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Enzymatic production of isoamyl acetate in an ionic liquid–alcohol biphasic system

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Abstract

A systematic investigation and optimization on the conditions for the biphasic enzymatic production of isoamyl acetate in ionic liquid solvents is reported. As a catalyst, immobilized *Candida antarctica* lipase B was used. As the best imidazolium type ionic liquid, [bmim]PF_6 was selected and other reaction conditions such as initial water content, acetic acid to isoamyl alcohol substrate ratio, [bmim]PF_6 to isoamyl alcohol solvent ratio, enzyme amount and temperature were optimized. The recyclability of the applied IL per se and together with enzyme were also studied and found to be reusable in 7 and 10 cycles, respectively. With the adjusted optimum conditions high isoamyl acetate yields (near 100%) were achieved.

Keywords: Biphasic system; Flavour; Isoamyl acetate; Ionic liquid; *Candida antarctica* lipase B

1. Introduction

While water is the traditional solvent for biocatalysis, around 1985 enzymes were shown to be active in media other than water, as well [1]. Although enzymatic transformations in ionic liquids (ILs) have only been considered since 2000 [2,3] a wide number of applications have already been tested [4] in this field. ILs have gained wide popularity not only in conventional synthetic chemistry, but also in biotransformations as solvents and catalysts [4].

Applying ILs for biotransformations in biphasic systems to replace the organic solvent [5–9], or together with organic solvents [5,10,11] or with s.c.CO_2 [10,12] enhanced stability, selectivity, enantioselectivity, product yield, initial reaction rate were achieved in several types of enzymatic reactions such as esterification [9,10,12], transcyanidation [11], oxidoreduction [5–7], hydrogenation [13], transglycosylation [8]. Lozano et al. used ILs not as real solvents, but coating immobilized enzyme to improve its stability [10]. They can be used as co-solvents as well, resulting enhanced stability [5,11].

Natural flavours obtained by extraction from fruits are too expensive for the commercial sector, therefore the demand for pure, high quality flavour compounds is gradually increasing nowadays. Isoamyl acetate is widely used in food industry because of its characteristic banana flavour [14,15]. It has been generally produced by free and immobilized lipases from various sources in organic solvents ([14], yield 80%). Novozym 435 from *Candida antarctica* gave the best results in hexane ([16], yield 192% for acetic anhydride), solvent-free systems ([17], yield 80% and [15], yield 75%) as well as in s.c.CO_2 ([18], yield >90%). Supercritical fluids have a number of advantages compared to organic solvents and have an attractive future due to their low environmental impact. Romero et al. have attained esterification to the extent of 100% with s.c.CO_2 [19] and in hexane [20] using acetic acid as acyl donor. The enzymatic production of isoamyl acetate has never been investigated in IL and/or biphasic systems. The purposes of this work were (1) to combine the advantages of ILs, solvent-free systems and biphasic productions (nonvolatility, stabilizing effect on enzyme, no added organic solvent, enzyme recovery, IL reuse, easy product

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separation) and eliminate their disadvantages (high price, high viscosity), (2) to focus our attention for scale up, separation and recirculation/reuse of compounds. Therefore, the first step was the optimization of the reaction conditions for enzymatic production of isoamyl acetate using Novozym 435 lipase B from *C. antarctica* (CALB) in IL–alcoholic biphasic system.

2. Experimental

2.1. Enzyme, ILs and other chemicals

Novozym 435 preparation is a commercial CALB (E.C. 3.1.1.3. *Triacylglycerol acylhydrolase*) immobilized on a macroporous acrylic resin with a water content of 1–2% (w/w). The nominal activity was approximately 7000 Propyl Laurate Units (PLU/g). The tested six ILs were as follows: 1-ethyl-3-methyl-imidazolium-toluolsulfonate ([emim]tosy), 1-butyl-3- methyl-imidazolium-hexafluorophosphate ([bmim]PF_6), 1-butyl-3-methyl-imidazolium-chloride ([bmim]Cl), 1-butyl-3-methyl-imidazolium-tetrafluoro-borate ([bmim]BF_4), 1-hexyl-3-methyl-imidazolium-hexafluorophosphate ([hmim]PF_6), and 1,3-dimethyl-imidazolium-dimethylphosphate [mmim] $(CH_3)_2PO_4$ from Ionic Liquid Technologies GmbH & Co. KG, Denzlingen, Germany. Isoamyl acetate, potassium hydroxide and *n*-hexane were purchased from Reanal, Hungary whereas isoamyl alcohol, ethyl alcohol and acetic acid were from Spectrum 3D, Hungary in analytical grade.

2.2. Determination of ester, water and acid contents

The GC analyses for the determination of ester concentration were carried out with a HP 5890 A gas chromatograph, with HP-FFAP column (Macherey-Nagel), split 70 kPa, N_2 19 cm^3/min, using a FID detector. The samples from the upper isoamyl alcoholic phase were diluted 10-fold with *n*-hexane and 0.5 μl was then injected. The isoamyl acetate concentration changes during the reaction were followed using toluene as internal standard.

The water content was determined by a Mettler DL 35 Karl-Fisher titrator. The acid content was measured by a conventional acid–base titration, using 0.1 M ethyl alcoholic solution of potassium hydroxide and phenolphthalein indicator. The ethanol was pre-treated as follows: 500 cm^3 of 96% of ethyl alcohol and 3 g of potassium hydroxide were refluxed for 1 h to bind all of the components in the ethanol, which consume potassium hydroxide, then it was distilled and used to prepare the suitable 0.1 M solution of potassium hydroxide.

2.3. Partitioning of components

7.34 mmol of acetic acid or 2.24 mmol of ester were incubated in 1 cm^3 of isoamyl alcohol and 1 cm^3 of [bmim]PF_6 for 1 h at 60 °C at 200 rpm. To investigate the distribution of isoamyl alcohol to IL 1 cm^3 of isoamyl alcohol and 1 cm^3 of [bmim]PF_6 were incubated for 1 h at 60 °C at 200 rpm. Then the isoamyl alcohol was extracted by 4×5 cm^3 hexane from the IL phase and measured by GC. To calculate the partition coefficients of acid/ester between the two phases and solubility of isoamyl alcohol in [bmim]PF_6 Eq. (1) was used [28]:

$$K_A = \left(\frac{[A]_{\text{alcohol(i)}} - [A]_{\text{alcohol(f)}}}{[A]_{\text{alcohol(f)}}}\right)\frac{V_{\text{alcohol}}}{V_{\text{IL}}} \tag{1}$$

where $[A]_{\text{alcohol(i)}}$ and $[A]_{\text{alcohol(f)}}$ are concentrations of solute before and after the extraction process in alcoholic phase; V_{alcohol} and V_{IL} are the volumes of alcoholic and IL phases, respectively.

2.4. Reaction conditions

Using the optimum reaction conditions, 54.23 mmol isoamyl alcohol, 7.34 mmol acetic acid, 3.72 mmol water, 2.83 mmol toluene, 1 cm^3 of [bmim]PF_6 and 38 mg Novozym 435 were shaken in a New Brunswick G24 incubator with 100 rpm at 60 °C for 24 h. In the scaled up reaction the above mentioned amounts were used in 6.5-fold increase, except enzyme amount, which was applied in 32.5-fold excess (1235 mg) to decrease the reaction time.

2.5. Downstream process for IL recovery in laboratory scale

At the end of each reaction cycle, the biphasic mixture was separated to an alcoholic and an IL phase containing the enzyme. The IL phase was then separated from the enzyme by glass filtration and finally treated at 60 °C in vacuum to remove the traces of volatile compounds.

2.6. Simultaneous reuse of enzyme and IL in a scaled up system

In a 50 cm^3 thermostated bioreactor reactions were carried out at 50 °C and 800 rpm stirring rate. After each cycle, the upper isoamyl alcoholic phase was pumped into a tank, and the lower IL phase containing the enzyme was used again 10 times in new reactions with fresh substrates.

3. Results and discussion

3.1. Selection of suitable IL

Six ILs of imidazolium type, considered as suitable solvents for esterification according to literature, were used in the experiments. The extremely ordered supramolecular structure of ILs maintains an active three-dimensional structure of the enzyme in aqueous nano-environments, and avoid the classical thermal unfolding [13,21]. This may be a reason why the enzyme is located in the IL phase in the case of two-phase systems. [bmim]BF_4, [bmim]PF_6 and [hmim]PF_6 formed two phases with the substrates. These ILs were considered more convenient because the lipase located in the IL phase could be recycled easier, while the product ester and the deactivating acetic acid were mainly present in the isoamyl alcoholic phase (see distribution results in Section 3.2). In a preliminary test reaction 90.5 and 89.7% ester yield were observed in [bmim]PF_6 and [hmim]PF_6, respectively. No reaction was observed in the

Table 1
Partition coefficients of substrates and product at different temperatures

T (°C)	$K_{\text{acetic acid}}$	$K_{\text{isoamyl acetate}}$	$K_{\text{isoamyl alcohol}}$[a]
30	0.354	0.782	0.007
40	0.392	0.146	0.007
50	0.428	0.050	0.008
60	0.443	0.542	0.007

[a] Maximal solubility of isoamyl alcohol in [bmim]PF$_6$.

hydrophilic [bmim]BF$_4$. Blank reactions with these ILs (i.e. without enzyme) did not give any conversion, consequently these ILs themselves did not catalyse the esterification, acting only as solvent in this reaction system. Based on these results, it was concluded that both [bmim]PF$_6$ and [hmim]PF$_6$ are suitable reaction media for the enzymatic production of isoamyl acetate. Considering its lower price, [bmim]PF$_6$ was used as solvent in the further experiments, which is a frequently used IL for esterification [22,23].

3.2. Partitioning of substrates

Since the reaction mixture is a biphasic system, the distributions of different substrates between the phases should be investigated, results are summarized in Table 1. Solubility of isoamyl alcohol in [bmim]PF$_6$ is very low at any temperature. Partitioning of acetic acid between the two phases does not depend on temperature, while ester does. For the most efficient product separation, 50 °C is the optimum temperature, since at this temperature the main quantity of the product is located in the upper isoamyl alcoholic phase.

3.3. Effect of initial water content

Water as a by-product of the esterification reaction can shift the equilibrium towards the direction of hydrolysis, therefore various methods were applied for removal of the water produced [24,25]. However, catalytic activity in non-conventional media strongly depends on the amount of water associated with the enzyme forming its native conformation [23].

In order to investigate the effect of the initial water content, different amounts of water were added to the reaction mixture. In Fig. 1, the initial reaction rates and final yields are plotted as a function of initial water content. Product yields reached a maximum between 1 and 4% (w/w) (of total mass in each case) water content. Initial water content up to 4% (w/w) had a positive effect on the enzyme activity by reducing the contact with the deactivating acetic acid molecules. At higher water content, water had a negative effect on the thermodynamic balance, shifting the equilibrium towards hydrolysis [26]. Above 4% (w/w) of initial water content, the initial reaction rate and the final yield decreased gradually and approached to zero at 10% (w/w). Since the best result was obtained in the range from 1 to 2% (w/w) initial water content, so further reactions were carried out with 1% (w/w). A similar optimum curve was obtained by Ghamgui et al. [26] with immobilized *Staphylococcus simulans*, while in the work of Hari Krishna et al. [14] the addition of buffer or water did not gave any beneficial result, because the used Lipozyme IM-20 enzyme contained high amount (∼10%, w/w) water.

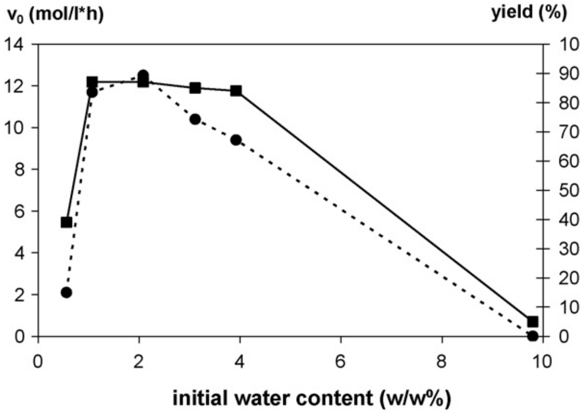

Fig. 1. Effect of initial water content on the yield after 24 h (continuous line) and initial reaction rate at 50 °C and 100 rpm in a shaking incubator (dashed line).

3.4. Influence of composition of reaction medium

To determine the optimal ratio of substrates, solvents and enzyme in the reaction mixture, to reach the highest yield and initial reaction rate, the following three parameters were studied.

At first the effect of isoamyl alcohol to acetic acid molar ratio on the yield was investigated at seven different ratios. When acetic acid was used in excess, one-phase system was obtained, which was not investigated further for the reasons described in Section 3.1. At 1:1.5 and 1:2 acid to alcohol molar ratio, low yield and reaction rate were observed (Fig. 2). This is in accordance with the observations of other authors [14,16,17,26,27]; acetic acid acts as a deactivating agent of lipases during ester synthesis regardless of the media used. Acetic acid is dissolved in the interfacial water of enzyme particles and alters its pH resulting in reduction of the enzyme activity. The yield sig-

Fig. 2. Effect of isoamyl alcohol to acetic acid molar excess on the yield after 48 h (unbroken line) and initial reaction rate (dashed line). The reaction mixture contained isoamyl alcohol in various amounts, 7.3 mmol of acetic acid, 5.8 mmol of water, 2.8 mmol of toluene, 38 mg of Novozyme 435 and 1 cm^3 of [bmim]PF$_6$ at 100 rpm in a shaking incubator at 50 °C.

Table 2
Influence of enzyme amount on reaction time to reach equilibrium ester yield (95%)

Weight of immobilized enzyme per milliliter of [bmim]PF$_6$ (mg)	38	193	378	750
Initial reaction rate (mol/l h)	2.9	3.8	5.2	5.2
Reaction time (h)	30	20	5	5

nificantly increased above four-fold molar excess of alcohol, reached almost 100% at seven-fold and higher excess. The maximum extent of acyl transfer is achieved at 1 to 7 acid to alcohol ratio, which is affected by the concentration of free alcohol available [14,16,17]. For further reactions, therefore, 1 to 7 acid to alcohol ratio was used. The initial reaction rate acts in a different way, as the maximal value was achieved in four-fold molar excess of isoamyl alcohol, which could be an ideal substrate ratio for future kinetic investigations (Fig. 2). The same phenomenon was observed in solvent-free system [17]. Above the critical two-fold excess of acid conversion rapidly decreased, while initial reaction rates slightly increased.

As a second step the IL to isoamyl alcohol volume ratio was investigated. Using IL in high excess (four-fold) one phase was obtained. In smaller excess (two-fold), there were two phases at the beginning of the reaction, but the upper alcoholic phase disappeared after some hours and the sampling was impossible. Alcohol excess had a significant positive influence on the reaction rate and final yield observed. Although the best result was obtained at the ratio of 1 to 1, isoamyl alcohol was used in higher excess in other experiments because of the high price of the IL. Lou et al. [11] and Okochi et al. [5] found 2–6% (v/v) and 5% IL content (respectively) as optimal, but they have used them as co-solvents to enhance the activity of enzyme.

Thirdly, lipase concentration was used in the range of 38–750 mg of immobilized enzyme per milliliter of [bmim]PF$_6$ in order to find the optimum amount of enzyme (Table 2). As it was expected the higher enzyme concentration was applied, the higher initial reaction rate was obtained and shorter time to reach equilibrium conversion. But above 378 mg of immobilized enzyme per milliliter of [bmim]PF$_6$ the increase in the initial reaction rate is weakening and finally vanishes, which could be a result of aggregate formation. It was literally reported [14] when enzyme is used in large excess, a part of its active sites are not exposed to the substrates and remain inside of the enzyme aggregates, without contributing significantly to the reaction. Nevertheless, the final yield appeared to be the same in each case after 30 h, thus the optimum amount of enzyme depends on the desired reaction time, and – from economical aspect – on the actual price of CALB. Therefore, in the scale up reaction an intermediate amount of enzyme was chosen.

Table 3a
Effect of recycling the ionic liquid in laboratory experiments

Number of cycles	1	2	3	4	5	6	7
Relative yield (%)	100	100	100	99	99	98	99
Loss of weight of IL (%)	0	11	11	14	19	19	21

After the downstream procedure 44.1 mmol of isoamyl alcohol, 14.7 mmol of acetic acid and 3.8 mmol of water were added at 100 rpm in a shaking incubator at 50 °C to the recycled [bmim]PF$_6$. The starting reaction mixture contained 400 mg of Novozyme 435 and 3.5 cm^3 [bmim]PF$_6$, in the later cycles the enzyme amount was decreased according to the loss of weight of IL.

3.5. Influence of temperature

Generally, the plot of the initial reaction rate vs. temperature shows a maximum [16,18,26] due to the superposition of the effects of temperature on enzyme reaction and inactivation, rate of inactivation increase exponentially with temperature. Investigating the effect of temperature, the synthesis was carried at four different temperatures: 30, 40, 50 and 60 °C. Initial reaction rate continuously increased within this range, with two-fold increase between 30 and 60 °C. The reaction reached equilibrium conversion at all temperatures in 24 h and gave a final yield over 90%. Initial reaction rate was found four times smaller, but the yield was 10–20% higher compared to the observations of Güvenc et al. [17] in solvent-free system, where the same final yield was reached regardless of temperature applied. The enzyme was stable even at 60 °C, due to the unique heat tolerant properties of Novozym 435 [16] and/or to the stabilization effect of IL on the enzyme. The [bmim]PF$_6$ was also stable at this temperature, unlike reported in literature where it was shown to be instable against hydrolysis especially at higher temperatures [28].

The activation energy was determined from initial reaction rates at different temperatures, based on the Arrhenius equation. It was found to be 27.3 kJ/mol, which is a higher value than the one measured in hexane by Romero et al. [16], but lower than the one obtained by Güvenc et al. [17] in a solvent-free system.

3.6. Recyclability of IL and enzyme

One of the most important properties of ILs is their reusability, which can determine their environmental friendly character and future industrial applications. There are two methods to investigate this key factor: recycling the IL itself or recycling the IL together with enzyme, which was first published by Itoh et al. [29]. In our work, both types of test were investigated for [bmim]PF$_6$ in biphasic system.

Firstly, the reusability of IL itself was investigated in laboratory scale in seven cycles using a special reaction mixture, which contained higher than optimum amount of [bmim]PF$_6$ to ensure the possibility of checking its weight loss during the

Table 3b
Simultaneous reuse of enzyme and IL in a bioreactor

Number of cycles	1	2	3	4	5	6	7	8	9	10
Relative yield (%)	98	96	99	100	99	98	100	96	98	99
Initial reaction rate (mol/l h)	5.2	4.9	5.0	5.2	5.2	5.1	5.2	4.9	5.1	5.1

Reaction conditions see in Section 2.4.

recycling procedure. In order to calculate this quantity, IL needs to be recovered after each cycle. The results are summarized in Table 3a. After seven cycles, nearly the same yields and initial reaction rate were kept in the purified IL with fresh enzyme. The weight of the IL somewhat decreased caused by downstream procedure and the final weight loss was 21%, what was decreased to zero after scale up (described below).

The reusability of enzyme in this reaction was studied by several research groups, where immobilized *S. simulans* kept its activity in four cycles from 10 in solvent-free system [26], Novozym 435 in each cycle from 10 in *n*-pentane [25] and during one month in s.c.CO_2 [19]. Itoh et al. [29] combined the recycling of enzyme with repeated use of ILs in a monophasic transesterification reaction, where the product and unreacted alcohol were extracted with diethyl ether. In a scaled up reaction the simultaneous reusability of Novozym 435 CALB and [bmim]PF_6 was investigated in our biphasic system, too. The grate advantage of the present of two phases was that the upper isoamyl alcoholic phase (containing the main part of product at reaction temperature, see in Section 3.2) was possible to remove into a tank for further separation process, while the lower phase including the enzyme and IL was used again in 10 cycles (Table 3b). The Novozym 435 kept its activity at the same level in 10 cycles, based on initial reaction rate and yield, so the stabilizing property of [bmim]PF_6 on CALB enzyme resulting a more compact enzyme conformation [21,30] was here also proven. Obviously loss in the weight of enzyme and IL was not observed.

4. Conclusions

The enzymatic production of isoamyl acetate from acetic acid and isoamyl alcohol substrates was investigated with Novozym 435 CALB, where the reaction medium was the biphasic mixture of excess of isoamyl alcohol and [bmim]PF_6 ionic liquid. It could be concluded that at optimum conditions excellent yield and initial reaction rate were reached. As far as we know, this is the first time when IL and enzyme were reused together in biphasic system. Since enzyme and IL are the most expensive compounds generally in an enzymatic reaction carried out in IL, biphasic systems seem more attractive from the viewpoint of practical application and environmental aspect. The green advantages both ILs and solvent-free systems appeared, because there was no loss in the amount of IL and enzyme, IL stabilizing the enzyme keeping its activity, the separation of product is easier from the upper phase. These advantages make this biphasic system favourable than monophasic reactions.

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