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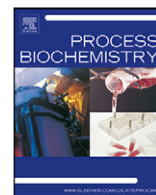

Isoamyl acetate synthesis in imidazolium-based ionic liquids using packed bed enzyme microreactor

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ABSTRACT

The acylation of isoamyl alcohol with acetic anhydride catalyzed by immobilized *Candida antarctica* lipase B was studied in ionic liquids (ILs) based on quaternary imidazolium cations with alkyl, alkenyl, alkynyl, benzyl, alkoxyl or *N*-aminopropyl side chains. Among the tested ILs, the highest enzyme activity together with the highest isoamyl acetate yield were obtained in [C_7mmim][Tf_2N].

No loss of lipase B activity was observed during one-month incubation in this hydrophobic IL without the presence of substrates. Isoamyl acetate synthesis using [C_7mmim][Tf_2N] as solvent was further studied in a continuously operated miniaturized enzymatic packed bed reactor at various flow rates and temperatures. Up to 92% isoamyl acetate yield could be obtained within 15 min by using 0.5 M acetic anhydride and 1.5 M isoamyl alcohol inlet concentrations at 55 °C, corresponding to the volumetric productivity of 61 mmol l^{-1} min^{-1}, which to the best of our knowledge is the highest reported so far for this reaction. No decrease in productivity was experienced during the subsequent runs of continuous microbioreactor operation performed within 14 consecutive days. The benefits of reactor miniaturization along with the green solvent application were therefore successfully exploited for the development of a sustainable flavour ester production.

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1. Introduction

Isoamyl acetate is one of the most industrially employed flavour esters, which is generally produced by chemical reaction of an alcohol with an organic acid in the presence of an acid catalyst or by extraction from natural sources. In recent years, biotechnological production by the application of lipases is becoming more and more attractive due to higher ecological acceptability when compared to chemical synthesis, or due to economic benefits when compared to extraction from natural sources [1–4]. Although lipase-catalyzed synthesis of isoamyl acetate in organic solvents has been successfully established, the use of solvent-free systems [4], supercritical carbon dioxide [5] and recently also ILs [6,7] as media for this enzyme-catalyzed process has been shown to be a promising environmentally friendly alternative. Among these alternatives, ionic liquids, nonflammable and nonvolatile organic salts which are in liquid state at room temperature, are of a particular interest, as there is a possibility of designing ionic liquids for a specific enzyme-catalyzed reaction in order to increase substrate/product solubility, enhance enzyme activity, selectivity and stability, or to tailor the reaction rate [8]. Fehér et al. [6] established very efficient Novozym 435-catalyzed production of isoamyl acetate in a biphasic mixture of excess of isoamyl alcohol and 1-butyl-3-methylimidazolium hexafluorophosphate. Pohar et al. [7] also developed a highly productive continuous isoamyl acetate synthesis in biphasic systems 1-butyl-3-methylpyridinium dicyanamide/*n*-heptane catalyzed by a dissolved *Candida antarctica* lipase B within a microfluidic system.

Microreactor technology is gaining importance in a broad range of areas, including chemistry and biochemistry. It is now clear that under the right conditions, microreactors can offer better selectivity, improved yields over shorter periods of time, increased process control, greater safety, flexible production, and the opportunity to tap into previously avoided or novel chemistries. Further benefits of miniaturization encompass the possibility of numbering up instead of the usually problematic scale-up. Process intensification by integration of small process units within lab-on-a-chip devices can result in smaller, faster responding, more flexible mini-plants with reduced capital and operating costs. Due to the small amount of chemicals needed, microreactors are also an extremely efficient tool for early (bio)process development stages relevant for large scale operation [9–12].

Continuously operated catalytic packed bed microreactors have recently attracted a lot of attention, since besides the above mentioned advantages they offer extended operational life of the catalyst, easier product recovery and, in specific cases, also reduced possibility for the reverse reaction [13,14]. Kulkarni et al. [13]

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have successfully demonstrated a relatively cheap, reproducible and reusable miniaturized fixed-bed reactor for the esterification of acetic acid and *n*-butanol employing Amberlyst-15 as a catalyst. Esterification of various fatty acids was accomplished in a microreactor packed with Novozym 435 [14], where higher productivity of the miniaturized flow reactor was attributed to the higher effective enzyme concentration within the packed bed reactor, unachievable in batch reactors. A continuous-flow mezzo-scale reactor packed with various immobilized lipases was significantly more favorable also for the kinetic resolution of 2-methylene-substituted cycloalkanols, when compared to its batch counterpart [15].

The aim of this work was to take advantages of ILs as biotransformation media and to harness the benefits of miniaturized packed bed reactors as an efficient tool for the development of a sustainable and environmentally friendly process of enzymatic ester synthesis. For this purpose, a series of alkyl-, alkenyl-, alkynyl-, benzyl- and *N*-alkoxyl-substituted imidazolium-based ILs were synthesized and screened together with some commercial imidazolium-based ILs for lipase-catalyzed esterification of isoamyl alcohol. Furthermore, the effect of temperature and flow rate of reactants on the reaction yield was investigated within a continuously operated miniaturized bioreactor packed with *C. antarctica* lipase B (Novozym 435), which was used to attain the optimization of process parameters in a very short time with low reagent consumption.

2. Materials and methods

2.1. Materials

Novozym 435 (lipase B from *C. antarctica*; immobilized on macro-porous polyacrylic resin beads, specific activity 10,000 propyl laurate unit (PLU)/g; water content 1–2%, w/w) was kindly provided by Novozymes, Bagsværd, Denmark. The six tested commercial ILs [C_2mim][Tf_2N], [C_2mim][BF_4], [C_4mim][PF_6], [C_4mim][BF_4], [C_4mim][Tf_2N], [C_5mim][Tf_2N] and [C_7mim][Tf_2N] were purchased from Ionic Liquid Technologies GmbH & Co. KG, Denzlingen, Germany (purity > 98%). Isoamyl alcohol, acetic anhydride, isoamyl acetate and *n*-heptane were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and were of analytical grade. All chemicals for ILs syntheses were purchased from Acros Organics. ^{1}H NMR and ^{13}C NMR spectra were recorded in a DMSO-d_6 on a VARIAN XL-GEM-300 MHz spectrometer. Chemical shifts were expressed in ppm values using TMS as an internal standard.

2.2. General procedure for the preparation of ionic liquids

Quaternization of 1-methylimidazole and 1,2-dimethylimidazole was performed according to standard procedures [16] or their modifications. Aliphatic or aromatic halide was added in 10% excess to the stirred solution of 1-methylimidazole or 1,2-dimethylimidazole in toluene at 0 °C and the reaction mixture was stirred for 24–48 h at 70 °C. The sedimented product was washed thoroughly with ethyl-acetate, dried under reduced pressure at 70 °C, and characterized by ^{1}H NMR and ^{13}C NMR (see ESI†) as specified above.

1-(3-Aminopropyl)-imidazole was used for the preparation of 1-(3-*N*,*N*-dimethylaminopropyl)-3-methylimidazolium iodide and 1-(3-aminopropyl)-3-ethylimidazolium iodide, and the preparation proceeded as described above.

For the synthesis of 1-methyl-3-pentoxyimidazolium bromide and 1-methyl-3-heptoxyimidazolium bromide, hydrogen peroxide (0.6 mol) was added dropwise to the stirred solution of 1-methylimidazole (0.2 mol). The reaction mixture was stirred for 5 h at room temperature, and the excess of reactants was removed under reduced pressure. The obtained yellow viscous oil that partially crystallized was 1-methylimidazole-*N*-oxide (74% molar yield). Further, to the stirred solution of 1-methylimidazole-*N*-oxide (0.2 mol) in acetonitrile (20 ml), 1-bromopentane or 1-bromoheptane (R-X, 0.22 mol) was added dropwise and the reaction mixture was stirred for 48 h at 60 °C. Acetonitrile was evaporated under reduced pressure and the slightly yellow viscous product was washed thoroughly with ethyl-acetate, dried under reduced pressure at 70 °C, and characterized by ^{1}H NMR and ^{13}C NMR (see ESI†) as specified above. All ILs were vacuum-dried prior to use.

The anion metathesis was accomplished by the treatment of the aqueous solution of quaternary imidazolium halides with a slight excess (10%) of $LiTf_2N$ and stirred for approximately 2 h [17]. The upper aqueous phase was decanted and the lower product portion was washed with water until chloride free, as determined by the silver nitrate test.

The IL was dried under high vacuum at 70 °C and characterized by ^{1}H NMR and ^{13}C NMR (see ESI†) as specified above.

2.3. GC analysis

Isoamyl acetate, isoamyl alcohol and acetic anhydride concentrations in the *n*-heptane phase were determined by a gas chromatograph HP 6890 (Hewlett-Packard, Palo Alto, USA) equipped with a hydrogen flame ionization detector and a HP-INNOWAX column (30 m × 0.25 mm i.d. × 0.25 mm). Nitrogen was used as a carrier gas at a flow rate of 29 ml/min. The temperature of the oven at the injection was 100 °C and was kept constant for 1 min. The linear increase in temperature to 200 °C was set by 358 min^{-1}, and was kept at 200 °C till the end of the analysis. Injector and detector temperatures were set at 250 °C [18]. Quantification of data was done by the calibration with standard samples. The retention time for isoamyl acetate and isoamyl alcohol were 1.57 and 1.73 min, respectively, while the retention time for acetic anhydride was 2.5 min.

2.4. Determination of partitioning coefficients for isoamyl acetate

According to our previous findings [7] the equilibrium concentration of isoamyl acetate within *n*-heptane/IL system was achieved in less than a minute using a vortex mixer, 1 ml of IL containing 0.5 M isoamyl acetate was incubated for 3 min with 1 ml of *n*-heptane at 1500 rpm at 25 °C. The concentration of isoamyl acetate in the *n*-heptane phase was determined by gas chromatography as stated above. The partitioning coefficient was evaluated as the ratio of isoamyl acetate concentration in *n*-heptane phase to its concentration in IL, where the latter was calculated from the initial isoamyl acetate concentration.

2.5. Determination of viscosities of ionic liquids

A rotational viscosimeter (Rotovisco RV 20, Haake, Karlsruhe, Germany) thermostated at 25 °C was used for determination of viscosities of [C_4mim][Tf_2N] and [C_7mmim][Tf_2N].

2.6. Screening of ionic liquids for enzymatic synthesis of isoamyl acetate

All experiments were carried out in thermostated test tubes at 25 °C placed on a vortex mixer at 2000 rpm. The reaction started by adding 20 mg Novozym 435 to 600 μl of IL or *n*-heptane containing 0.8 M acetic anhydride and 2.4 M isoamyl alcohol. Reactions without the enzyme were also performed. When monitoring reactions in ILs, isoamyl acetate was recovered at specified time intervals via liquid extraction using *n*-heptane until no further raise in yield was detected. The biphasic mixture containing 50 μl of IL-phase aliquots and 50 μl of *n*-heptane was strongly shaken for 3 min and analyzed by a gas chromatograph as stated above. Isoamyl acetate concentration was calculated by taking into account the partitioning coefficient for isoamyl acetate in the *n*-heptane/IL system. For reactions performed in *n*-heptane, aliquots of 20 μl were directly analyzed by a gas chromatograph. All experiments were carried out in duplicates and the average values were calculated from the results.

2.7. Enzyme stability in [C_7mmim][Tf_2N]

Novozym 435 (2 mg) was incubated in [C_7mmim][Tf_2N] (393 μl) at 25 °C. At selected intervals within one month of incubation, enzyme reactions were initiated by adding acetic anhydride isoamyl alcohol into the lipase–IL mixture in amounts to yield final concentrations 0.5 M and 1.5 M, respectively and the reactions were monitored as stated above. The relative activity (%) was calculated from the initial reaction rate obtained by the enzyme after incubation in IL, compared to the one obtained without previous exposure to IL.

2.8. Continuously operated isoamyl acetate synthesis within a microreactor

A miniaturized continuous flow reactor made of PMMA and an olefin-based laboratory film assembled in a sandwich-like structure providing microchannel structure was used for achieving continuous esterification (Fig. 1). One layer of Novozym 435 beads (70 mg) was incorporated into the channel of 1 cm width, 450 μm height and 75 mm length [19]. [C_7mmim][Tf_2N] containing 0.5 M of acetic anhydride and 1.5 M of isoamyl alcohol was pump-driven through the packed bed microreactor by means of a high pressure syringe pump (Harvard Apparatus, Holliston, USA) at flow rates between 5 and 90 μl min^{-1}. The reaction was carried out at 25, 35, 45 and 55 °C. The reaction temperature was controlled by embedding the enzyme microreactor into a thermostated bath. After reaching a steady state, the outflow of the microreactor was collected and analyzed at selected time intervals by gas chromatography as stated above. The developed miniaturized reactor was repeatedly used every day over consecutive 14 days in order to evaluate system stability. After each continuous-flow experiment performed at 25 °C as stated above, the flow through the microreactor was stopped and the system was stored at 25 °C till further use.

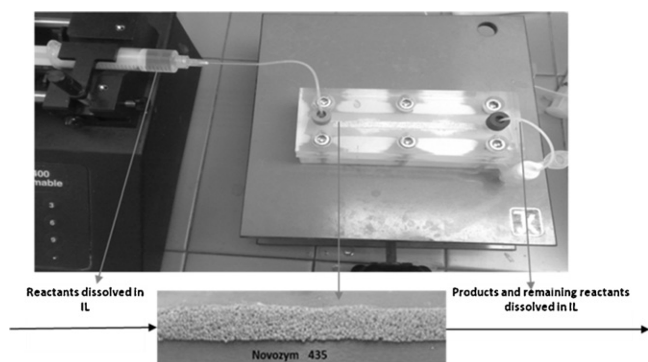


Fig. 1. Miniaturized packed bed reactor used for the investigation of various process parameters on lipase-catalyzed esterification.

3. Results and discussion

3.1. Screening of ionic liquids for enzymatic synthesis of isoamyl acetate

In total, 19 ILs based on imidazolium cation, shown in Table 1, have been evaluated as media for the *C. antarctica* lipase B-catalyzed isoamyl acetate synthesis accomplished by acylation of isoamyl alcohol with acetic anhydride (Scheme 1), and compared with the reaction performed in *n*-heptane. 12 of the tested ILs associated with [Tf_2N] anions have been synthesized by preparation of bromide imidazolium salts followed by anion exchange reaction.

As shown in Table 1, all ILs synthesized in our lab were liquid at room temperature and were not miscible with water. Amino-functionalized ILs ([mapim][Tf_2N], [eapim][Tf_2N]) could not be further characterized and used for biotransformation due to their very high viscosity. Miscibility of the tested ILs with isoamyl alcohol was found to be related to the length of the alkyl chain of the cation (Table 1). Addition of acetic anhydride to mixtures of alcohol and ILs promoted solubilisation of isoamyl alcohol, resulting in homogeneous solutions in all cases except in mixtures with ethyl-, allyl- and propargyl-substituted ILs, which gave stabile emulsions with both reactants.

Based on previous studies on the type of acyl donor and the ratio of reactants for the lipase-catalyzed isoamyl acetate synthesis [1,2,7], acetic anhydride was used in 1:3 molar ratio with isoamyl alcohol. Blank experiments without the enzyme were performed in all tested ILs and no conversion was observed within the reaction times used in this study. The time-courses of esterifications in various ILs, shown in Fig. 2, reflect the complex kinetic behavior of the synthesis, occurring in two steps (Scheme 1). First, one acyl group is directly employed to form ester (main reaction, fast) while the other one leads to the formation of an acetic acid molecule which may also react with the alcohol (secondary reaction, slow). Thus, at the beginning of esterification, acetic anhydride promptly reacted with alcohol, while acylation by the produced acetic acid did not occur until acetic anhydride was completely consumed [3]. This resulted in fast ester formation at the beginning of the batch process, followed by significantly slower esterification rate in all media tested within this study.

As evident from Fig. 2a, the esterification process was proved to be highly dependent on both cation and anion nature of the solvent. The influence of anion structure on esterification performed in ILs with [C_4mim] cation, represented in Fig. 2a, revealed the benefit of using [Tf_2N] anion over [PF_6] and especially [BF_4], which resulted also in much lower product yield at the end of the experiment. Besides, initial reaction rates were notably higher in all ILs with [Tf_2N] and [PF_6] anions compared to [BF_4]-associated ILs (Fig. 3), which is in accordance with other reports on the effect

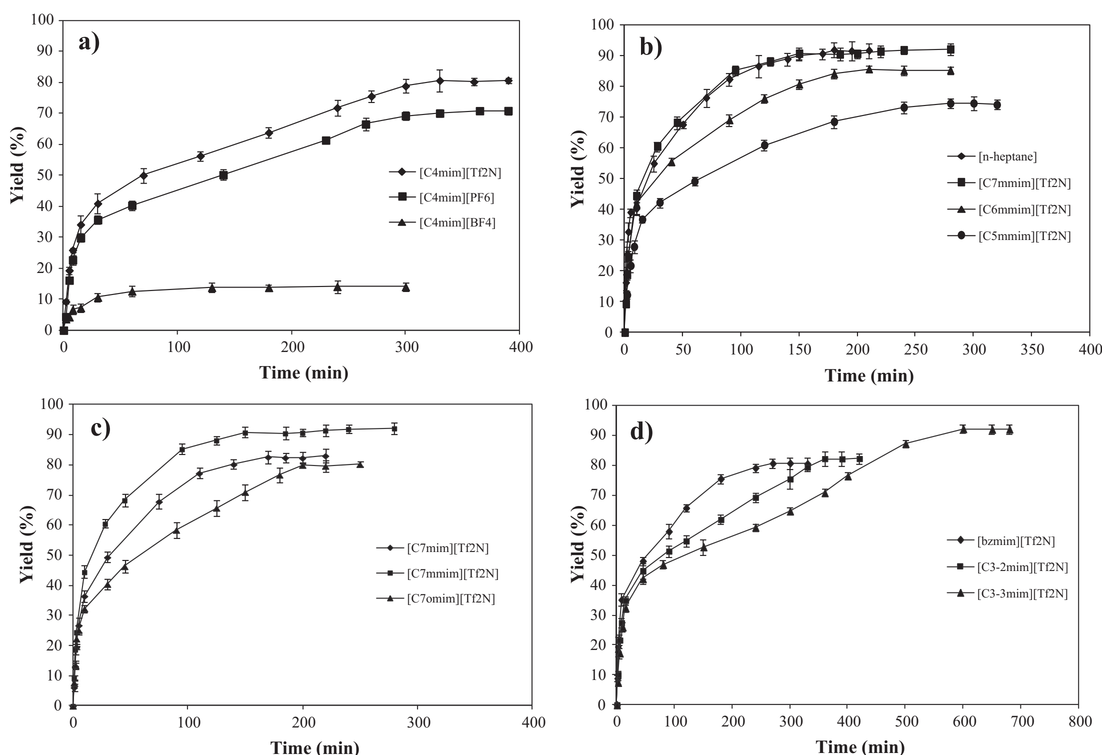
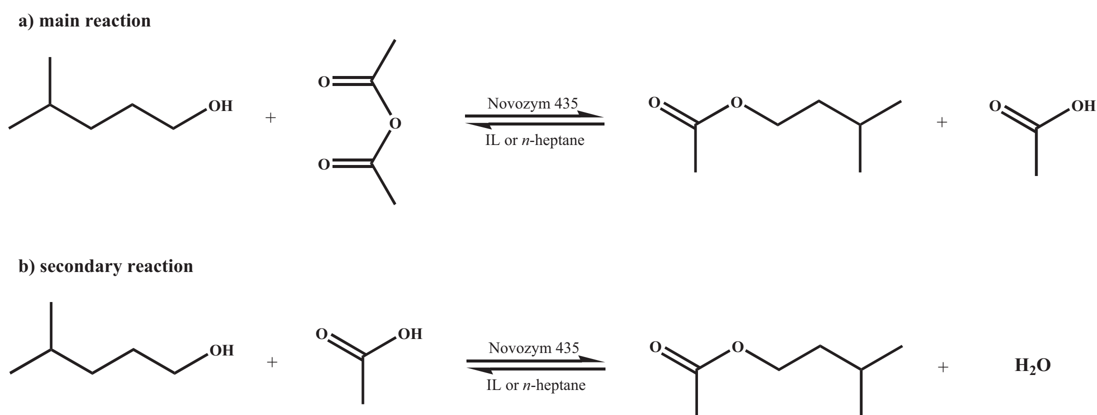


Fig. 2. Time courses of lipase-catalyzed isoamyl acetate syntheses within (a) ILs with [C_4mim] cation, but various anions; (b) *n*-heptane and ILs with [Tf_2N] anion and cations with N-3 and C-2-methyl-substituted imidazolium ring containing various lengths of N-1-substituted alkyl chains; (c) ILs with [Tf_2N] anion and various cations with heptyl- or heptoxy-substituted imidazolium rings; (d) ILs with [Tf_2N] anion and allyl-, propargyl- or benzyl-substituted imidazolium-based cations. Reaction conditions: 0.8 M acetic anhydride, 2.4 M isoamyl alcohol, 20 mg Novozym 435, 25 °C. Each point represents the average of two experiments with standard deviations indicated as vertical bars.



Scheme 1. Lipase-catalyzed synthesis of isoamyl acetate from acetic anhydride and isoamyl alcohol. (a) Main reaction and (b) secondary reaction.

of anion nature on esterification [20–22]. As suggested by Lee et al. [22], whose work on lipase-catalyzed transesterification of benzyl alcohol with vinyl acetate revealed the same anion order regarding initial rates, these results can be partially explained by the hydrophobicity of ILs associated with $[PF_6]$ and $[Tf_2N]$ anions and water solubility of those associated with $[BF_4]$ anion; since esterification reactions were carried out under almost anhydrous conditions, the hydrophobic character of water-immiscible ILs allowed the preservation of the essential water layer in the active site of enzyme, and thus minimized direct protein-ion interactions. Several authors imply that anion nucleophilicity should also be considered to explain such poor lipase performance in ILs associated with the $[BF_4]$ anion, which has the highest nucleophilicity among the tested anions [20,21,23].

Higher ester yields in water-immiscible ILs (anion dependent) could be related to the separation of water produced in the secondary reaction (Scheme 1) from the hydrophobic solution, which shifted the chemical equilibrium towards product formation, as observed also by Jiang et al. [24].

The comparison of esterifications performed in ILs containing the same anion $[Tf_2N]$ and different cations revealed that both the reaction rate and yield increased with the alkyl chain length at the N-1 of the imidazolium ring (Fig. 2b). High ester yields obtained in $[C_7mmim][Tf_2N]$ and $[C_6mmim][Tf_2N]$, as well as in $[C_7mim][Tf_2N]$ (Fig. 2c) could be related to their capability to dissolve isoamyl alcohol, as discussed above and shown in Table 1, which positively influenced the reaction by shifting the chemical equilibrium towards product formation. Similar correlation between lipase activity and alkyl chain length of the cation was observed also in some other literature reports on lipase-catalyzed esterifications [25,26], while Ha et al. [27] reported that the activity of lipase generally decreased with increasing alkyl chain length

Table 1
List of ILs used for screening and their selected physical properties.

Name	Abbrev.	Type	R	X^-	Miscibility			K_p[a]
					H_2O	AA	IA	
1-Ethyl-3-methylimidazolium tetrafluoroborate	$[C_2mim][BF_4]$[b]	I	CH_2CH_3	BF_4^-	Yes	Yes	No	4.43
1-Ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_2mim][Tf_2N]$[b]	I	CH_2CH_3	$(CF_3SO_2)_2N^-$	No	Yes	No	1.94
1-Butyl-3-methylimidazolium hexafluorophosphate	$[C_4mim][PF_6]$[b]	I	$(CH_2)_3CH_3$	PF_6^-	No	Yes	No	3.95
1-Butyl-3-methylimidazolium tetrafluoroborate	$[C_4mim][BF_4]$[b]	I	$(CH_2)_3CH_3$	BF_4^-	Yes	Yes	No	5.62
1-Butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_4mim][Tf_2N]$[b]	I	$(CH_2)_3CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	No	1.15
1-Pentyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_5mim][Tf_2N]$[b]	I	$(CH_2)_4CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	No	1.04
1-Heptyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_7mim][Tf_2N]$[b]	I	$(CH_2)_6CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	Yes	0.91
1-Allyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_{3-2}mim]$ $[Tf_2N]$	I	CH_2CHCH_2	$(CF_3SO_2)_2N^-$	No	Yes	No	1.33
1-Propargyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_{3-3}mim]$ $[Tf_2N]$	I	CH_2CCH	$(CF_3SO_2)_2N^-$	No	Yes	No	1.27
1-Benzyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[bzmim][Tf_2N]$	I	$CH_2C_6H_5$	$(CF_3SO_2)_2N^-$	No	Yes	No	1.55
1-Pentyl-2,3-dimethylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_5mmim][Tf_2N]$	II	$(CH_2)_4CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	No	1.79
1-Hexyl-2,3-dimethylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_6mmim][Tf_2N]$	II	$(CH_2)_5CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	Yes	1.13
1-Heptyl-2,3-dimethylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_7mmim][Tf_2N]$	II	$(CH_2)_6CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	Yes	0.96
1-Allyl-2,3-dimethylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_{3-2}mmim][Tf_2N]$	II	CH_2CHCH_2	$(CF_3SO_2)_2N^-$	No	Yes	No	1.82
1-Propargyl-2,3-dimethylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_{3-3}mmim][Tf_2N]$	II	CH_2CCH	$(CF_3SO_2)_2N^-$	No	Yes	No	1.29
1-Pentoxy-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_5omim][Tf_2N]$	II	$O(CH_2)_4CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	No	1.13
1-Heptoxy-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[C_7omim][Tf_2N]$	II	$O(CH_2)_6CH_3$	$(CF_3SO_2)_2N^-$	No	Yes	Yes	1.14
1-(3-*N*,*N*-Dimethylaminopropyl)-3-methylimidazolium bis(trifluoromethanesulfonyl)imide	$[mapim][Tf_2N]$[c]	III	CH_3	$(CF_3SO_2)_2N^-$	No	N.D.	N.D.	N.D.
1-(3-Aminopropyl)-3-ethylimidazolium bis(trifluoromethanesulfonyl)imide	$[eapim][Tf_2N]$[c]	IV	CH_2CH_3	$(CF_3SO_2)_2N^-$	No	N.D.	N.D.	N.D.

N.D., not determined; AA, acetic anhydride; IA, isoamyl alcohol.
[a] Partitioning coefficient (K_p) for isoamyl acetate within *n*-heptane/IL system.
[b] Commercial ILs.
[c] Highly viscous ILs (esterification could not be conducted).

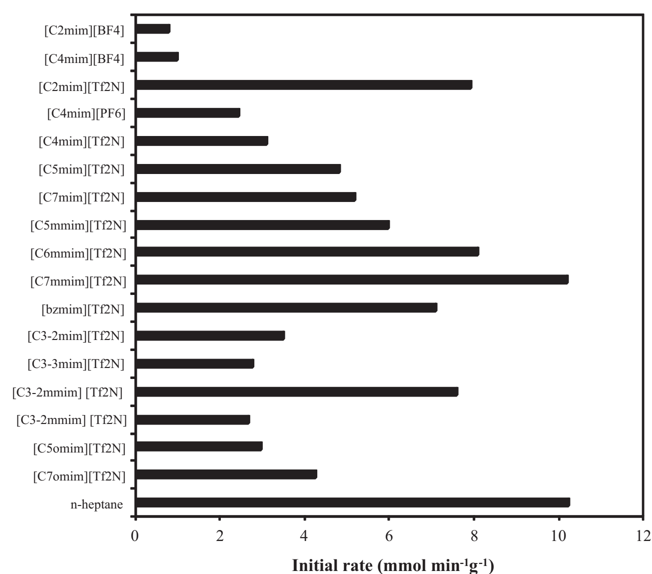


Fig. 3. Initial rates of Novozym 435-mediated isoamyl acetate syntheses in various ILs and *n*-heptane. Reaction conditions: 0.8 M acetic anhydride, 2.4 M isoamyl alcohol, 20 mg Novozym 435, 25 °C. Initial reaction rates were calculated from the linear parts of the plots of product concentration vs. reaction time and expressed per mass of Novozym 435.

on the cation in ILs. Contradictory results of Lee et al. [22] regarding lipase activity in ILs with various alkyl chain lengths at the N-1 of the imidazolium ring were explained by the increase of viscosity and hydrophobicity with increasing alkyl chain length of the cation, which appeared to have a contradictory effect on lipase activity. In our case, slower mass transfer due to higher viscosity of [C_7mmim][Tf_2N], which was 0.17 Pa s at 25 °C, as compared to the viscosity of e.g. [C_4mim][Tf_2N] with 0.05 Pa s at 25 °C, obviously did not prevail over the positive effect of higher hydrophobicity of ILs with longer alkyl chain length of the cation on the process. The increase in the initial reaction rate and ester yield in ILs with cations containing a methyl group at the C-2 position of the imidazolium ring is evident from Figs. 2c and 3. Specifically, the initial reaction rate in [C_7mmim][Tf_2N] was approx. two-fold higher with respect to that obtained in [C_7mmim][Tf_2N], while ester yield was improved by approx. 10%. Ha et al. [27] also observed higher lipase activity and stability in [Tf_2N]-based IL and attributed it to the lowered acidity of this IL related the removal of the proton at C-2-position on the imidazolium cation.

The influence of functionalized side chains at the N-1 position of the imidazolium ring was also investigated. ILs with alkoxy functional group ILs, [C_5omim][Tf_2N] and [C_7omim][Tf_2N], were found to be slightly less favorable for the studied lipase-catalyzed esterification compared with their non-functionalized ILs analogues [C_5mim][Tf_2N] and [C_7mim][Tf_2N], respectively (Figs. 2c and 3). Poorer lipase performance in these ILs could be related to the nucleophilic character of oxygen in the alkoxy group, which may interact with the positively charged sites in the enzyme molecule, leading to conformational changes and consequently to changes in enzyme activity. When ILs with unsaturated (allyl, propargyl) or aromatic (benzyl) side chains were compared, esterification rate of lipases followed the order benzyl-functionalized > allyl-functionalized > propargyl-functionalized ILs (Figs. 2d and 3). To explain this effect, electron density in the functionalized part of the cation should be considered, since electron density in the carbon–carbon triple bond of propargyl side-chains is much higher than in the carbon–carbon double bond of the allyl side-chain. The same is valid also for the aromatic ring of benzyl side-chain where the negative charge is distributed over many carbon atoms through

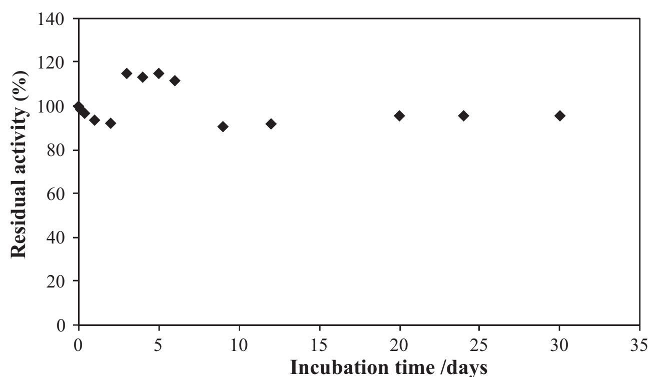


Fig. 4. Relative activity of Novozym 435 after incubation in [C_7mmim][Tf_2N] at 25 °C without substrate addition. The relative activity (%) refers to the percentage of the initial reaction rate obtained by the enzyme after incubation in IL as compared to the one obtained without previous exposure to IL.

resonance (lower overall negative charge), leading to lower interaction with the enzyme due to weaker nucleophilic character.

This screening revealed that [C_7mmim][Tf_2N] was the best solvent among the tested ILs according to initial reaction rate (10.2 mmol min^{-1} g^{-1}) and isoamyl acetate yield (92%). The reaction in [C_7mmim][Tf_2N] was comparable to that performed in *n*-heptane (Figs. 2b and 3), which suggested that this IL was a promising reaction media for the studied biotransformation instead of highly volatile organic solvents.

3.2. Enzyme stability in [C_7mmim][Tf_2N]

In order to acknowledge the suitability of [C_7mmim][Tf_2N] for lipase-catalyzed isoamyl acetate synthesis, the influence of [C_7mmim][Tf_2N] on Novozym 435 stability was studied by incubating the enzyme in the chosen IL at 25 °C. As seen in Fig. 4, Novozym 435 showed excellent stability in the selected IL within a one-month period, which is presumably related to high hydrophobicity of this IL and low nucleophilicity of its anion. Namely, previous studies on the stability of free and immobilized lipases in various ILs revealed the benefit of using highly hydrophobic ILs with anions of low nucleophilicity, which coated and thus protected the layer of essential water surrounding the enzyme [24,26–30]. The activity of Novozym 435 was well maintained during two days of incubation in hydrophobic and water-immiscible ILs containing [Tf_2N] or [PF_6] anion at 70 °C, while it remarkably decreased in hydrophilic ILs [24]. Yuan et al. [30] suggested that the excellent enzyme stability in hydrophobic ILs could also be explained by electrostatic interactions between the IL and the protein, resulting in a more rigid molecular structure, which needs to overcome a higher kinetic barrier to unfold as compared to the enzyme suspended in a non-ionic organic solvent.

The results of our investigations on enzyme stability also demonstrated an increase in enzyme activity after two days of incubation. Enhancement of activity was also reported for free and immobilized lipase after incubation in various ILs [22,29–31]. To explain this unusual data, Kaar et al. [29] suggested that ILs probably caused a permanent activating conformational change or an increase in active site concentration.

3.3. Continuous flow esterification within enzyme microreactor

Continuous isoamyl acetate production within the selected [C_7mmim][Tf_2N] was further studied in a miniaturized packed bed bioreactor. 0.5 M acetic anhydride and 1.5 M isoamyl alcohol dissolved in [C_7mmim][Tf_2N] were pumped through the microbioreactor by means of a high-pressure syringe pump,

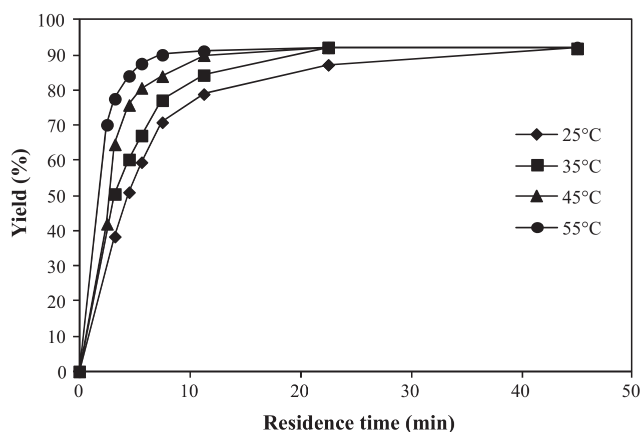


Fig. 5. The influence of flow rate and temperature on isoamyl acetate yield at the outlet of a miniaturized continuous-flow packed-bed reactor at steady-state conditions. Reaction conditions: $[C_7mmim][Tf_2N]$, 0.5 M acetic anhydride, 1.5 M isoamyl alcohol, 70 mg Novozym 435, 25 °C.

providing constant flow without disturbances. The continuous process was governed at various flow rates of inlet substrate solution and at temperatures ranging from 25 to 55 °C and the outflow of the microreactor was analyzed after reaching the steady state.

The effect of residence time and temperature on isoamyl acetate synthesis in the packed bed microreactor is shown in Fig. 5. As expected, the increase of the flow rate through the miniaturized bioreactor and thereby shortening of the residence time resulted in a decrease in ester concentration at the microchannel outlet. When the reaction was conducted at 25 °C and at 70 μl min^{-1} (residence time 3.2 min), only 29% ester yield was obtained, while at 5 μl min^{-1} (residence time 45 min), a maximum 92% ester yield was achieved, which would give the volumetric productivity of 20 mmol l^{-1} min^{-1} and specific biocatalyst productivity of 0.064 mmol g^{-1} min^{-1}, calculated per g of Novozym 435 preparation. The unrivalled volumetric productivity of the continuous process as compared to the batch process was confirmed, since batch esterification within the same solvent at 25 °C and at even higher substrate concentration could yield 92% ester in 150 min, giving the volumetric productivity of 9.1 mmol l^{-1} min^{-1}. Although the calculated specific biocatalyst productivity of 0.273 mmol g^{-1} min^{-1} was higher in batch operation, the high enzyme loads obtained in packed bed reactors could not be achieved within the batch reactor, preventing high ester yields within the given time and space, as discussed also by Woodcock et al. [14]. Moreover, the continuous operation is much more efficient due to the lack of downtimes, essential in the batch mode. Furthermore, the very small void volume of the miniaturized packed bed reactor resulted in very short diffusion paths within the fluid stream and consequently led to very efficient mass transfer and mixing. Therefore much lower energy input was required for achieving mass transfer of reactants and products to and from the active sites of enzyme molecules, which for Novozym 435 was found to be in a 80–100 μm thick outer shell of resin beads [32], within the pressure-driven microfluidic reactor as compared to the vigorously shaken batch reactor.

By elevating the temperature to 35, 45 and 55 °C, the expected increase in lipase activity was observed (Fig. 5). By using 0.5 M acetic anhydride and 1.5 M isoamyl alcohol inlet concentrations at 55 °C, the maximal 92% isoamyl acetate yield could be obtained in 15 min, giving volumetric productivity of 61 mmol l^{-1} min^{-1} and specific biocatalyst productivity of 0.196 mmol g^{-1} min^{-1}. Our results indicated the highest reported productivity for lipase-catalyzed isoamyl acetate synthesis as compared to other studies, summarized by Pohar et al. [7]; the highest stated productivity of 22.3 mmol l^{-1} min^{-1} was achieved in our previous study using an ionic-liquid/*n*-heptane two phase system within a Ψ-shaped microchannel with free *C. antarctica* lipase B. Therefore we could claim that the packed bed system reported in this study significantly improved process efficiency and confirmed process intensification within the developed microstructured device.

Furthermore, multiple runs of continuous biotransformation were done over a period of two weeks and no change in steady-state outlet product concentration was observed, indicating no loss of lipase activity. This proved the selection of $[C_7mmim][Tf_2N]$ to be a suitable medium for isoamyl acetate synthesis.

4. Conclusions

Screening of 19 imidazolium ILs for lipase-catalyzed isoamyl acetate synthesis confirmed that both catalytic activity and maximum ester yield were highly dependent on the intrinsic characteristics of the IL used as a solvent. Highly hydrophobic ionic liquids with long carbon chains as substituents on imidazolium ring of the cation were shown to be very efficient media for the studied esterification. Furthermore, continuous esterification within a pressure-driven miniaturized reactor packed with Novozym 435 beads was successfully accomplished using $[C_7mmim][Tf_2N]$, revealing the highest volumetric productivity for lipase-mediated isoamyl acetate synthesis reported so far in an environmentally benign process without the presence of volatile organic solvents. The microbioreactor presented in this study could also serve as a useful tool for process parameters evaluation for heterogeneous enzyme-catalyzed processes of industrial importance, as a wide range of conditions could be tested over a very short period of time and at low costs.

Further integration with a miniaturized continuous separator that exploited ionic liquids' high boiling point compared to those of products/substrates might be achieved, as recently proved in our studies on a lipase-catalyzed transesterification [19], which would enable simultaneous and efficient cleaning of ionic liquid for its reuse. Thus the integration of new environmentally friendly chemical routes and technical innovation was established in order to achieve process intensification and green process development. However, the greenness of the IL used should be assessed on an entire basis, including its synthesis and recovery, using the life-cycle analysis method [33] and possible improvements should be made. Furthermore, a detailed process economics analysis such as recently presented by Tufvesson et al. [34] would be required to select the most cost-effective production system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2012.04.028.

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