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Recent Progress on Biocatalysis and Biotransformations in Ionic Liquids*

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Abstract Ionic liquids have negligibly low vapor pressure, high stability and polarity. They are regarded as green solvents. Enzymes, especially lipases, as well as whole-cell of microbe, are catalytically active in ionic liquids or aqueous-ionic liquid biphasic systems. Up to date, there have been many reports on enzyme-exhibited features and enzyme-mediated reactions in ionic liquids. In many cases, remarkable results with respect to yield, catalytic activity, stability and (enantio-, regio-) selectivity were obtained in ionic liquids in comparison with those observed in conventional media. Accordingly, ionic liquids provide new possibilities for the application of new type of solvent in biocatalytic reactions.
Keywords ionic liquids, biocatalysis and biotransformations, enzymatic catalysis

1 INTRODUCTION

Ionic liquids(ILs), a new class of non-aqueous but polar solvents, consist of low melting point (<100℃) salts that are liquids at room temperature. The thermal stability and the negligible vapor pressure diminish the emission of the volatile organic compounds. Thus ILs are environmentally attractive compared with the conventional organic solvents. Recoverability and recyclability also make ILs a practical solution to industrial environmental concerns. As solvents for chemical processes, ILs display excellent physical characteristics including the ability to dissolve highly polar and nonpolar organic, inorganic and polymeric compounds[1]. By appropriate modification of the anion or the substituents in the cation or both, it is possible to drastically alter the chemical and physical properties (hydrophobicity, polarity, viscosity, density and miscibility with other solvents) of ILs. This feature plays a key role in manipulating the solvent properties of ILs, which allows ILs to be designed for specific reaction system. Therefore, they are often regarded as engineered solvents[2].

The replacement of volatile organic solvents by ILs as green reaction medium for biocatalysis and biotransformations has recently gained increasing attention and the ILs used for this purpose are mainly composed of dialkylimidazolium or *N*-alkylpyridium cations and a non-coordinating anion (Table 1). Cull *et al.*[3] first reported the biotransformation of 1,3-dicyanobenzene to 3-cyanobenzamide mediated by whole cells (*Rhodococcus* R312) in a biphasic [BMIm][PF_6]-water system. This work established ILs as a potential alternative to organic solvents for multiphase biotransformations. The first enzyme-catalyzed reaction in an IL, described by Erbeldinger *et al.*[4], involved the thermolysin-catalyzed synthesis of *Z*-aspartame in buffer-saturated [BMIm][PF_6] medium with 5% (by volume) water. This work showed that enzyme activity was comparable to that observed in conventional organic solvents such as ethyl acetate and that the enzyme maintained very high stability in the IL. Since this work, there have been many reports of enzymatic catalysis in ILs, including the alcoholysis, ammonolysis, and perhydrolysis reactions catalyzed by free and immobilized *Candida antarctica* lipase B (CaLB) in [BMIm] [PF_6] or [BMIm] [BF_4] medium[5] and the lipase-catalyzed transesterification in [BMIm] [PF_6] and [EMIm] [BF_4][6]. More recently, Nara *et al.*[7] described the lipase-mediated regioselective hydrolysis and alcoholysis of 3,4,6-tri-*O*-acetyl-D-glucal in tetrahydrofuran and two different ILs, such as [BMIm][PF_6] or [BMIm][BF_4]. A marked regioselectivity towards the formation of 4,6-di-*O*-acetyl-D-glucal was observed in [BMIm][PF_6] with 84% yield after 6h with 98% selectivity in hydrolysis and 48% yield after 8h with 98% selectivity in alcoholysis.

The above-mentioned examples suggest that ILs have great potential as alternative reaction media for biocatalysis and biotransformations with reviews now available[8–10]. A number of investigations on biocatalysis and biotransformations in ILs have been successfully carried out[11–13]. Herein, the recent important progress on properties of ILs, the applications of ILs in biocatalysis and biotransformations, product isolation, ILs and biocatalysts recycling, and their potential and challenges are reviewed and discussed.

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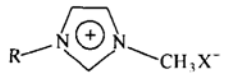
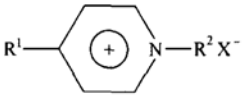
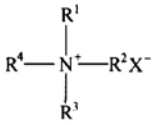
Table 1 Ionic liquids in biocatalysis and biotransformations

1-Alkyl-3-methylimidazolium cations		1-Alkylpyridinium cations				Alkylammonium cations					
											
Abbreviation	R	X	Abbreviation	R^1	R^2	X	Abbreviation	R^1	R^2	R^3	R^4	X
[MMIm][$MeSO_4$]	CH_3	CH_3OSO_3	[EPy] [TFA]	H	C_2H_5	CF_3COO	[$EtNH_3$] [NO_3]	C_2H_5	H	H	H	NO_3
[BMIm] [PF_6]	n-C_4H_9	PF_6	[BMPy] [BF_4]	CH_3	n-C_4H_9	BF_4						
[BMIm][BF_4]	n-C_4H_9	BF_4										
[EMIm][Tf_2N]	C_2H_5	$(CF_3SO_2)_2N$										
[BMIm][$MeSO_4$]	n-C_4H_9	CH_3OSO_3										
[OMIm][BF_4]	n-C_8H_{17}	BF_4										
[BMIm][TfO]	n-C_4H_9	CF_3SO_3										
[BMIm][NO_3]	n-C_4H_9	NO_3										
[BMIm][lactate]	n-C_4H_9	$CH_3CH(OH)COO$										
[EMIm][$EtSO_4$]	C_2H_5	C_2H_5 OSO_3										
[MOEMIm][BF_4]	$CH_3OC_2H_4$	BF_4										
[BMIm][Cl]	n-C_4H_9	Cl										
[OMIm][PF_6]	n-C_8H_{17}	PF_6										

2 THE PROPERTIES OF ILS

In general, ILs are regarded as highly polar solvents and able to dissolve many polar compounds, such as (poly)saccharides, amino acids and nucleotides. The polarity of ILs has been investigated by several groups[14,15]. On Reichardt's normalized polarity scale, which sets tetramethylsilane at 0 and water at 1[16], ILs' polarity lies in the range of 0.6—0.7, similar to that of methanol, *N*-methylformamide or 2-chloroethanol. The impact of the alkyl group on the imidazole ring (C_4—C_8) and the anion [tetrafluoroborate, hexafluorophosphate, bis(trifluoromethanesulfonyl)amide] on the IL's polarity seems to be slight. Although polar organic solvents inactivate enzymes, surprisingly, ILs do not. This feature extends enzyme catalyzed reactions to a solvent polarity range that was previously inaccessible. The ability to use solvents with higher polarity boosts the solubility of polar substrates, thus resulting in higher enzymatic activity. For example, IL enhanced remarkably the conversion (83%) of CaLB-mediated acylation of ascorbic acid with oleic acid in comparison with typical results in organic solvents (50%)[17] due to the higher solubility of ascorbic acid in the IL.

The miscibility of ILs and water varies widely and unpredictably. [BMIm][BF_4] and [BMIm][$MeSO_4$] are water-miscible but [BMIm][PF_6] and [EMIm][Tf_2N], which are of similar polarity to [BMIm][BF_4], are water-immiscible. The miscibility behavior of ILs and organic solvents is not well documented either. A relationship with the dielectric constant was proposed for lower alcohol and ketones, dichloromethane and THF thio phene (ε = 7.58) mixing with, for example, [BMIm][Tf_2N]. Alkanes and ethers do not follow the relationship with ethyl acetate probably at the borderline. Supercritical carbon dioxide ($scCO_2$) does not mix with ILs, such as [BMIm][PF_6] and [OMIm][BF_4], but is absorbed in the ionic liquid phase to the amounts up to molar fraction of 0.7. No IL dissolves in the CO_2 phase. Thus, less volatile or non-volatile products could be isolated from ILs by extraction in combination with organic solvents or supercritical CO_2.

3 APPLICATIONS OF ILS IN BIOCATALYTIC REACTIONS

3.1 Enzymatic hydrolysis in ILs

We employed [BMIm][BF_4] as the reaction medium for the asymmetric hydrolysis of D,L-*p*-hydroxyphenylglycine methyl ester to enantio-pure L-hydroxyphenylglycine with 5 proteases and 2 lipases. Papain gave the best results with respect to activity and enantioselectivity for this reaction. It has been found that the activity and the enantioselectivity of papain were clearly dependent on the [BMIm][BF_4] content of reaction medium. L-Hydroxyphenylglycine with the enantiomeric excess (e.e.) of 95.6% and the yield of 47.4% could be obtained with the aqueous solution of 12.5% (by volume) [BMIm][BF_4]. To further improve the yield and the product e.e., the hydrolysis was conducted under reduced pressure to remove the product formed of methanol *in situ*. Thus, higher yield (49.8%) and product e.e. (98.1%) were achieved. The comparative studies on the hydrolytic activity, the enantioselectivity and the stability of papain in [BMIM][BF_4] and molecular solvents have also been made. It was found that the significant enhancement

Table 2 Comparison of the activity, the enantioselectivity and the stability of papain for asymmetric hydrolysis of D,L-*p*-hydroxyphenylglycine methyl ester in different media

Media	hydrolytic activity, $U \cdot g^{-1}$	yield, %	e.e. ①, %	*E*-value ②	$t_{1/2}$ ③, h
BMIM·BF_4 (12.5%, by volume) ④	3.09	48.3	96.9	665	169
phosphate buffer	1.74	40.5	92.3	118	16
2-propanol (20%, by volume) ④	1.91	43.7	93.5	173	18
acetonitrile (15%, by volume) ④	1.45	38.9	90.8	89	10

① The enantiomeric excess of the product; ② The enantiomeric ratio; ③ Half-life time;
④ The optimum concentration for this reaction.

in the activity, the enantioselectivity and the stability of papain at 45℃ was observed with the aqueous solution of 12.5% (by volume) [BMIm][BF_4] as compared to phosphate buffer, 2-propanol and acetonitrile for asymmetric hydrolysis of D,L-*p*-hydroxyphenylglycine methyl ester (Table 2). Subtilisin Carlsberg mediated enantioselective hydrolysis of *N*-acetyl-amino acid esters into the corresponding enantiopure amino acids in ILs instead of conventional organic solvents was reported by Zhao *et al.*[18]. The enantioselectivity and catalytic activity of the enzyme were enhanced clearly using [EPy][TFA]-water (15:85, by volume) as the reaction medium in place of the acetonitrile-water (15:85, by volume). Very recently, soluble epoxide hydrolase was shown to catalyze the hydrolysis of epoxides using the ILs [BMIm][PF_6], [BMIm][BF_4] and [BMIm][Tf_2N] as reaction medium. Reaction rates were generally comparable with those observed in buffer solutions[19].

3.2 Enzymatic transesterification in ILs

Lipase-catalyzed transesterification for preparation of polyesters has received considerable attention. CaLB (*Candida antarctica* lipase B) mediates polyester synthesis in [BMIm][BF_4] or [BMIm][PF_6] at 60℃[20] but the molecular weight of the product is low compared with the free solvent system[21], perhaps owing to the high viscosity of ILs. Transesterification of racemic phenylglycine methyl ester with ethanol was studied with four different formulations of CaLB: free enzyme (Novo SP525), enzyme immobilized on a support (Novozym 435), crosslinked enzymes crystals (CLEC) and crosslinked enzyme aggregates (CLEA)[11]. It was observed that the catalytic activities of CaLB in [BMIm][PF_6] and [BMIm][TfO] were comparable to those in *tert*-butanol. In contrast, no reaction (< 5% conversion) was detected in [BMIm][NO_3],[BMIm][lactate], [EMIm][$EtSO_4$] and [$EtNH_3$][NO_3]. Similar results were obtained in the transesterification of ethyl butyrate with *n*-butanol in the same solvents. The ILs in which low or, even no activities, are observed contain more strongly coordinating anions: lactate, nitrate and ethylsulfate. Thus, it suggests that a plausible explanation for this is that coordination of these anions to the enzyme surface, be it in the free or immobilized form, gives rise to conformational changes in enzyme, leading to a loss of activity. Recently, many investigations on lipase-mediated transesterifications in ILs have been carried out to explore the effect of IL media on these biotransformations. For example, Nara *et al.*[12] reported a comparative study on lipase-catalyzed transesterifications of 2-hydroxymethyl-1,4-benzodioxane in [BMIm][BF_4] and [BMIm][PF_6] and different organic solvents. The remarkable influence of the IL as an additive in an organic solvent on this reaction has been observed, and the enzyme in ILs can be recycled for several runs without substantial diminution in the transesterification activity of the lipase. Kim *et al.*[6] described that the use of ILs enhanced significantly the enantioselectivity of lipase in the transesterification of several chiral alcohols. It was found that lipases were up to 25 times more enantioselective in ILs than in organic solvents, such as toluene or tetrahydrofuran.

Lozano *et al.* compared the α-chymotrypsin-catalyzed transesterification of *N*-acetyl-L-tyrosine ethyl ester in different ILs and in 1-propanol. Despite the fact that in the ILs tested the enzyme activity reached only 10% to 50% of the value in 1-propanol, the boosted stability resulted in higher yield of the product. The transesterification rates in [BMIm][PF_6] and [OMIm][PF_6] media were of the same magnitude as those in isooctane or acetonitrile. However, in [EMIm][Tf_2N] the rate was nearly an order of magnitude higher than those in isooctane or acetonitrile. Many investigations on protease-mediated transesterification in ILs have also been carried out by other groups[22,23]. It has been proven that ILs can boost markedly the transesterification activity of protease at low water activity.

3.3 Enzymatic ammonolysis in ILs

Primary fatty acid amides are important commodities that can be prepared under mild conditions *via* lipase-mediated ammonolysis of carboxylic esters with ammonia. Lau *et al.*[5] compared the CaLB-catalyzed ammonolysis of ethyl octanoate in an IL medium with that in *tert*-butyl alcohol under otherwise identical reaction conditions. The conversion into octanamide was remarkably lower in [BMIm][BF_4]

than in *tert*-butyl alcohol. The several preparations of CaLB (immobilized on Lewatit E, free and immobilized on EP 100) show different performances with the free enzyme showing the lowest activity in this system (38% conversion after 24 h). It was found that the ammonolysis of octanoic acid with ammonia in the presence of Novozym 435 at 40℃ in [BMIm][BF_4], proceeded to complete conversion in 4 days compared to 90%—100% conversion in 17 days using ammonium carbamate in methylisobutyl ketone. Currently, we are attempting to conduct ammonolysis of unnatural amino acid esters with various ammonium salts in ILs using lipase or protease as biocatalysts, which might be a potential new route for the preparation of enantiopure unnatural amino acid amides, such as D-*p*-hydroxyphenglycine amide, D-phenylglycine amide and D-allylglycine amide.

3.4 Enzymatic acylation in ILs

Lipase-catalyzed enantioselective acylation for the resolution of chiral alcohol is one of the major industrial applications of lipases[24], and thus has gained increasing attraction. Recently, many investigations on the resolution of aryalkanols with lipases, especially CaLB and *Pseudomonas cepacia* lipase (PcL) in ILs, have been successfully carried out[25,26]. For instance, Schöfer *et al.*[25] reported the kinetic resolution of racemic 1-phenylethanol *via* asymmetric acylation with different lipases in organic solvents (methyl *tert*-butyl ether, MTBE) and ILs, such as [BMIm][PF_6], [BMIm][TfO], [BMIm][Tf_2N] and [BMIm][BF_4] (Table 3). Lipases showed good activity and, in some cases, improved enantioselectivity when employed in such ILs. Another advantage is that the ILs are nonvolatile and it is, therefore, possible to remove the product by distillation and repeat the catalytic cycle after adding fresh substrate. Lipases suspended in ILs could be reused for three batches with the enantioselectivity not influenced. Reetz *et al.*[27] described the lipase-catalyzed acylation of chiral secondary alcohols in ILs in combination with supercritical carbon dioxide for continuous selective extraction of the desired product, which provides a new approach for the separation of enantiomers. Excellent enantioselectivities were achieved with [BMIm][Tf_2N] as the reaction medium. Very recently, a variety of racemic secondary alcohols with different structure were resolved *via* enantioselective acylation using succinic anhydride as acyl donor catalyzed by *Pseudomonas cepacia* lipase supported on celite in [BMIm][PF_6][28]. Surprisingly, organic base, namely triethylamine as an additive in IL was found to improve the rate of the reaction greatly without significant change in the enantioselectivity. The rate enhancement in IL might have taken place due to the removal of one of the products, an acid, by the added organic base in the form of an ion-pair. It has been demonstrated in our experiment that aminoacylase I from *Aspergillus sp.* could catalyze enantioselective acylation of unnatural organosilyl alcohols, such as racemic 1-trimethylsilylethanol with vinyl acetate as acyl donor and the results will be reported elsewhere.

Lipase-mediated enantioselective acylation of amines with carboxylic acids was also performed in ILs or in a non-solvent system. The reaction equilibrium was shifted toward amide synthesis by the removal of water formed under reduced pressure in ILs owing to their negligible vapor pressure, and thus the product yield and the product e.e. increased greatly[29].

CaLB-catalyzed acetylation of glucose showed more regioselective in ILs than in organic solvents because glucose is up to one hundred times more soluble in ILs[30]. Acetylation of insoluble glucose in organic solvents afforded the more soluble 6-*O*-acetylglucose, which underwent further acetylation to give 3,6-*O*-diacetylglucose [(2-3):1 mixture]. However, acetylation of glucose in ILs yielded only the desired product 6-*O*-acetylglucose (Table 4).

3.5 Biocatalytic reduction in ILs

Biocatalytic redox reactions are frequently conducted with whole cells of microbes as biocatalysts due to the necessity of recycling the redox cofactors. The organic phase, which is often used to store the sparingly soluble reactants and products, can be replaced by an IL, which is much less toxic to cell membranes

Table 3 Lipase-mediated enantioselective acylation of racemic 1-phenylethanol in ILs

Solvent	Comparison of various lipases					
	candida antarctica lipase B		*pseudomonas cepacia sp.*		*alcaligenes sp.*	
	conversion, %	e.e., %	conversion, %	e.e., %	conversion, %	e.e., %
MTBE	43	> 98	53	84	98	0
[BMIm][PF_6]	< 5	—	0	—	44	77
[BMIm][BF_4]	< 5	—	7	53	60	81
[HMIm][BF_4]	10	> 98	0	—	68	14
[OMIm][BF_4]	41	> 98	< 5	—	50	> 98
[BMIm][TfO]	50	> 98	50	> 98	70	82
[BMIm][Tf_2N]	50	> 98	47	> 98	89	15

Table 4 Regioselective CaLB-catalyzed acylation of glucose in ILs and in organic solvents

Solvent	Conversion, %	Yield of 6-*O*-acyl-D-glucose, %	Yield of 3,6-*O*-diacyl-D-glucose, %
[EMIm][BF_4]	50	50	—
[MOEMIm][BF_4]	99	93	6
[BMIm][BF_4]	78	69	8.7
acetone	72	55	17.7
tetrahydrofuran	99	53	47

than a conventional organic solvent, such as toluene. It has been proven that baker's yeast[31] as well as *Rhodococcus* R312 and *E. coli* could maintain their activity in ILs containing no or a very small amount of water. Thus, a variety of prochiral ketones were enantioselectively reduced by immobilized yeast in a [BMIm][PF_6]-water (10:1, by volume) biphasic medium. The performance of the system was, in general, comparable with that in a conventional organic-water medium.

To broaden the field of biocatalytic reactions in ILs, isolated reductase-catalyzed enantioselective reduction of prochiral ketones to the corresponding optically active alcohols has also been investigated[32]. Formate dehydrogenase from *Candida boidinii* was found to be stable and active in mixtures of [MMIm][$MeSO_4$] and buffer solution. Up to date, there has been, however, no report on the catalytical performance of alcohol dehydrogenase in ILs. We are now exploring horse-liver alcohol dehydrogenase catalyzed asymmetric reduction of acetyltrimethylsilane to enantiomerically pure (*S*)-1-trimethylsilylethanol with ILs as the reaction media. The results will be reported later.

3.6 Enzymatic oxidation in ILs

Hinckley *et al.*[33] reported oxidative enzymes, laccase C from *Trametes sp.* and horseradish and soybean peroxidases, catalyzed oxidation reactions in systems with varied concentrations of ILs. The catalytic activity of enzymes was decreased by adding a water-miscible [MBP][BF_4](4-methyl-*N*-butylpyridinium tetrafluoroborate), or by suspending enzymes in a water-immiscible [BMIm][PF_6]. However, for the oxidation of anthracene catalyzed by laccase C and assisted by a number of mediators, the addition of [MBP][BF_4], instead of *tert*-butanol, increased the yield of the oxidation product by several folds. Subsequently, Laszlo *et al.*[34] demonstrated that three peroxidases such as hemin, cytochrome *c* and microperoxidase-11 displayed high activity in ILs ([BMIm][PF_6], [OMIm][PF_6], [BMIm][Tf_2N]) when activated by an electron acceptor. Okrasa *et al.*[35] used the IL {[BMIm][PF_6]} as a new reaction medium for oxidase-peroxidase-catalyzed sulfoxidation. That work showed that IL markedly enhanced the operational stability as well as the stereoselectivity of these enzymes because of the perfect solubility of the substrate of glucose oxidase (glucose) and the substrate of peroxidase (sulfide) in IL systems.

4 IL AND BIOCATALYST RECYCLING AND PRODUCT ISOLATION

The key to open the door of the application of IL's into the biocatalytic processes in an industrial scale is their efficient recovery, product isolation and biocatalyst reuse owing to their higher costs than that of conventional organic solvents. Likewise, these are also the key issues regarding the "greenness" of ILs. Recognizing the importance of these issues, some researchers have focused on the methods that would be suitable for industrial scale operation, such as extraction with benign solvents like water or supercritical carbon dioxide, pervaporation or distillation (Table 5). As ILs are nonvolatile, volatile products such as alcohol and water can be removed by evaporation under reduced pressure, as was shown in the reduction of parachiral ketones by baker's yeast in [BMIm][PF_6]. Nonvolatile products can also be efficiently separated from ILs *in situ* in combination with supercritical CO_2, as was described in lipase-mediated kinetic resolution of chiral secondary alcohols and enantiomer separation using IL (an working phase)/supercritical CO_2 (an extractive phase). The evaporation or extraction of the product from ILs with negligible vapor pressure can be used to drive the equilibrium towards complete conversion. For example, Itoh *et al.*[26] removed methanol from the CaLB-catalyzed transesterification using a methyl ester as an acyl donor, so the conversion was enhanced significantly.

Table 5 Separation of products from the systems with ILs

Isolation method	Comments	References
supercritical CO_2	above 95% of yield, limited to nonpolar substrate	[27, 36]
column chromatography	conventional technique in organic chemistry	[6,33]
extraction	typically hexane or ether may not be suitable for polar substrates	[7,22,31,37]
distillation	85℃, 0.06 millibar volatile substrates only	[25]

As mentioned earlier, ILs, such as [BMIm][PF_6], do not mix with ether or supercritical CO_2. This unconventional behavior was advantageously used by extracting the products and the unconverted reactant from the reaction mixture with ether or supercritical CO_2, while the biocatalyst remained suspended in the IL phase to be recycled. The reaction could also be restarted after only addition of reactants.

5 POTENTIAL AND CHALLENGES

The ILs have a tremendous potential as reaction media for biocatalysis and biotransformations. This is being increasingly recognized. A variety of biocatalysts are eminently capable of performing biocatalytic conversions in ILs with activity, (enantio-, regio-) selectivity and stability being generally comparable with or higher than those observed in conventional organic solvents. Thus, the use of ILs in enzymatic catalysis demonstrates possibilities for non-aqueous enzymology following the pioneering work of Klibanov[38] for the use of enzymes in pure organic solvents. ILs have obvious benefits of carrying out biotransformations of highly polarized substrates, which otherwise is impossible in water owing to equilibrium limitations or in organic solvents due to poor solubility of the substrate. To broaden the applications of ILs in biocatalytic processes, further investigations are however required. Firstly, efficient approaches for less volatile or non-volatile product separation from the expensive IL need to be developed. Secondly, their stability needs to be demonstrated so that they can be recycled over a prolonged periods of time under reaction conditions.

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