinyl acetate. Despite the fact that ILs have low log*P* values (below zero), which seem to suggest that they are highly hydrophilic in nature and would likely inactivate enzymes,[3] immobilized CALB retained its activity in all assayed ILs. The enzyme activity increased with the decrease in log*P* values of ILs. There was almost no difference in conversion obtained after 5 h when reaction was performed in [emim][NTf_2] and [bmim][PF_6]. Obtained conversions for reactions performed in [bmim][PF_6] and in [emim][NTf_2] were 47.6% and 47.9%, respectively. However, the highest conversion (49.7%) of (*R*)-1-phenylethanol to (*R*)-1-phenylethyl acetate after 5 h of reaction performance was obtained, when the reaction was carried out in hydrophilic IL [bmim][BF_4] with log*P* value -2.44.

Kaar et al.,[22] who studied the enzyme activity in different ILs, suggested that enzyme activity in ILs is anion depended. Furthermore, the studies from Sheldon et al.[27] has shown that anions, such as [NO_3^-], [$CH_3CO_2^-$] and [$CF_3CO_2^-$], are more nucleophilic than for example [PF_6^-] and may coordinate more strongly to positively charged sites in the lipases structure causing conformation changes in the enzymes structure, leading to a loss of activity. This coincide well with results published by Toral and coworkers,[28] who studied the influence of ILs on enantioselective acylation of (*R,S*)-1-phenylethanol with vinyl acetate, catalyzed with CALB, adsorbed and cross-linked on a polypropylene carrier at atmospheric pressure. They discovered that the weakly coordinating ILs, such as [bmim][PF_6] and [bmim][NTf_2], gave comparable or higher reaction rates and enantioselectivities than reactions, performed in conventional solvents. Reactions, performed in strongly coordinating ILs, such as 1-butyl-3-methylimidazolium nitrate [bmim][NO_3], 1-butyl-3-methylimidazolium lactate [bmim][lactate], and 1-butyl-3-methylimidazolium dicyanamide [bmim][DCA], were quite slow.

Based on previously reported studies,[22,27,28] we concluded that in our case the immobilized CALB retained its activity in assayed ILs, also due to their low hydrogen-bond basicity of the enzyme-compatible anions, since the [BF_4^-] spreads its negative charge over four fluorine atoms, the [PF_6^-] over six fluorine atoms and the [NTf_2^-] over five atoms.[8]

Compared to conventional organic solvents, ILs are much more viscous. Viscosity of the ILs can control the enzyme activity by affecting the mass-transfer limitations and therefore, a lower reaction rate would be expected in an IL with higher viscosity.[3] In our case, when the reaction was carried out in [emim][NTf_2], [bmim] [BF_4] and [bmim][PF_6] with viscosities of 34 cP, 154 cP and 430 cP,[3,29] respectively, a reduction in the initial reaction rate was corresponding to an increase in the viscosity of the

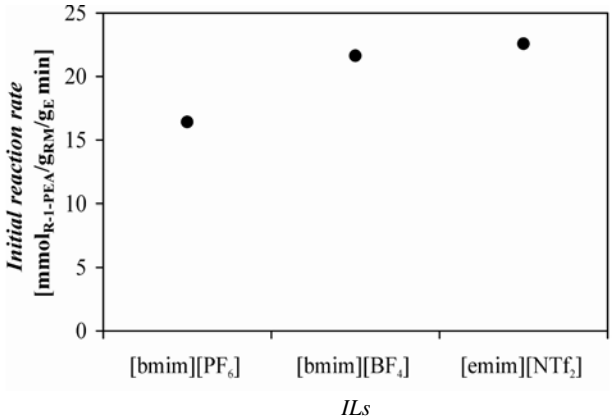


Figure 3. Influence of three different ILs on initial reaction rate. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol ILs, 100 mg immobilized CALB, 313.15 K, 600 rpm.

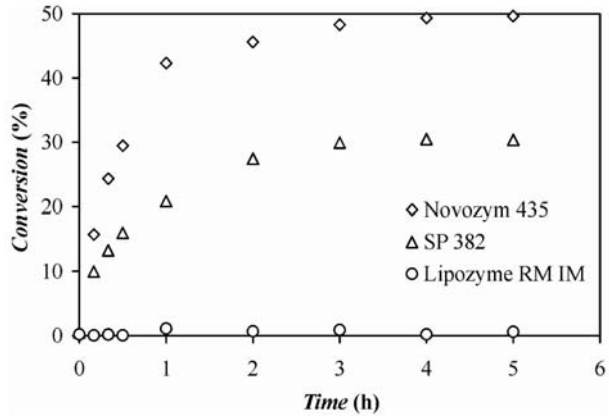


Figure 4. Influence of three different lipases on acylation of (*R,S*)-1-phenylethanol with vinyl acetate during the 5 h of reaction performance. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol ILs, 100 mg of immobilized lipase, 313.15 K, 600 rpm.

ILs (Figure 3). Furthermore, according to visual observations, hydrophobic IL [bmim][PF_6] formed a layer around the enzyme and enzyme could be considered as being included into the media. Therefore, the contact between substrate and the active site of the lipase was limited, what resulted in lower conversion of (*R*)-1-phenylethanol to (*R*)-1-phenylethyl acetate.

Although, the highest initial reaction rate of acylation of (*R,S*)-1-phenylethanol with vinyl acetate was achieved in [emim][NTf_2], all further studies were done using hydrophilic [bmim][BF_4] as a reaction medium. Mainly because of [bmim][BF_4] lower price, because the highest degree of conversion of (*R*)-1-phenylethanol in (*R*)-1-phenylethyl acetate after 5 h of reaction performance was achieved in [bmim][BF_4] and the initial reaction rate was just for 4.13% lower in [bmim][BF_4] compared to one obtained in [emim][NTf_2].

3. 2. Screening of three Different Immobilized Lipases for Acylation of (*R,S*)-1-phenylethanol with Vinyl Acetate in [bmim][BF_4]

Lipase-catalyzed transesterification of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4] was performed at 313.15 K and stirred with a rotational speed of 600 rpm with (*R,S*)-1-phenylethanol/vinyl acetate/[bmim][BF_4] molar ratio of 1. Reactions were catalyzed with three different kinds of immobilized lipases, e.g. lipase B from *Candida antarctica* (Novozym 435), lipase A and B from *Candida antarctica* (SP 382) and lipase from *Rhizomucor miehei* (Lypozyme RM IM).

Figure 4 and figure 5 shows the influence of three different lipases on acylation of (*R,S*)-1-phenylethanol during the 5 h of reaction performance and on initial reaction rate, respectively.

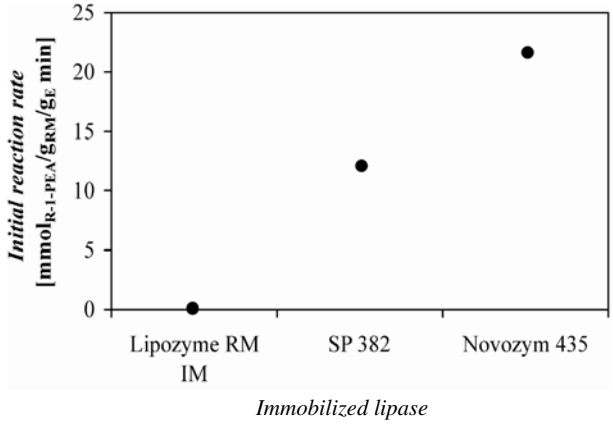


Figure 5. Influence of three different immobilized lipases on initial reaction rate. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol ILs, 100 mg of immobilized lipase, 313.15 K, 600 rpm.

Immobilized lipases from *Candida antarctica* were capable to catalyze the reaction. Both of them showed good preference to (*R*)-enantiomer in rac-1-phenylethanol, while the (*S*)-enantiomer remained unchanged. The highest conversion (42.34%) and initial reaction rate (21.65 mmol$_{R-1-PEA}$/g$_{RM}$/g$_E$min) were obtained with immobilized lipase B from *Candida antarctica* Novozym 435. Considerably lower conversion (20.85%) and initial reaction rate (12.09 mmol$_{R-1-PEA}$/g$_{RM}$/g$_E$min) were obtained with lipase from *Candida antarctica* SP 382. That could be due to the composition of enzyme preparation SP 382, which is a mixture of lipase A and lipase B, and only lipase B catalyses the reaction. The lipase A is active in a non-specific manner towards triglycerides. In contrast, the lipase B is less active towards large triglycerides but very active towards a broad range of amides, thiols and esters.[30] Furthermore, it is well known that lipase B is highly spe-

cific for kinetic resolution of sec-alcohols due to its good enantioselectivity.[31–33] Namely, the active site of lipase B contains a small cavity called the stereospecificity pocket, in which secondary alcohols have to orient one substituent during catalysis, which gives CALB a high enantioselectivity towards chiral secondary alcohols.[34]

Lipase from *Rhizomucor miehei* (Lypozyme RM IM) did not catalyze the acylation of (*R,S*)-1-phenylethanol with vinyl acetate after stirred for 5 h at 313.15 K. Therefore, it can be concluded that Lypozyme RM IM is not adequate biocatalyst for abovementioned reaction.

3. 3. Influence of Enzyme Concentration on Acylation of (*R,S*)-1-phenylethanol with Vinyl Acetate in [bmim][BF_4]

Enzyme concentration, used for enzyme-catalyzed reaction, is a crucial economical factor for successful industrial applications. For that purpose, the influence of enzyme concentration on the acylation of (*R,S*)-1-phenylethanol was optimized. Reactions were performed to determine the minimum concentration of immobilized CALB that maximizes the concentration of formed product during the reaction performance. Experiments were conducted varying immobilized CALB concentration in the range from 0 mg_E/mL_{RM} to 201 mg_E/g_{RM} at 313.15 K, (*R,S*)-1-phenylethanol/vinyl acetate/[bmim][BF_4] composition with molar ratio of 1:1:1, stirrer rate of 600 rpm and at atmospheric pressure.

The influence of concentration of CALB on conversion of (*R*)-1-phenylethanol to (*R*)-1-phenylethyl acetate is shown in Figure 6.

The rate of an enzyme-catalyzed reaction depends on the concentrations of enzyme and substrate. In order to optimize the enzyme concentration, 0–201 mg_E/g_{RM} of immobilized CALB was used for acylation of (*R,S*)-1-phenylethanol with vinyl acetate. As expected, at enzyme conentration of 0 mg_E/g_{RM} the product (*R*)-1-phenylethyl acetate was not formatted, consequently [bmim][BF_4] themselves did not catalyze the acylation, acting only as solvent in this reaction system. At lower enzyme concentrations there was presented the insufficient amount of the enzyme and therefore the reaction did not proceed as fast as it was when the enzyme concentrations were higher, because there was not enough enzyme for all of the reactant molecules. As the enzyme concentration was increased from 24.80 mg_E/g_{RM} to 201.00 mg_E/g_{RM}, the reaction rate increased as well. For the reactions catalyzed with 75.09, 100.40, 150.60 and 201.00 mg_E/g_{RM}, 50% conversion was obtained after 2 h of reaction performance and for reaction catalyzed with 50.48 mg_E/g_{RM} after 4 h.

At the highest enzyme concentration of 201.00 mg_E/g_{RM}, the highest initial reaction rate was obtained (Figure 7). On the other hand, by halving the enzyme concentration to 100.40 mg_E/g_{RM}, a lower initial reaction rate was obtained, but conversion of 50% was obtained after 2 h of reaction performance just like with higher enzyme concentration. An increase in the enzyme concentration in the reaction mixture leads to an increase in the conversion and in the initial reaction rate, since more successful collisions between enzyme and substrate molecules occurs. But after a certain limiting concentration of enzyme, the rate of reaction and final conversion will no longer depend upon this increase. In the case if the initial reaction rate and final conversion remains approximately the same although the enzyme concentration was increased, it means that not all enzyme particles are exposed to the substrates and the excess of enzyme present in the reaction mixture is not actively involved in the reaction (increased amount of the enzyme does not act as a catalyst but as ballast).[35]

Even though higher reaction rate was obtained with enzyme concentration of 201.00 mg_E/g_{RM}, all further stu-

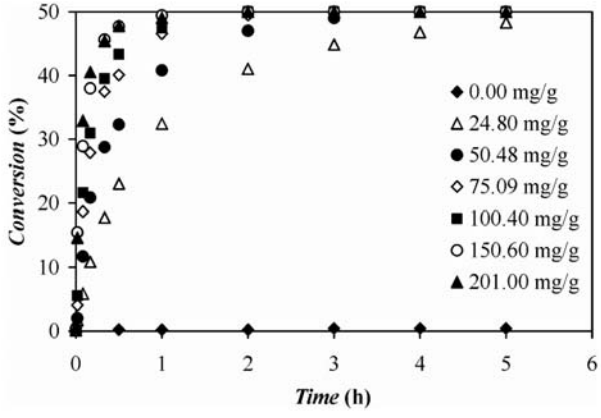


Figure 6. Influence of enzyme concentration on acylation of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4]. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol [bmim][BF_4], 313.15 K, 600 rpm, 5 h of reaction performance.

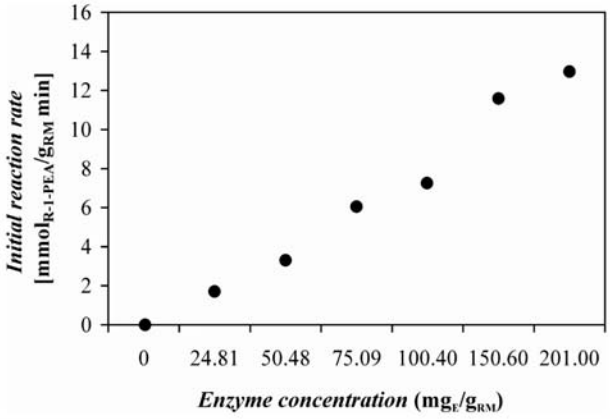


Figure 7. Influence of enzyme concentration on initial reaction rate for acylation of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4]. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol [bmim][BF_4], 313.15 K, 600 rpm, 5 h of reaction performance.

dies were done with enzyme concentration of 100.40 mg_E/g_{RM}. Mainly because there was no changes in the final yield of formatted product when the reactions were catalyzed with enzyme concentration of 100.40 mg_E/g_{RM} and 201.00 mg_E/g_{RM} and due to the problems related with sampling since the reaction mixture was saturated with enzyme.

3. 4. Influence of Temperature on Acylation of (*R,S*)-1-phenylethanol with Vinyl Acetate in [bmim][BF_4]

To determine optimal process temperature, its influence of temperature on immobilized CALB activity was studied. Experiments were conduced in equimolar mixture (5.0 mmol) of (*R,S*)-1-phenylethanol, vinyl acetate and IL [bmim][BF_4], with enzyme concentration of 100.40 mg_E/g_{RM} and the rotational speed of the stirrer was 600 rpm. Reactions were performed at 6 different temperatures in the range between 303.15 K and 373.15 K. The results are summarized in Figure 8 and figure 9.

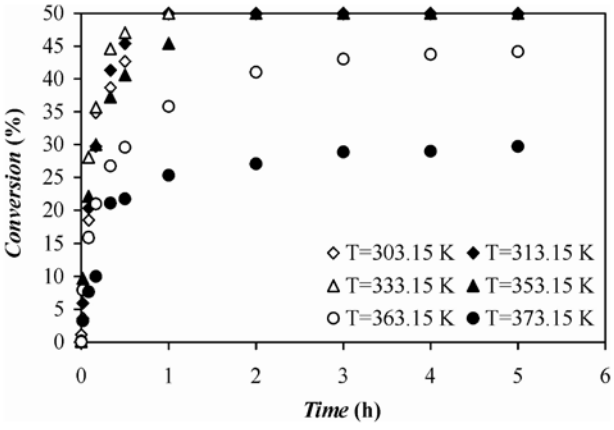


Figure 8. Influence of temperature on acylation of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4]. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol [bmim][BF_4], 100.40 mg_E/g_{RM} immobilized CALB, 600 rpm, 5 h of reaction performance.

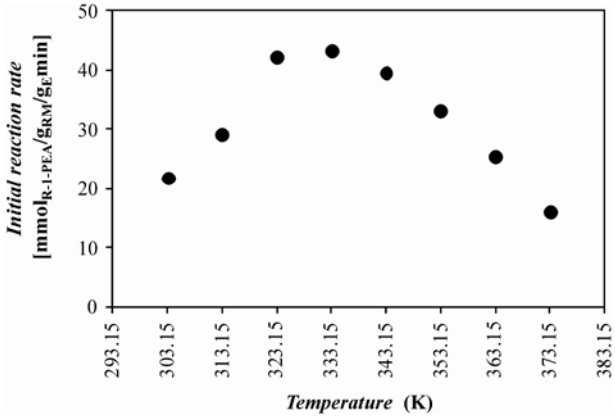


Figure 9. Influence of temperature on initial reaction rate for acylation of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4]. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol vinyl acetate, 5.0 mmol [bmim][BF_4], 100.4 mg_E/g_{RM} immobilized CALB, 600 rpm, 5 h of reaction performance.

Each enzyme has an optimum temperature. As the temperature increases, molecular motion increases resulting in more molecular collisions. If, however, the temperature rises above a certain point, the heat will denature the enzyme, causing it to lose its three-dimensional functional shape by denaturing its hydrogen bonds. Low temperatures, on the other hand, slow down the enzyme activity by decreasing molecular motion.[36]

In our case, the lowest temperature studied was 303.15 K, which resulted in 50% conversion obtained after 1 h of reaction performance. As expected, the initial reaction rates were found to increase from 21.778 $mmol_{R\text{-}1\text{-}PEA}/g_{RM}/g_E min$ to 43.326 $mmol_{R\text{-}1\text{-}PEA}/g_{RM} min$ (Figure 9) with increasing temperature from 303.15 K to 333.15 K. Optimum temperature for immobilized CALB used as biocatalyst for acylation of (*R,S*)-1-phenylethanol with vinyl acetate was found to be 333.15 K, since at temperatures higher than 333.15 K, such as 353.15 K, 363.15 K and 373.15 K a decrease in reaction rate and in conversion was observed, which could happen due to enzyme deactivation. The interesting fact in this situation was that even at 373.15 K immobilized CALB showed some activity.

The influence of temperature on acylation of (*R,S*)-1-phenylethanol with vinyl acetate, was studied by Eckstein and coworkers,[37] as well. They performed the acylation of (*R,S*)-1-phenylethanol, catalyzed by a lyophilized lipase from *Pseudomonas* sp. in IL [bmim][NTf_2]. The temperature was varied between 298.15 K and 363.15 K. The conversion reached the maximum at 343.15 K, at the temperature above 343.15 K the decrease in conversion was observed. A lyophilized lipase from *Pseudomonas* sp. showed some activity even at temperature of 363.15 K. As far as the authors of this paper are aware, the influence of temperature on immobilized CALB-catalyzed acylation of (*R,S*)-1-phenylethanol, performed in [bmim][BF_4] at atmospheric pressure have not been reported yet.

Activation energy E_a and deactivation enthalpy ΔH_d for immobilized CALB were calculated from the Arrhenius plot (Figure 10), in which ln v_i is graphed against 1/T giving a straight line with a slope of $-E_a/R$ for temperatures from 303.15 K to 333.15 K and another straight line with a slope approximately equal to $\Delta H - E_a/R$ for temperatures above 333.15 K. In the approach of the simple model of reversible thermal deactivation, assuming that the enzyme exists in inactive and active forms in equilibrium, with equilibrium constant K_d, deactivation entropy ΔS_d and deactivation Gibb's free energy ΔG_d, were estimated as well.[36]

At temperatures above 333.15 K, the deactivation enthalpy change is higher than the energy of activation (26.76 kJ/mol and 51.51 kJ/mol, respectively). At this

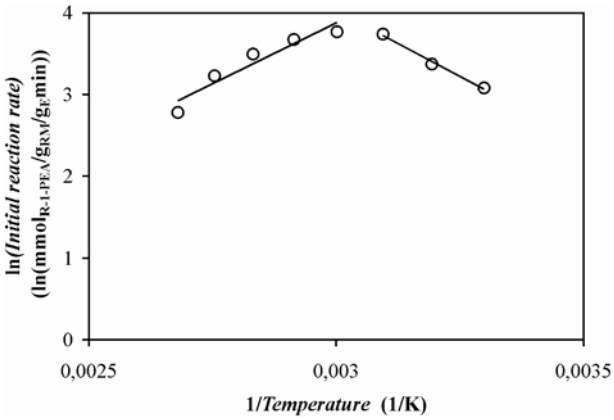


Figure 10. Arrhenius Plot for Acylation of (*R,S*)-1-phenylethanol with Vinyl Acetate in [bmim][BF_4], Catalyzed with Immobilized CALB.

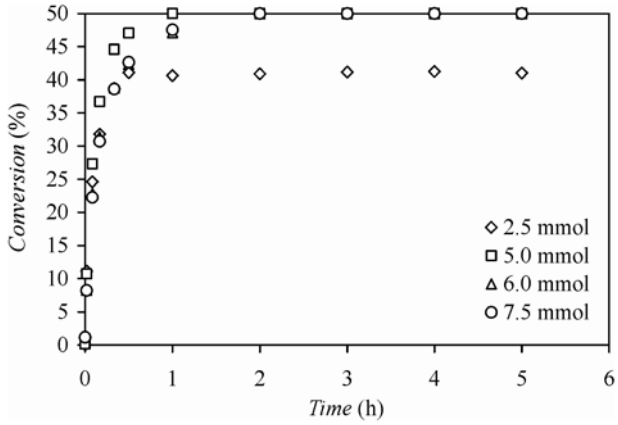


Figure 11. Influence of vinyl acetate concentration on acylation of (*R,S*)-1-phenylethanol in [bmim][BF_4]. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol [bmim][BF_4], 100.40 mg_E/g_{RM} immobilized CALB, 600 rpm, 5 h of reaction performance.

temperature, enzyme deactivation predominates in the system, causing a great decrease of the activity with temperature rise from 343.15 K to 373.15 K. The constant of enzyme deactivation K_d, which is described as a ratio between inactive and active form of enzyme was 1.33. Therefore, the preparation of immobilized CALB contained more inactive form of enzyme. Other thermodynamic parameters, such as deactivation Gibb's free energy and deactivation entropy, were calculated and determined to be –0.764 kJ/mol and 167 J/molK, respectively. Any natural process occurs spontaneously if the associated change in Gibb's free energy for the system is negative. Negative Gibb's free energy during deactivation indicates that thermal deactivation occurres at temperatures above 333.15 K. The use of higher temperatures causes an increase in enzyme activity and a decrease in stability, taking in account the period of time the enzyme is used.

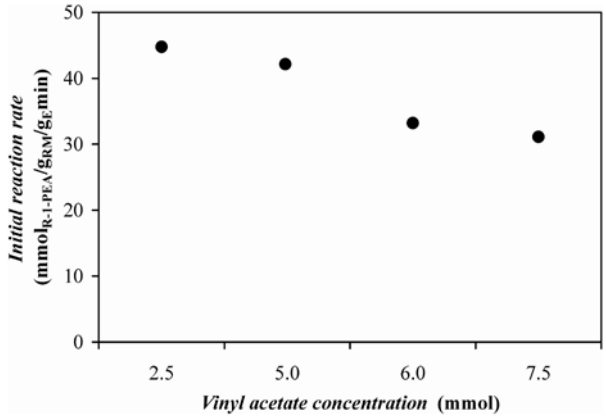


Figure 12. Influence of vinyl acetate concentration on initial reaction rate for acylation of (*R,S*)-1-phenylethanol at constant (*R,S*)-1-phenylethanol concentration. Reaction conditions: 5.0 mmol (*R,S*)-1-phenylethanol, 5.0 mmol [bmim][BF_4], 333.15 K, 100.40 mg_E/g_{RM} immobilized CALB, 600 rpm, 5 h of reaction performance.

3. 5. Influence of Substrates Ratio on Acylation of (*R,S*)-1-phenylethanol with Vinyl Acetate in [bmim][BF_4]

Because the enzyme activity and reaction rates of an enzyme-catalyzed reaction depend on the substrate concentrations, the behavior of immobilized CALB in the presence of different substrate concentrations was studied. The influence of (*R,S*)-1-phenylethanol and vinyl acetate was investigated by varying one of the substrates concentration, while the concentration of other substrate was held constant. Other reaction parameters, such as concentration of biocatalyst, temperature and rotational speed of the stirrer were held constant.

Firstly, the vinyl acetate concentration was varied from 2.5 mmol to 7.5 mmol at the fixed (*R,S*)-1-phenylethanol concentration (5.0 mmol) (Figure 11 and figure 12).

The increase in vinyl acetate concentration from 2.5 mmol to 7.5 mmol led to decrease in initial reaction rate (Figure 12) from 44.76 $mmol_{R-1-PEA}/g_{RM}/g_E$min to 31.11 $mmol_{R-1-PEA}/g_{RM}/g_E$min, which could be explained by the inhibition effect of the vinyl acetate at high concentration. Despite the fact that the initial reaction rates were decreasing with increasing the vinyl acetate concentration from 2.5 to 7.5 mmol, 50% conversion was achieved after 1 h of reaction performance when 5.0 mmol of vinyl acetate was used and after 2 h of reaction performance when 6.0 and 7.5 mmol of vinyl acetate were used for the reaction. For reaction performed with 2.5 mmol of vinyl acetate only 41% conversion was achieved after 5 h of reaction performance and the reason for such decrease in conversion could be the insufficient amount of the (*R*)-1-phenyletha-

nol in the reaction mixture, which could be converted to (*R*)-1-phenylethyl acetate.

Secondly, the (*R,S*)-1-phenylethanol concentration was varied from 1.0 mmol to 10.0 mmol while the vinyl acetate concentration was fixed at 5.0 mmol. Figure 13 and figure 14 show the influence of (*R,S*)-1-phenylethanol concentration on reaction performance and on initial reaction rate for acylation of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4], respectively.

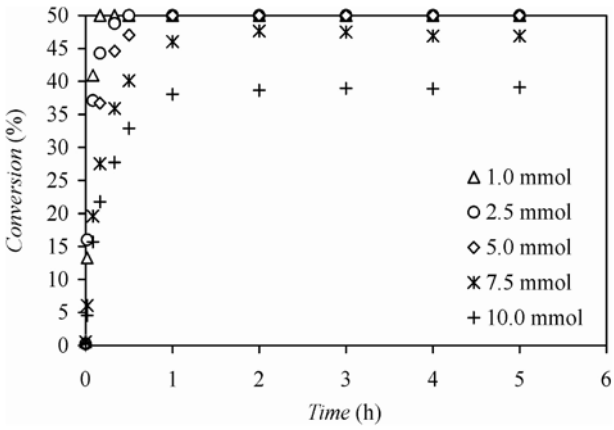


Figure 13. Influence of (*R,S*)-1-phenylethanol concentration on acylation of (*R,S*)-1-phenylethanol with vinyl acetate in [bmim][BF_4]. Reaction conditions: 5.0 mmol vinyl acetate, 5.0 mmol [bmim][BF_4], 100.40 mg_E/g_{RM} immobilized CALB, 600 rpm, 5 h of reaction performance.

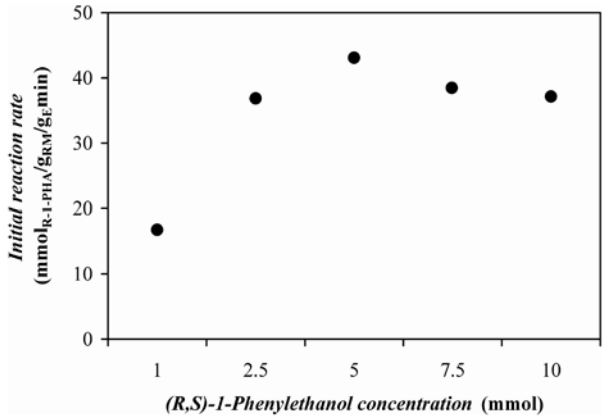


Figure 14. Influence of (*R,S*)-1-phenylethanol concentration on initial reaction rate for acylation of (*R,S*)-1-phenylethanol at constant vinyl acetate concentration. Reaction conditions: 5.0 mmol vinyl acetate, 5.0 mmol [bmim][BF_4], 333.15 K, 100.40 mg_E/g_{RM} immobilized CALB, 600 rpm, 5 h of reaction performance.

At constant vinyl acetate concentration (5.0 mmol) the increase in (*R,S*)-1-phenylethanol concentration from 1.0 mmol to 5.0 mmol resulted in higher initial reaction rate and 50% conversion of (*R*)-1-phenylethanol to (*R*)-1-phenylethyl acetate for 1.0 mmol, 2.5 mmol and 5.0 mmol of (*R,S*)-1-phenylethanol was obtained after 0.16 h, 0.5 h and after 1 h of reaction performance, respectively. With further increasing in (*R,S*)-1-phenylethanol concentration the decrease in initial reaction rates and equilibrium conversions was observed. This could probably happen due to insufficient amount of acyl donor, which would be needed for the increased amount of (*R,S*)-1-phenylethanol. The effect of alcohol inhibition should be considered, as well. Furthermore, enzyme deactivation could occur due to relatively low log*P* value for the (*R,S*)-1-phenylethanol (1.62), since the enzyme activity is affected by the polarity of organic media, which can be measured by the log*P* value (partition coefficient of a given compound in the octanol and water two-phase system). Organic solvents with a log*P* lower than 2 are considered hydrophilic in nature and often ineffective. On the other hand, the high hydrophobic solvents with log*P* higher than 4 are considered the most suitable solvents for biocatalysis, since they generally support enzymatic activity.[22]

4. Conclusion

ILs are promising media for enzymatic reactions. Besides potential environmental benefits, ILs permit enzyme-catalyzed reactions in a solvent polarity range that was previously inaccessible. The acylation of (*R,S*)-1-phenylethanol was successfully carried out, when immobilized lipases from *Candida antarctica* were used as biocatalyst. Only immobilized lipase from *Rhizomucor miehei* proved to be unsuitable biocatalyst for acylation of (*R,S*)-1-phenylethanol performed in [bmim][BF_4]. Moreover, the acylation of (*R,S*)-1-phenylethanol, catalyzed with CALB was successfully carried out in all assayed ILs, e.g. [bmim][BF_4], [bmim][PF_6], and [emim][NTf_2]. IL [bmim][BF_4] has been shown to act as an excellent non-aqueous media, since the highest reaction rate and 49.7% conversion of (*R*)-1-phenylethanol into the enantiopure (*R*)-1-phenylethyl acetate after 5 h of reaction performance were achieved.

Furthermore, the study of the (*R,S*)-1-phenylethanol acylation, catalyzed by immobilized CALB in IL [bmim][BF_4] was aimed to obtaining optimum conditions for the mentioned reaction. Since the price of the biocatalyst largely contributes to the overall process costs, the biocatalyst concentration for enzyme-catalyzed acylation of (*R,S*)-1-phenylethanol was optimized. Because the reaction rate of enzymatic reaction depends on substrate concentration, the influence of (*R,S*)-1-phenylethanol and vinyl acetate was investigated as well by varying one of the substrates concentration, while the other was held constant. Furthermore, enzymes are proteins, which can deactivate at temperatures higher than optimal temperature and for that reason temperature was optimized. The highest conversion for the acylation of (*R,S*)-1-phenylethanol with vinyl acetate at atmospheric pressure was achieved with

100.40 mg_E/g_{RM} of biocatalyst at 333.15 K and with rotational speed of 600 rpm.

In the researches published to date, the optimization of different reaction parameters (biocatalyst concentration, temperature, substrate concentration ...) for enzyme-catalyzed acylation of (*R,S*)-1-phenylethanol at atmospheric pressure have been studied particularly in organic solvents.[38] Eckstein et al.[37] studied the influence of temperature on acylation of (*R,S*)-1-phenylethanol with vinyl acetate in 1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl)]amide.

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Povzetek

Ionske tekočine predstavljajo zanimiv razred reakcijskih topil za katalizo, ki se uspešno uporabljajo za encimsko katalizirane reakcije. V našem delu smo sintetizirali tri različne ionske tekočine, in sicer 1-butil-3-metilimidazolijev tetrafluoroborat, 1-butil-3-metilimidazolijev heksafluorofosfat in 1-etil-3-metilimidazolijev bis[(trifluorometil)sulfonil)]amid, z namenom, da določimo najprimernejšo ionsko tekočino kot topilo za encimsko katalizirano acilacijo (*R,S*)-1-feniletanola z vinil acetatom in za optimizacijo reakcijskih parametrov (koncentracije biokatalizatorja, temperature, ...) za omenjeno reakcijo, ki smo jo izvedli v mešalnem šaržnem reaktorju. Proučili smo tudi vpliv treh različnih imobiliziranih lipaz na potek reakcije acilacije.

Najvišjo začetno hitrost reakcije in 49.7% presnovo po 5 h reakcije smo dosegli, ko smo kot biokatalizator uporabili imobilizirano lipazo Novozym 435 iz *Candida antarctica* in hidrofilno ionsko tekočino 1-butil-3-metilimidazolijev tetrafluoroborat kot reakcijski medij. Tako smo vpliv reakcijskih parametrov, kot so koncentracija biokatalizatorja, temperatura in koncentracija substratov, na acilacijo (*R,S*)-1-feniletanola, optimirali v 1-butil-3-metilimidazolijevem tetrafluoroboratu.