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Enhanced enantioselectivity of *Candida rugosa* lipase in ionic liquids as compared to organic solvents

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Abstract

Candida rugosa lipase has shown to retain catalytic activity in ionic liquids. In this work, enantioselectivity of this enzyme in the esterification of 2-substituted-propanoic acids and 1-butanol is compared in ionic liquids and organic solvents. The role of solvent hydrophobicity (log *P*), water content and the effect of substituents are evaluated. Optimal water concentration in the reaction media was determined, where the enzyme shows maximal activity and enantioselectivity. Enantioselectivity can be improved when chlorine substituent was replaced by slightly bigger size bromine. Contrary to reactions in common organic solvents, there was no need for purification steps following the reaction in ionic liquids in order to recycle the enzyme. In 1-butyl-3-methyl-imidazolium-hexafluoro-phosphate ([bmim]PF_6) and 1-octyl-3-nonyl-imidazolium-hexafluoro-phosphate ([onim]PF_6) ionic liquids, *C. rugosa* lipase could be recycled five times without appreciable activity or enantioselectivity losses.

Keywords: Ionic liquid; *Candida rugosa* lipase; Enantioselective esterification; Water activity; Hydrophobicity

1. Introduction

The number of the possible biocatalytic pathways leading to a given product has been multiplied since it turned out that enzymes are able to work not only in aqueous systems (their natural media), but in organic solvents, as well. Some of the enzymes showed enhanced stability, activity and selectivity in the new non-conventional reaction media, thus the application of biocatalysts has been considerably widened [1]. Application of organic solvents—as the most often used non-conventional reaction media—has, however, numerous disadvantages: they may be flammable, toxic, volatile, which makes the separation more difficult and the hazard of environmental pollution increases due to solvent loss. Usage of ionic liquids as green solvents seems to eliminate these drawbacks, while it is possible to keep the advantages of common organic solvents or even assure better conditions, since many enzymes have higher stability, activity and selectivity in ionic liquids than in organic solvents [2–6].

Beyond the fact that ionic liquids are considered as green solvents, one of their further advantages is that—unlike organic solvents—they can be "tailored" by selecting the proper cation and anion species. In case of organic solvents, the properties of the media can only be modified by applying solvent mixtures or some kind of additives, like water-mimicking additive (e.g. ethylene-glycol (EG), polyethylene-glycol (PEG)) [7]. For enzymatic reactions, in particular, this is extremely important, since it is well known that the nature of reaction media has significant effect on the activity and selectivity of enzymes. Varying the solvent, the substrate specificity [8] and enantioselectivity [9] of the enzyme may turn.

One of the most studied properties of the solvents is their hydrophobicity (log *P*). In general, it is not possible to predict how it influences the enantioselectivity of the enzymes. In some papers, higher enantioselectivity in hydrophilic solvents was reported [10], but examples for its opposite could

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also be found [11]. There are even special cases, where no correlation between the log *P* value and enzyme enantioselectivity was detected [12]. In addition to this, dipole moment and dielectric constant of the solvents have been found in some systems to provide correlation to enzyme enantioselectivity [11]. So far, a general mechanism on the effect of solvents has not been accepted, although various models were elaborated [13].

The amount of free water available for the enzyme can be estimated by the water activity of the reaction medium [14]. It has great influence both on the reaction rate and the selectivity. Therefore, in order to study the effect of solvents, the most accurate method is to keep the water activity at a constant level during reactions in each solvent.

However, the great drawback of the methods applied for keeping the water activity at a constant level is that additives may be necessary in the reaction medium (molecular sieves, salt-hydrates [14], salt solutions [4,15]). As a result, product separation and enzyme recycling may become troublesome. Moreover, the application of salt-hydrates and salt solutions is limited by the temperature, since many salts become unstable at elevated temperatures. For these reasons, either transesterification reactions have been studied, where water formation is not occurring (having no effect on water activity in the reaction mixture) [4,16], or in case of esterification reactions water concentration is kept at a constant level instead of water activity [17].

Earlier the changes in *Candida rugosa* lipase activity in esterification reaction of racemic 2-chloro-propanoic acid and 1-butanol have been studied measuring the reaction rate and conversion in different ionic liquids and common organic solvents [18]. In this work—as a continuation of our research program—enantioselectivity and enzyme recycling in ionic liquids and organic solvents are compared during the esterification of different 2-substituted-propanoic acids (Fig. 1).

2. Materials and methods

2.1. Materials and enzyme

C. rugosa lipase (E.C. 3.1.1.3.) (nominal activity: 943 U/mg_{enzyme}) was from Sigma (St. Louis, MI, USA). Racemic 2-chloro-, and 2-bromo-propanoic acids were obtained from Merck (Darmstadt, Germany), other 2-substituted-propanoic acids (2-metoxy-, 2-ethoxy-, 2-propoxy-, 2-isopropoxy-, and 2-phenoxy-propanoic acids were synthesised from 2-chloro-propanoic acid, through the patent developed by us [19]. Their purity, which was analysed by gas chromatography, exceeded 98% in all cases. Ionic liquids [bmim]PF_6 (1-butyl-3-methylimidazolium hexafluorophosphate), [onim]PF_6 (1-octyl-3-nonylimidazolium hexafluorophosphate) and [bmim]BF_4 (1-butyl-3-methylimidazolium tetrafluoroborate) were from Solvent Innovation GmbH (Cologne, Germany). 1-Butanol and all the other solvents were products of Reanal Ltd. (Budapest, Hungary).

2.2. Esterification without water control

In a typical experimental procedure, 2-chloro-propanoic acid (0.2 M) and 1-butanol (1.2 M) were placed into screw-capped vials and dissolved in the appropriate amount of solvent. Reaction mixtures were shaken in a New Brunswick G 24 horizontal incubator shaker at 30 °C and the reaction was started by adding lyophilised enzyme powder (0.4 g/100 cm^3) to the reaction mixture. Water content was controlled and adjusted using a Mettler DL35 automatic Karl–Fischer titrator.

2.3. Esterification with water control

Water produced during esterification reaction was removed in a standard laboratory pervaporation unit (Fig. 2) [20]. The reaction mixture (liquid) was circulated on the primary side of the pervaporation cell, while the permeate (vapour) was condensed in cold traps cooled by dry-ice. Hydrophilic membrane PERVAP® 2201 from Sulzer Chemtech GmbH, Germany with an effective membrane area of 10 cm^2 was used. The pervaporation experiments were carried out at 30 °C (temperature of the reaction mixture) and a permeate pressure of 10 kPa, the feed volume used was 30 cm^3/min throughout all experiments.

2.4. GC analysis

Esterification reaction was followed by an HP 5890A type GC using a 25 m FS-LIPODEX E chiral capillary

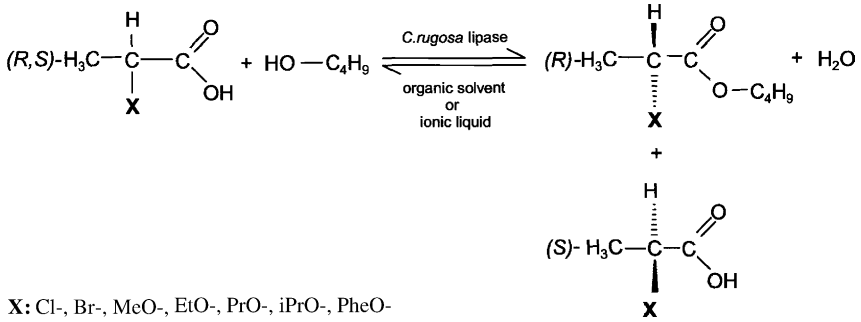

Fig. 1. Lipase-catalysed enantioselective esterification of 2-substituted-propanoic acids with 1-butanol.

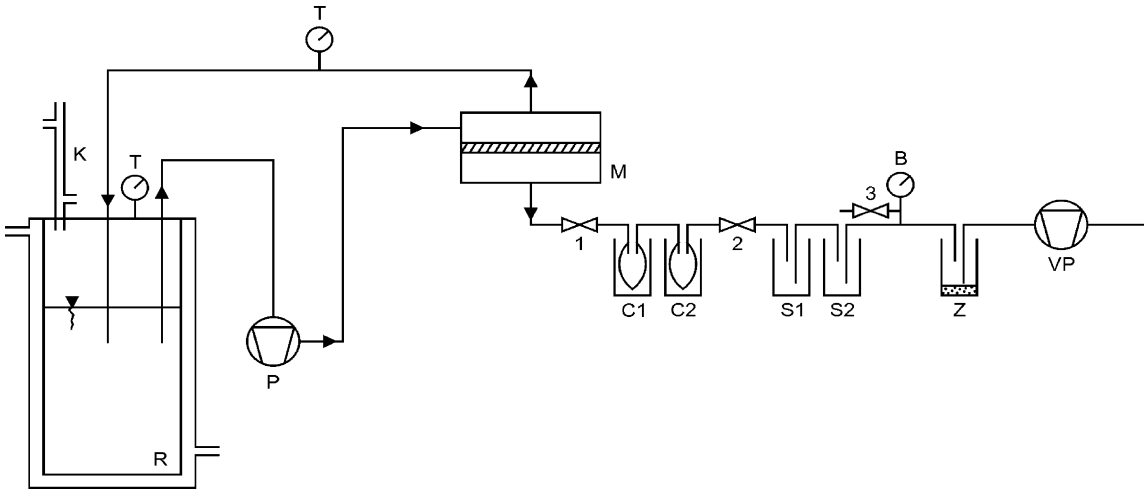

Fig. 2. Schematics of the apparatus for continuous water removal by pervaporation R – reactor, T – thermometer, K – condenser, P – pump, M – membrane module, C1, C2 – cold traps, S1, S2 – silicone, Z – zeolite, VP – vacuum pump, 1, 2, 3 – valves, B – pressure gauge.

column from Macherey-Nagel (Aachen, Germany). The samples from organic solvents were directly injected to the capillary column. In case of ionic liquid containing reaction mixtures, products and unreacted substrates were extracted by 3 × 2 ml of *n*-hexane, and then injected to the GC column. The activity of the lipase was characterised by the amounts of (*R*)- and (*S*)-ester products. The enantioselectivity of *C. rugosa* lipase characterised by the enantiomeric ratio was calculated by the method of Chen et al. [21] for reversible reactions. All experiments were conducted twice and the maximum deviations were <6%.

2.5. Enzyme recycling

After reaction in *n*-hexane, toluene and tetrahydofuran, enzyme preparation was filtered off from the reaction mixture, washed, dried and then used in the same esterification reaction. However, in case of reactions in ionic liquids where enzyme preparation remained in the ionic liquid phase, it was enough to separate the hexane phase containing the products and unreacted substrates from the ionic liquid phase. Then fresh substrates were added to the ionic liquid containing the enzyme preparation, and the water content was measured and adjusted. After reaction, these procedures were repeated in four more cycles.

3. Results and discussion

3.1. Role of solvents

As reaction media for the esterification of (*R*,*S*)-2-chloropropanoic acid and 1-butanol, [bmim]PF_6, [onim]PF_6 and [bmim]BF_4 ionic liquids and *n*-hexane, toluene and tetrahydrofuran were selected due to the wide log *P* range of these solvents (Table 1). In order to study the clear effect of these solvents on enzyme activity, reaction rate data obtained at the same water activity (same hydration of the enzyme) must be compared. Therefore, first water amount necessary in the reaction media for optimal hydration of *C. rugosa* lipase was determined in each solvent by adjusting different water concentrations (0.08–0.77 mol/dm^3 water concentrations according to solvent log *P*) in the reaction mixtures prior to starting the reactions. Instead of using continuous water activity control during esterifications, only short-term experiments were carried out. Reactions were followed up to only 10% conversion or for 5 h reaction time and the limiting acid substrate was applied in high dilution (low concentration). Under these conditions, up to 10% conversion of the acid substrate, the increase in the initial water concentration and the amount of 2-chloro-propanoate butyl ester product as well as the decrease in the 2-chloro-propanoic acid and 1-butanol

Table 1
Yield and enantioselectivity in the esterification of (*R*,*S*)-2-substituted-propanoic acids at initial water contents c_w (yield), and $c_w(E)$

Solvent	log *P*	c_w(yield) (mol/dm^3)	Yield (%)	*E*	$c_w(E)$ (mol/dm^3)	Yield (%)	*E*
[bmim]BF_4	-2.44 ± 0.23[a]	0.54	4.6	5	0.46	4.0	6
[bmim]PF_6	-2.38 ± 0.25[a]	0.38	29.0	18	0.31	26.2	20
[onim]PF_6	-2.19 ± 0.24[a]	0.31	13.4	24	0.23	8.9	25
Tetrahydrofuran	0.5[b]	0.38	4.3	4	0.31	3.2	5
Toluene	2.5[b]	0.23	13.9	3	0.15	10.1	5
n-Hexane	3.5[b]	0.15	36.1	8	0.08	31.7	10

Reaction time: 2 h.
[a] log *P* values were measured by the "shake flask" method [23].
[b] Literature data.

concentration was only 0.02 mol/dm^3. These small changes in the composition of the substrates and products had no strong influence on the polarity characteristics of the whole reaction mixture. As a consequence, at the initial stage of the esterification, water activity can be considered constant.

Based on data obtained up to 10% conversion, reaction rates and enantioselectivities were calculated. Both the enzyme activity and the enantioselectivity as a function of water concentration varied along bell shape curves. The maxima of reaction rate versus water concentration curves determine the water concentrations where the esterification of 2-chloropropanoic acid and 1-butanol proceeds with the highest reaction rate. Similarly, the maxima of E values versus water concentration curves determine the water concentrations where the highest E value can be obtained at these particular reaction conditions. The former water concentration providing optimal hydration for the enzyme from activity aspect will be referred as c_w(yield) while the latter one providing optimal hydration for the enzyme from enantioselectivity aspect as $c_w(E)$ throughout this paper. In each solvent, $c_w(E)$ was tended to be lower than c_w(yield) (Table 1). It can be explained by the fact that lower hydration level enhance the rigidity of enzymes and so improve the enantioselectivity.

The slope of reaction rate versus water concentration curves (Fig. 3) shows how small changes in the water concentration effect the reaction rate. In order to confirm that the effect of 0.02 mol/dm^3 water concentration increase as a result of water produced up to 10% conversion is negligible, the variation in the reaction rate as the effect of 0.02 mol/dm^3 water produced up to 10% conversion was calculated for each solvent.

Among the reaction rate versus water concentration curves (Fig. 3) the slope is highest in case of *n*-hexane, which means that a unit increase in water concentration causes the largest change in reaction rate in case of *n*-hexane solvent. When the initial water concentration is 0.155 mol/dm^3 (c_w(yield) in hexane), 10% conversion of the acid will increase the water concentration of the reaction mixture with 12.9%, i.e. the actual water concentration will be 0.174 mol/dm^3. The actual reaction rate can be determined by interpolation as 8.6 mol/h·g, which is only 4.4% lower than the reaction rate at 0.155 mol/dm^3 water concentration. Thus, it can be stated that the effect of water concentration change caused by the water produced up to 10% conversion on the enzyme activity is negligible.

In Table 1, two sets of yield and E values are summarized: yield and enantioselectivity data in the left were observed at c_w(yield) water concentrations (assuring optimal hydration from activity aspect), however data in the right side were obtained at $c_w(E)$ water concentrations (assuring optimal hydration from selectivity aspect). It can be seen, that the yield decreased when lower hydration ($c_w(E)$) was provided for the enzyme. Similarly, when higher water concentration (c_w(yield)), was provided for the enzyme where the highest enzyme activity could be obtained, the E decreased. It shows again, that *C. rugosa* lipase requires different conditions depending on that its activity or selectivity are preferred in the particular reaction.

Enzyme activity observed in ionic liquids and common organic solvents was in the same order of magnitude. The highest yield could be reached in *n*-hexane (36.1%) followed by [bmim]PF_6 ionic liquid (29.0%) at water concentrations of 0.15 and 0.38 mol/dm^3, respectively (Table 1). Contrary to the activity, remarkable higher enantioselectivities were obtained in [bmim]PF_6 and [onim]PF_6 ionic liquids compared to that obtained in the organic solvents. In [bmim]PF_6 (E = 20) and [onim]PF_6 (E = 25) ionic liquids at $c_w(E)$ water concentrations, E was higher than in *n*-hexane (E = 10) by 2 and 2.5 times, respectively.

In order to evaluate the role of solvents, Park and Kazlauskas have studied the relation between Reichardt's polarity of some ionic liquids and common organic solvents and the activity of *Pseudomonas cepacia* lipase in a recent paper [22]. They showed that in common organic solvents with Reichardt's polarity lower than 0.5 the activity of *Pseudomonas cepacia* lipase is in inverse proportion to solvent polarity (enzyme activity decreased with the increase of solvent polarity) while in the more polar ionic liquids (Reichardt's polarity between 0.6 and 0.8), enzyme activity is in direct proportion to solvent polarity. Similarly, we studied the

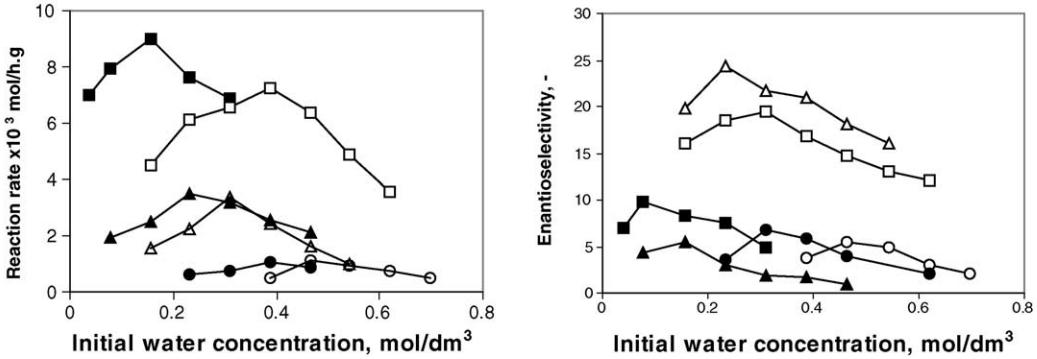

Fig. 3. Effect of water concentration on the reaction rate and enantioselectivity in the esterification of (*R,S*)-2-chloro-propanoic acid (10% conversion, 30 °C): (▲) toluene; (■) *n*-hexane; (●) tetrahydrofuran; (□) [bmim]PF_6; (△) [onim]PF_6; (◯) [bmim]BF_4.

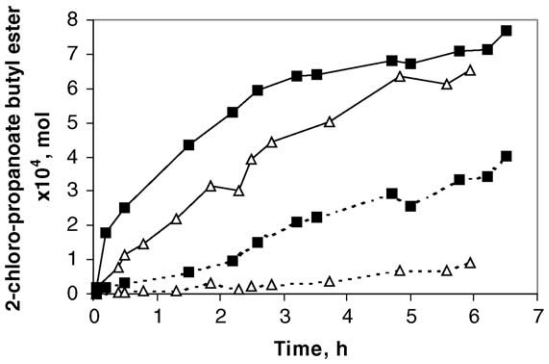

Fig. 4. Formation of (*R*)-, and (*S*)-2-chloro-propanoate butyl ester over time (T = 30 °C, $c_w = c_w(E)$ = constant): (■) *n*-hexane; (△) [onim]PF_6. Continuous line curves show the formation of (*R*)-, while the broken line curves show the formation of (*S*)-ester isomers.

possible connections between the log *P* values of the ionic liquids and the activity, enantioselectivity of *C. rugosa* lipase. So far, the correlation between the octanol/water partition coefficient of ionic liquids and the performance of different enzymes has not been widely published yet. Moreover, data only on the log *P* of [bmim]PF_6 (-2.39 ± 0.27) could be found in the literature [23]. We measured the $\log P_{\text{octanol/water}}$ of [bmim]PF_6, [onim]PF_6 and [bmim]BF_4 ionic liquids by the so-called "shake flask" method which utilizes the fact that imidazolium ring present in these ionic liquids absorbs strongly at 211 nm [23]. For all ionic liquids, the average of three parallel measurements were calculated (Table 1). Despite the highly hydrophilic character of ionic liquids (log *P* is below zero), $c_w(E)$ and c_w(yield) in organic solvents and ionic liquids are in the same range. This fact together with the variation of yield and *E* with solvent log *P* indicates that solvent hydrophobicity cannot be correlated with activity or enantioselectivity of *C. rugosa* lipase.

Since *C. rugosa* lipase showed only very low activity in tetrahydrofuran and [bmim]BF_4 ionic liquid (Fig. 3) these solvents were not involved in further studies. In [bmim]PF_6, [onim]PF_6, toluene and *n*-hexane solvents long-term experiments were conducted. In order to decrease the hydrolysis side reaction occurring as a result of water producing during esterification, continuous water removal was applied. In each solvent, water concentration optimal from enantioselectivity aspect were adjusted and controlled by pervaporation. The formation of (*R*)-, and (*S*)-2-chloro-propanoate butyl esters were followed (Fig. 4) and the reaction rate as well as the *E* were calculated. As a consequence of shifting the esterification equilibrium, reaction yield increased (Table 2). As it was expected, equilibrium concentrations of (*R*)-, and (*S*)-propanoate esters were enhanced, therefore, *E* was not influenced by continuous removal of water as by-product.

3.2. Role of substituents

It is well known, that the size and the e-acceptor/donor character of the substituent(s) in the chiral substrates have influence on the enzyme performance [24]. In order to study the reactions of chiral propanoic acids containing different ligands in α-position, six other 2-substituted-propanoic acids were selected and involved in the studies. Enzyme activity and enantioselectivity in the esterification of (*R,S*)-2-Br-, MeO-, EtO-, PrO-, *i*PrO- and PheO-propanoic acids in [bmim]PF_6 ionic liquid and *n*-hexane were compared. In *n*-hexane 0.08 mol/dm^3, while in [bmim]PF_6 ionic liquid 0.31 mol/dm^3 water concentrations were adjusted and controlled according to data in Table 1.

Esterification of all the six racemic 2-substituted-propanoic acids proceeded with measurable reaction rate. Electron acceptor halo substituents containing propanoic acids showed higher reactivity than the alkoxy- and phenoxy-propanoic acids (Table 3). Furthermore, in case of the bigger size bromine substituent containing (*R,S*)-2-bromo-propanoic acid, enzyme activity was lower but the enantioselectivity was higher compared to 2-chloro-propanoic acid. It can be, therefore, concluded that electron acceptor and bigger size substituent in α-position of the chiral propanoic acid is beneficial in terms of enantioselectivity.

Based on literature data, and data observed by our group, esterifications of chiral 2-substituted-propanoic acids should be discussed by dividing these acids into two groups: *profens* containing aryl substituents and *non-profens* containing other substituents in the chiral acid molecule. The esterification reactions of the two groups differ significantly taken into account reaction rate and enantioselectivity. Esterification of chiral propanoic acids containing halo, halophenoxy and alkoxy substituents takes place approximately three order

Table 2
Effect of water removal on the yield in the esterification of (*R,S*)-2-chloro-propanoic acid catalysed by *C. rugosa* lipase

Solvent	Yield (%)	
	Without water removal	With water removal (constant water content)
[bmim]PF_6	37.6	48.2
[onim]PF_6	23.8	31.8
Toluene	21.9	30.5
n-Hexane	40.3	50.3

5 h, T = 30 °C, $c_w(E)$ initial water concentration in each solvent as it is listed in Table 1.

Table 3
Yield and enantioselectivity in the esterification of different (*R,S*)-2-substituted-propanoic acids

X substituent	Conversion (%)		*E*	
	n-Hexane	[bmim]PF_6	*n*-Hexane	[bmim]PF_6
Chlorine	31.7	26.2	10	20
Bromine	24.5	20.5	18	29
Methoxy	29.2	21.3	16	25
Ethoxy	21.6	17.7	13	21
Propoxy	17.5	15.0	7	19
Isopropoxy	10.0	10.8	7	14
Phenoxy	5.2	6.2	4	10

2 h, $c_w(E)$ water concentration in each solvent as it is listed in Table 1.

of magnitude higher rate under the same reaction conditions, and applying same *C. rugosa* lipase concentration, but the selectivity of the esterification reactions is much lower. The enantiomeric ratio was 12 in the resolution of racemic 2-(*p*-chloro-phenoxy)propanoic acid with 1-butanol (with same substrate and *C. rugosa* lipase concentration) in *iso*-octane [21]. In the esterification reaction of 2-chloro-propanoic acid and 1-butanol, *E* varied between 3 and 25 depending on the solvent. In case of profens much higher enantioselectivity (*E*) can be achieved in the similar reactions because >98% ee_p value can be obtained at 50% conversion [25].

3.3. Enzyme recycling

When enzyme preparation is to be used repeatedly in reactions in common organic solvents, first, enzyme preparation must be filtered off, then washed and dried before applied for the catalysis of another reaction. In ionic liquids, however, following product extraction by *n*-hexane, enzyme preparation remains in the ionic liquid phase, therefore, there is no need for further purification of the enzyme. It can be used again for the catalysis of the same reaction, only the water content must be adjusted and controlled. Because of this feature of ionic liquids, solvent and enzyme losses, process time as well as money can be saved by eliminating the treatment steps before recycling.

In order to evaluate the possibility of enzyme recycling in ionic liquids, enzyme preparation was used in five consecutive esterification reactions. The activity of recycled lipase (relative activity) is expressed as the percentage of its initial activity (100%) obtained in the first reaction. In order to avoid the effect of water by-product, pervaporation was used again. Surprisingly, *C. rugosa* lipase retained 92 and 95% of its original activity after recycling five times in [bmim]PF_6 and [onim]PF_6 ionic liquids (Fig. 5). While in *n*-hexane and toluene, initial activity decreased to 55 and 50%, respectively. Moreover, relative enantioselectivity of recycled *C. rugosa* lipase in [bmim]PF_6 and [onim]PF_6 ionic liquids decreased only by 10 and 15% compared to *n*-hexane, where more than 50% lower *E* value was obtained when the enzyme was used in the fifth recycling.

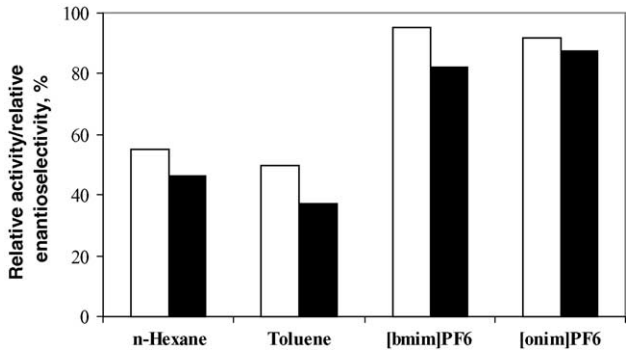

Fig. 5. Relative activity and enantioselectivity of *C. rugosa* lipase in the fifth recycling (t = 5 h, c_w(yield) and c_w(*E*) water concentrations, T = 30 °C): (■) relative activity; (□) relative enantioselectivity.

4. Conclusions

In our previous work, *C. rugosa* lipase showed comparable or higher activity in the esterification of (*R*,*S*)-2-chloro-propanoic acids in ionic liquids compared to that in *n*-hexane [18]. Here, the range of 2-substituted-propanoic acids esterified in ionic liquids has been extended. This study demonstrated that application of [bmim]PF_6 and [onim]PF_6 ionic liquids is beneficial from not only enzyme activity, but enantioselectivity aspect as well. In the esterification of (*R*,*S*)-2-chloro-propanoic acid, 98 and 150% higher enantioselectivity could be achieved in [bmim]PF_6 and [onim]PF_6 ionic liquids than in *n*-hexane.

One of the great advantage of ionic liquids is that enzyme recycling is much more easy compared to common organic solvents. The purification steps before reuse of the enzyme preparation can be eliminated, therefore, enzyme, solvent and time can be saved. *C. rugosa* lipase maintained its activity after recycling five times in ionic liquids. Moreover, during the fifth reuse of the lipase in [bmim]PF_6 and [onim]PF_6 ionic liquids, enantioselectivity decreased only by 10 and 15%, respectively, while the enzyme performed only 50% of its initial enantioselectivity in *n*-hexane.

Although the *E* values throughout this study were not high enough for an industrial application the significant increase in the *E* values in PF_6 anion containing ionic liquids is promising especially when consider that in these experiments free lipase preparation was applied without any purification or treatment. Since additives (for water removal or to improve *E*) were not used, ionic liquids as well as the enzyme preparation itself can be easily recycled. As a consequence, solvent and enzyme losses could be markedly decreased.

In order to find explanation for the improved enzyme performance in ionic liquids, further experiments are necessary. It is worth investigating other ionic liquids and substrates as well in order to find relation between the structure of ionic liquids, substrates and enzyme catalysis.

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