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SCIENTIFIC PAPER

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IONIC LIQUIDS AS (CO)SOLVENTS FOR ENZYMATIC REACTIONS

Ionic liquids are low melting point salts that represent an exciting new class of reaction solvents. Many reactions show advantages when carried out in ionic liquids, either with regard to enhanced reaction rates, improved selectivity, or easier reuse of catalysts. To ascertain the influence of ionic liquids on the enzyme activity, three different ionic liquids, 1–butyl–3–methylimidazolium chloride ([bmim] [Cl]), 1–butyl–3–methylimidazolium hexafluorophosphate ([bmim] [PF_6]) and 1–butyl–3–methylimidazolium tetrafluoroborate ([bmim][BF_4]) were synthesized and investigated as potential media for the hydrolysis of carboxymethyl cellulose, catalyzed by non–immobilized cellulase from Humicola insolens (Celluzyme 0,7T) and for ester synthesis, catalyzed by immobilized lipase from Rhizomucor miehei (Lipozyme RM IM). Enzyme–catalyzed reactions were performed in a batch stirred reactor at atmospheric pressure. Celluzyme 0,7T showed better activity in hydrophobic ionic liquid ([bmim] [PF_6]), as compared to hydrophilic ionic liquid ([bmim] [BF_4]). In the case of Lipozyme RM IM, the synthetic activity of the enzyme was strongly reduced by incubating the enzyme in ionic liquids.

Key words: Ionic liquids, [bmim] [Cl], [bmim] [PF_6], [bmim] [BF_4], Hydrolysis, Esterification, Cellulase, Lipase.

Ionic liquids (ILs) are organic salts composed of cations and anions, which are usually in the liquid state at room temperature [1–4]. They are "designer solvents", as their physical properties such as melting point, viscosity, density and hydrophobicity can be modified according to the nature of the desired reactions by altering the nature of their cations and anions [5,6]. ILs mainly comprise organic cations such as 1–alkyl–3–methylimidazolium, tetraalkylammonium, tetra-alkyl– phosphonium,... The common anions which lead to neutral and stoichiometric ILs are: bis[(trifluoromethyl) sulfonyl]a mide, hexafluorophosphate, tetrafluoroborate,... The common anions which lead to neutral and stoichiometric ILs are: bis[(trifluoromethyl)sulfonyl]a mide, hexafluorophosphate, tetrafluoroborate,... Common cations and anions involved in ILs are shown in Figure 1 [5,7–9].

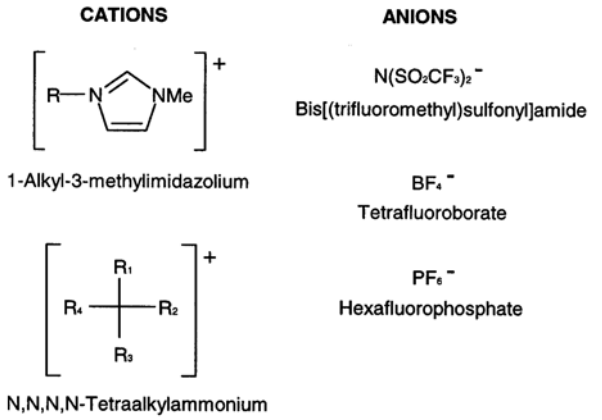

Figure 1. Common ions involved in ionic liquids

ILs are an attractive alternative to conventional organic solvents because of their unique properties. Contrary to organic solvents, they have essentially no vapour pressure, which reduces emission to the environment and working exposure hazards. In addition, they also have suitable densities and viscosities. Therefore, they may replace toxic, flammable, and polluting volatile organic solvents, such as toluene, hexane and dichloromethane. ILs posses good thermal stability and do not decompose over a large temperature range, thereby making it feasible to carry out reactions requiring high temperature conveniently in ILs. ILs are able to dissolve a wide range of organic, inorganic and organometallic compounds and they serve as a good medium to solubilise gases such as hydrogen, carbon monoxide, carbon dioxide and oxygen. Many reactions are now being performed using ILs and supercritical carbon dioxide [1,7].

Although water is the traditional solvent for biocatalysis, some reactions work only in organic solvents or work better in organic solvents than in water. ILs can replace these organic solvents. ILs are used in enzyme systems as co–solvents in the aqueous phase, as pure solvents and as two–phase systems together with other solvents. Different enzymes such as hydrolases (proteases and lipases) and oxidoreductases (peroxidases and dehydrogenases) retain their activity when suspended in ILs. Not all ILs are suitable for biocatalysis. Enzymes are usually active in ILs containing tetrafluoroborate, hexafluorophosphate and bis[(trifluoromethyl)sulfonyl]amide anions, but not in ILs containing chloride, nitrate, trifluoroacetate or acetate anions [5,10–12]. Some potential advantages of

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enzymatic reactions in ionic liquids, such as high activity, the thermal and operational stability of biocatalysts, and good enantioselectivity of biotransformation in comparison with conventional media have been reported [13].

In the present research, three different ILs, [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], were synthesized. In order to ascertain their influence on enzymes, the stability of non–immobilized cellulase from *Humicola insolens* and immobilized lipase from *Rhizomucor miehei* was investigated by using ILs as potential media for enzyme–catalyzed reactions and as media for incubating the enzymes. The reactions were performed in a batch stirred reactor at atmospheric pressure.

MATERIALS AND METHODS

Enzymes and chemicals

Two different enzymes; non–immobilized cellulase from *Humicola insolens* (Celluzyme 0,7T) and immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) were kindly donated by Novo Nordisk AS (Copenhagen, Denmark). Carboxymethyl cellulose (CMC), 3,5–dinitrosalicinic acid, Na–K tartrate tetrahydrate and glucose were supplied from Sigma (Germany). Sodium hydroxide solution (0,1 mol/L), oleic acid, chlorobutane, 1–methylimidazole and anhydrous magnesium sulfate were purchased from Merck (Darmstadt, Germany). Ethanol, 1–octanol and sodium tetrafluoroborate were supplied by Aldrich Chemical Co. (Diesenhofen, Germany). Phenolphthalein and sodium hydrogen orthophosphate were from Kemika (Zagreb, Croatia). Citric acid was obtained from Mariborske lekarne (Maribor, Slovenia).

Synthesis of ionic liquids

Three different ILs, [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], were synthesized according to the procedure of Lewandowski et al [14].

1–Butyl–3–methylimidazolium chloride ([bmim] [Cl])

A chlorobutane was mixed with 1–methylimidazole and stirred at 60 °C for 70 hours. The resulting yellow, viscous liquid mixture was cooled to room temperature, washed three times with portions of ethyl acetate and the [bmim] [Cl] crystals were dried under vacuum at 60 °C for 24 hours. [bmim] [PF_6] and [bmim] [BF_4] were synthesized using this IL.

1–Butyl–3–methylimidazolium hexafluorophosphate ([bmim] [PF_6])

An aqueous solution of hexafluorophosphoric acid HPF_6 was added drop–wise into a solution of [bmim] [Cl] in water and stirred at room temperature for 36 hours. The two–phase system was separated, and the lower phase (IL [bmim] [PF_6]) was washed with water portions to neutral pH. The light yellow liquid salt was dried under vacuum at 80 °C for 24 hours.

1–Butyl–3–methylimidazolium tetrafluoroborate ([bmim] [BF_4])

A solution of sodium tetrafluoroborate $NaBF_4$ in water was added drop–wise to a solution of [bmim][Cl] D in water and stirred for 12 hours; the reaction vessel was cooled with ice. The two–phase system was separated and the lower phase (IL [bmim] [BF_4]) was washed twice with portions of dichloromethane and twice with portions of water. The IL [bmim] [BF_4] was first dried with an addition of anhydrous magnesium sulfate $MgSO_4$ and after filtration under vacuum at 60 °C for 4 hours.

Enzyme incubation in ionic liquids

The enzymes Celluzym 0,7T and Lipozyme RM IM were incubated in three different ILs, [bmim] [Cl], [bmim] [PF_6] and [bmim] [BF_4], at room temperature for 24 hours. After incubation the enzymes were used as biocatalysts for the hydrolysis of CMC (cellulase) and the esterification of 1–octanol and oleic acid (lipase), respectively. The enzyme activities were determined.

General procedure for enzyme–catalyzed reactions

ILs, [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], were used as potential media for two different enzyme–catalyzed reactions (the hydrolysis of CMC and the esterification of 1–octanol and oleic acid).

In the hydrolysis experiments, 10 mL of phosphate–citrate buffer of pH 4.6 containing ionic liquid (50 %) and enzyme cellulase from *Humicola insolens* (0.4 g) were added to the substrate carboxymethyl cellulose (0.15 g). In the synthesis of n–octyl oleate, ionic liquid (5 mL) and enzyme Lipozyme RM IM from *Rhizomucor miehei* (0.5 g) were added to the substrate (equimolar (63 mmol) mixture of 1–octanol and oleic acid (10 mL)).

The reactions were performed in a 100 mL round bottom flask, immersed in a water bath, heated to the desired operating temperature and stirred by a magnetic stirrer. Samples were taken from the reaction mixture at defined time periods and analyzed.

Enzymes preincubated in ionic liquids were also used as biocatalysts for the hydrolysis of CMC and the esterification of 1–octanol and lauric acid.

Analysis

The hydrolysis of carboxymethyl cellulose performance was monitored by UV determinations on the basis of reduced glucose production. 3,5–dinitrosalicinic acid (1.5 mL) was added to the sample (0,5 mL). All the samples were placed in a hot water bath for five minutes and after cooling to room

temperature in a cold water bath, the absorbance was recorded by using a UV spectrophotometer at 540 nm [15].

For the esterification of 1–octanol and oleic acid the ester content was quantified by measuring the amount of residual fatty acid in the reaction mixture, which was determined by the volumetric method. A sample of the reaction mixture (0.1 g) was diluted in 20 mL of 0.1wt% phenolphthalein solution in absolute ethanol and then titrated with standardized aqueous 0.1 mol/l sodium hydroxide solution [16].

RESULTS AND DISCUSSIONS

Preliminary research on the enzyme–catalyzed hydrolysis of carboxymethyl cellulose and enzyme–catalyzed esterification of 1–octanol and oleic acid performed in ILs, was carried out to determine the influence of different ILs on enzyme activity and stability. The hydrolysis of carboxymethyl cellulose and esterification of 1–octanol and oleic acid were also performed in a non–IL system, [17,18].

Hydrolysis of carboxymethyl cellulose

The hydrolysis of carboxymethyl cellulose was catalyzed with preincubated enzyme in ILs. Cellulase from *Humicola insolens* (1 g) was incubated in 1 mL of three different ILs, [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], at room temperature for 24 hours. After incubation, the substrate carboxymethyl cellulose (0.15 g) and phosphate–citrate buffer of pH 4.6 (10 mL) were added to the enzyme with IL and the reaction was started. The hydrolysis of carboxymethyl cellulose, catalyzed by non–incubated cellulase, was performed in aqueous medium for comparison. The results are presented in Figure 2.

Cellulase from *Humicola insolens* retained its activity when incubated in [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl]. The activity of the incubated enzyme in comparison to the non–incubated enzyme was comparable. Therefore, it seemed that all the assayed ILs had stabilized the non–immobilized cellulase. Despite the fact that [bmim] [Cl] is not suitable for biocatalysis [10,12], good results with enzymes incubated in [bmim] [Cl] were also obtained. An explanation for this could be found in the high solvent power. Cellulose dissolves in [bmim] [Cl] up to 25 % weight, but not in [bmim] [PF_6] or [bmim] [BF_4]. The key to the solubility is hydrogen bonding between the cellulose and chloride to compensate for breaking the strong interstrand hydrogen bonds in insoluble cellulose [10]. In this case [bmim] [Cl] maintains the enzyme activity and dissolves carboxymethyl cellulose.

Furthermore, the ILs [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], were also used as co–solvents. In order to investigate the effect of ILs on the cellulase activity, hydrolysis with an IL:buffer ratio of 1:1 was carried out. The volume fraction of the IL phase was adjusted to 50 % of the 10 mL reacting volume. The reaction was performed with an enzyme concentration of 40 g/L, a CMC concentration of 15 g/L, at 40 °C and a stirring rate of 500 rpm. From the results illustrated in Figure 3, the assayed ILs proved as adequate media for the non–immobilized cellulase–catalyzed hydrolysis of carboxymethyl cellulose.

Non–immobilized cellulase from *Humicola insolens* showed good activity for hydrolysis under biphasic conditions, i.e. with the hydrophobic IL, [bmim] [PF_6], as compared to the hydrophilic IL, [bmim] [BF_4], which provided a monophasic environment. After six hours of reaction a glucose concentrations of 2.015 g/L and 1.47 g/L were obtained when the ILs [bmim] [PF_6] and [bmim] [BF_4] were used as the reaction media, respectively. Similar results were published for the hydrolysis of butyl 2–(4–chlorophenoxy)propionate, catalyzed by lipase from *Candida rugosa* [19]. When the hydrolysis of carboxymethyl cellulose was performed in aqueous medium, a glucose concentration of 1.95 g/L

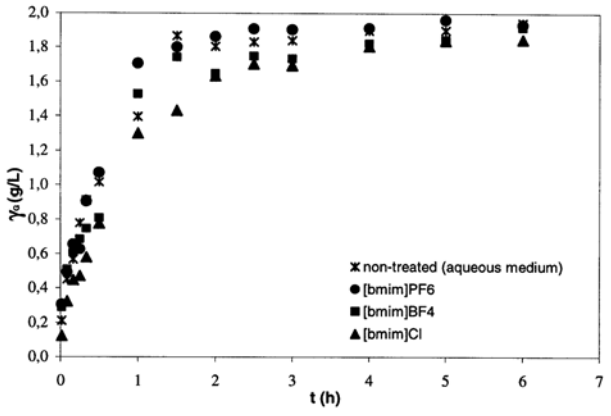


Figure 2. Hydrolysis of carboxymethyl cellulose, catalyzed with preincubated cellulase in ILs. Reaction conditions: CMC concentration 15 g/L, temperature 40°C and stirring rate 500 rpm

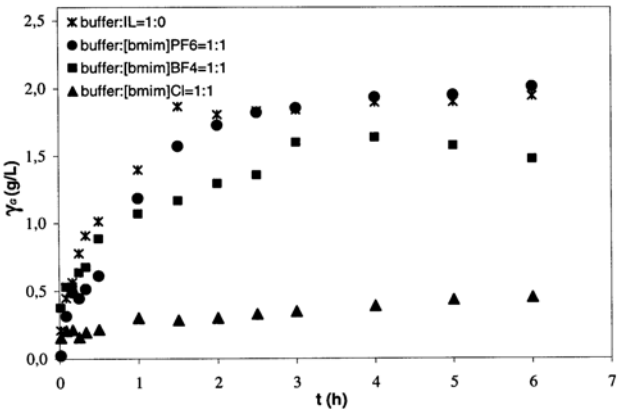


Figure 3. Effect of ILs on the activity of non–immobilized cellulase from Humicola insolens. Reaction conditions: enzyme concentration 40 g/L, CMC concentration 15 g/L, 5 mL of ILs, temperature 40°C and stirring rate 500 rpm

was obtained. That value is 3.3% lower compared to the glucose concentration obtained in the IL [bmim] [PF_6]. Deactivation of the enzyme occured in the IL [bmim] [Cl].

Enzymes are described as usually active in IL containing [PF_6] and [BF_4] anions, but not in IL containing Cl anions. A possible reason for this difference is the hydrogen–bond basicity of enzyme–compatible anions. The [PF_6] anion spreads its negative charge over six fluorine atoms and the [BF_4] anion over four fluorine atoms. The lower hydrogen–bond basicity minimizes the interference with the internal hydrogen bonds of an enzyme. Consistent with this notion, enzymes are inactive in [bmim] [Cl], which has high hydrogen–bond basicity [10]. Interestingly, while these ILs have the same cation, they display quite different properties. They are characterized by different polarity and viscosity. These properties may be of primary importance in enzyme–catalyzed reactions, since they are capable of affecting the conformation of the enzymes and, consequently, their reactivity [12]. In our case, the activity of non–immobilized cellulase decreased with increasing polarity of the ILs. We obtained the best results with less polar, hydrophobic [bmim] [PF_6].

Because ILs are more expensive than organic solvents (~800 times more in the Fluka catalogue) [10], experiments were performed to determine the minimum IL concentration which maximizes the enzyme activity during the reaction. The hydrolysis of carboxymethyl cellulose was performed in phosphate–citrate buffer containing different volume fractions of [bmim] [PF_6] (10% – 50% of the total reaction volume). The results illustrated in Figure 4, exhibit a comparable biocatalyst activity in all the assayed [bmim] [PF_6] concentrations.

By using 10% and 50% of [bmim] [PF_6] 1.99 g/L and 2.01 g/L of glucose were obtained, respectively. This is important from the economic point of view, because smaller amounts of IL may be used to obtain similar results than with higher amounts of IL.

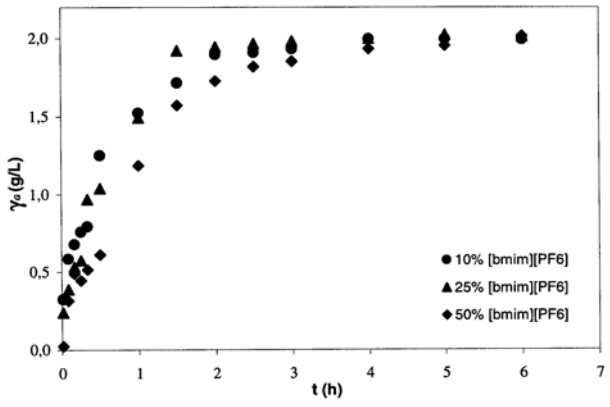
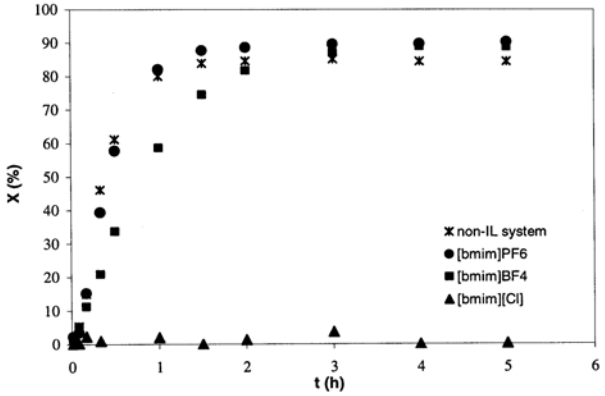


Figure 4. Hydrolysis of carboxymethyl cellulose in phosphate–citrate buffer containing 10 % – 50 % of ILs. Reaction conditions: enzyme concentration 40 g/L, CMC concentration 15 g/L, temperature 40 °C and stirring rate 500 rpm

Esterification of 1–octanol and oleic acid

The synthesis of a long chain fatty acid ester is the reverse reaction of hydrolysis [16]. The effect of the ILs, [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], as a reaction medium on the immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) was investigated by performing the synthesis of n–octyl oleate. Ester synthesis was carried out with an equimolar (63 mmol) mixture of 1–octanol and oleic acid (10 mL), 0.5 g of enzyme, 5 mL of IL, at 50 °C and a stirring rate of 500 rpm. The conversion profiles are presented in Figure 5.



The reactions performed with and without ILs were compared. As can bee seen, the ILs [bmim] [PF_6] and [bmim] [BF_4] were suitable media for lipase–catalyzed

Figure 5. Effect of ILs on the activity of immobilized lipase from Rhizomucor miehei. Reaction conditions: equimolar (63 mmol) mixture of 1–octanol and oleic acid, 5 mL of IL, 0,5 g enzyme, temperature 50 °C and stirring rate 500 rpm

n–octyl oleate synthesis. When [bmim] [PF_6] or [bmim] [BF_4] were added to the reaction mixture, the conversion was 90% and 88%, respectively. A lower yield (84%) of n–octyl oleate was obtained in a non–IL system. The low activity shown by immobilized lipase from *Rhizomucor miehei* in [bmim] [Cl] could be related with the hydrogen–bond basicity of the enzyme–compatible anions. The lower hydrogen–bond basicity minimizes interference with the internal hydrogen bonds of the enzyme. Consistent with this notion, enzymes are inactive in [bmim] [Cl], which has high hydrogen–bond basicity [10].

The synthesis of n–octyl oleate, catalyzed with enzyme, preincubated in ILs, was also performed. Lipase from *Rhizomucor miehei* (0.5 g) was incubated in 1.3 mL of three different ILs, [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl], at room temperature for 24 hours. After 24 hours of incubation, an equimolar mixture of substrates, 1–octyl oleate and oleic acid (10 mL), was added to the lipase with IL. The synthesis of n–octyl oleate, catalyzed by non–incubated lipase was performed in a non–IL system for comparison. The reaction was performed at

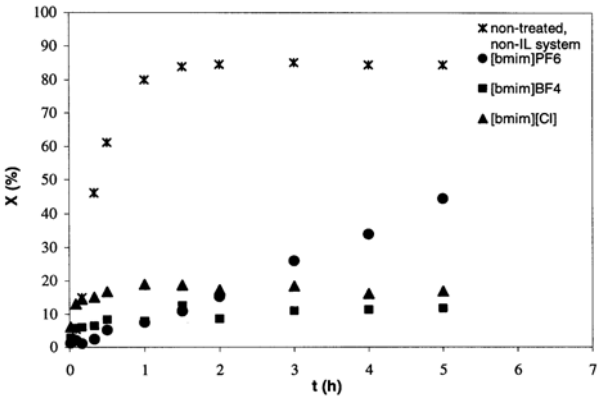


Figure 6. n–Octyl oleate synthesis, catalyzed by preincubated cellulase in ILs. Reaction conditions: equimolar (63 mmol) mixture of 1–octanol and oleic acid, temperature 50 °C and stirring rate 500 rpm

50°C and a stirring rate of 500 rpm. The results are presented in Figure 6.

The assayed ILs strongly reduced the synthetic activity of Lipozyme RM IM. In the two ILs, [bmim] [BF_4] and [bmim] [Cl], the enzyme synthetic activity was comparable, whereas the conversion in [bmim] [BF_4] after five hours of reaction was much lower (only 11 %), than in [bmim] [PF_6] (44 %). An explanation for this behaviour could be that the hydrophilic IL [bmim] [BF_4] is prone to desorb water from the enzyme surface and decrease the activity of the enzyme [13]. Enzymes need a certain amount of water for their activity and if it is stripped away, the activity of the biocatalysts falls [20].

The incubation of immobilized lipase from *Rhizomucor miehei* in [bmim] [PF_6], [bmim] [BF_4] and [bmim] [Cl] resulted in a progressive loss of activity, comparable with that observed when non–treated enzyme was used as the biocatalyst in an IL system. That means that the stability of lipase from *Rhizomucor miehei* is poor in all the assayed ILs. Similar results were published for *Candida antarctica* lipase B incubated in [bmim] [PF_6] at 80 °C [2].

CONCLUSIONS

The *Humicola insolens* cellulase–catalyzed hydrolysis of carboxymethyl cellulose in phosphate–citrate buffer with ILs as co–solvents was demonstrated. In view of the cellulose activity, hydrophobic IL [bmim] [PF_6] was observed to be a more efficient co–solvent than the hydrophilic IL [bmim] [BF_4]. Addition of the IL [bmim] [Cl] caused enzyme deactivation. The hydrolysis of carboxymethyl cellulose, catalyzed by incubated cellulase in ILs was also performed. All the assayed ILs stabilized non–immobilized cellulase.

ILs [bmim] [PF_6] and [bmim] [BF_4] were successfully used as reaction media for the *Rhizomucor miehei* lipase–catalyzed synthesis n–octyl oleate. The enzyme showed comparable conversion yields in reactions without IL and when [bmim] [PF_6] and [bmim] [BF_4] were used as the reaction media. By incubating the enzyme in ILs, the enzyme synthetic activity was strongly reduced.

ILs show great promise as reaction media for enzyme–catalyzed reactions. Therefore, the study of ILs in biocatalysis will be continued.

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IZVOD

JONSKE TEČNOSTI KAO KO–SOLVENTI ZA ENZIMSKE REAKCIJE

(Naučni rad)

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Jonske tečnosti imaju niske temperature mržnjenja i kao takve predstavljaju novu klasu rastvarača koji se mogu iskoristiti u mnogim reakcijama. Očigledne su prednosti kada se hemijska reakcija izvodi uz prisustvo jonskih tečnosti, bilo kod ubrzanja brzine hemijske reakcije, povećane selektivnosti ili jednostavnije ponovne upotrebe katalizatora. U cilju utvrđivanja uticaja jonskih tečnosti na aktivnost enzima, sintetizovane su i ispitane tri različite tečnosti, 1–butil–3–meilimidazolijum hlorid ([bmim][Cl]), 1–butil–3–metilimidazolijum heksafluorofosfat ([bmim][PF6]) i 1–butil–3–metilimidazolijum tetrafluoroborat ([bmim][BF4]) kao potencijalni rastvarači u reakcijama hidrolize karboksimetil celuloze koja je katalizovana neimobilisanom celulazom iz *Humicola insolens* (Celluzyme 0,7T) i sintezama estara katalizovanim imobilisanom lipazom iz *Rhizomucor miehei* (Lipozyme RM IM). Enzimski katalizovane reakcije su izvedene u šaržnom reaktoru pod atmosferskim pritiskom. Pokazalo se da Celluzyme 0,7T ima veću aktivnost u hidrofobnoj jonskoj tečnosti [bmim][PF6]), u odnosu na hidrofilnu ([bmim][BF4]). U slučaju Lipozyme RM IM, aktivnost enzima je značajno smanjena inkubacijom enzima u jonskoj tečnosti.

Ključne reči: Jonske tečnosti, [bmim][Cl], [bmim][PF6], [bmim][BF4], Hidroliza, Esterifikacija, Celulaza, Lipaze.