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Review

Biocatalytic reactions in hydrophobic ionic liquids

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ABSTRACT

Medium engineering for biocatalytic reactions is imperative approach for the synthesis of biologically active compounds. Biocatalytic reaction media holding water, organic solvent and supercritical fluids have become well established for commercial applications. Non-aqueous biocatalytic reactions carry significant advantages that non-polar substrate can be specifically reacted and/or product recovered efficiently. On the other hand, usage of conventional organic solvents as non-aqueous medium affects green chemistry aspects. Hydrophobic ionic liquids (ILs) provide desirable environment for many enzymatic reactions as conventional organic solvents do but without emission of volatile organic compound (VOCs). To extend hydrophobic ILs laboratory performance into commercial process requires the creation of a brief database for biocatalytic reactions. In light of the growing relevance of this theme, the current review intends to address the important biotechnological applications of the frequently employed hydrophobic 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim] PF_6) IL. The review also attempts to describe the comparison of various whole cell biocatalytic reactions using supercritical carbon dioxide ($scCO_2$) biphasic systems for product extraction with potentially competitive ILs and other conventional solvents.

Contents

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1. Introduction

More than 13,000 enzyme catalyzed reactions have been successfully demonstrated in laboratory scale [1]. Enzymes offer advantages in stereo and regioselectivity for the preparation of new drugs, food additives and organic chemical products. Enzymatic reactions can performed under preferred conditions with minimized yield of the undesired side products [2], which is often troublesome in traditional chemically catalyzed methods. On the other hand, the majority of organic compounds are not easily soluble in aqueous media. Meanwhile, diminished yields, selectivity, and poor solubility of substrates in aqueous medium may require the enzymatic reactions to be carried out in non-aqueous medium. In this regard, prevalent substituents such as organic solvents [3], supercritical carbon dioxide ($scCO_2$) [4], ionic liquid (IL), fluorous phase media [5] and solventless process [6] have been adopted to overcome the hurdles of aqueous reaction medium. However, increasing environmental demands and law restrictions necessitated the development of new types of green solvents and innovations towards more sustainable enzymatic processes.

Liquid phase organic salts or ILs [7] are composed of bulky asymmetric cations, and small inorganic anions, which received immense attention as non-volatile reaction medium. Additionally, ILs are easily modified with respect to the organic cation, inorganic anion and the length of the side chain attached to the cation. Besides, theoretically 10^{18} numerous composition of ILs will be possible. Furthermore, the simple versatile IL possess many attracting properties such as miscible/immiscible, inexpensive to prepare, easy to recycle, non-existence of vapor pressure and merely liquid at and/or around room temperature [8,9]. The pioneering works of Cull et al. [10] and Erbeldinger et al. [11] have shown that biocatalysts remain active not only in organic solvents and supercritical fluids (SCFs), but also in ILs. Consequently, ILs have been used as a pure solvent, co-solvent and biphasic media for biochemical and chemical reactions. Interestingly, most of the reactions display quite different properties than in other non-aqueous solvents such as SCFs and organic solvents.

Overviews of extensive studies carried out in enzymatic reactions using ILs are available in excellent publications and reviews [10–44]. Hydrophobic IL is well known for its capability towards biocatalytic applications. Particularly hydrophobic medium possessing hexafluorophosphate (PF_6), bis(trifluoromethanesulfonyl)imide (Tf_2N) anions are capable of multitude usage in different biochemical and chemical reactions. In order to develop the possibilities of commercial application and usability of ILs towards bioprocess requires the construction of detailed database of each IL for different biocatalytic reactions and related process. In particular, this review chooses a typical hydrophobic IL, 1-butyl-3-methylimidazolium ([Bmim]) PF_6 as a model system. Fig. 1 shows the expansion of research papers published with the subject of [Bmim] PF_6 applications in recent years. [Bmim] PF_6 is regarded as one of the example for 'alternative reaction media' and a promising solvent used in enzymatic transesterification, alcoholysis, aminolysis, hydrolysis, and oxidation. Table 1 gives a list of enzymes that have been used for the biocatalytic reactions in hydrophobic ILs. The chemical and physical properties of [Bmim] PF_6 are shown in the Supplementary information. In this review we attempt to summarize the effect of reaction conditions involving model hydrophobic IL [Bmim] PF_6 on biocatalytic reactions and illustrate its expanding use in various biological applications.

2. Biocatalytic reactions

Hydrophobic ILs like other organic solvents were employed for carrying out biocatalytic reactions (i) as a pure solvent, (ii) as a co-solvent, (iii) in a biphasic system involving organic solvent or

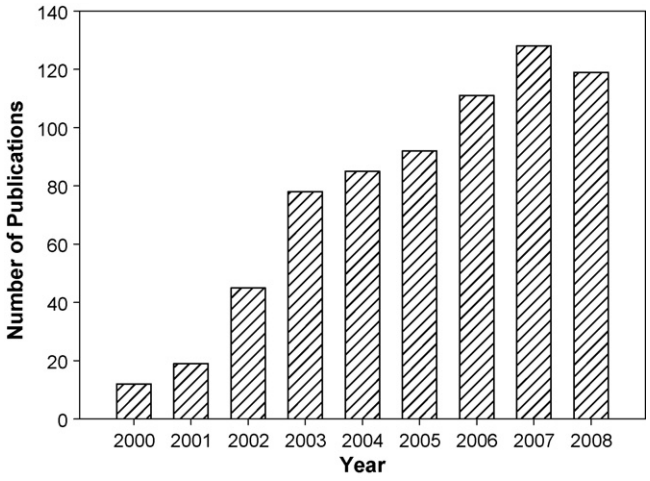

Fig. 1. Number of publications containing '[Bmim] PF_6' in the topic found from the ISI Web of Science, in the period 2000–2008.

$scCO_2$. Erbeldinger et al. [11] demonstrated the first preparative enzyme catalysis in IL by soluble thermolysin. *Z*-aspartame was synthesized from the coupling of carbobenzoxy-L-aspartate and L-phenylalanine methyl ester hydrochloride in [Bmim] PF_6 with 5% H_2O at 37 °C. The activity of thermolysin was well retained after incubation in [Bmim] PF_6 at 37 °C for 144 h, whereas the same treatment in ethyl acetate resulted in the loss of almost half of the enzyme activity. On the other hand, lipases were the mostly studied enzyme for the biocatalytic reactions in ILs. Generally, lipases and proteases catalyze the hydrolytic reactions. However, the catalytic reaction can be reversed and changed into esterfication/transesterfication, alcoholysis or aminolysis by medium engineering or water content of the medium. The recently studied biocatalytic reactions in hydrophobic ILs are summarized in Table 2 and discussed in the following sections.

2.1. Transesterification

Sheldon and co-workers [12] reported enzyme catalyzed reactions using pure [Bmim] PF_6 and [Bmim] tetrafluoroborate (BF_4) at 40 °C. Novozym 435 catalyzed transesterfication of ethyl octanoate with propan-2-ol in [Bmim] PF_6 demonstrated lower conversion

Table 1
List of enzymes that have been used for the biocatalytic reactions in ionic liquids.

Enzymes	Reference
Aldolase	[107]
Cytochrome C	[92]
Esterase	[13,49]
Hemin	[92]
Horseradish peroxidise	[91,99]
Laccase C	[91]
Lipase	[12–14,18,34,35,45–48,50,51,53,54,58,77,86,87,89,90,98,100,102]
Morphine dehydrogenase	[106]
Microperoxidase	[92]
Penicillin G acylase	[81,85]
Soybean peroxidise	[91]
Subtilisin	[52,102,108]
Thermolysin	[10]
Yeast alcohol dehydrogenase	[96]
α-Chymotrypsin	[16,46]

Table 2
Summary of biocatalysis reactions in 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim] PF_6).

Substrate	Enzyme	Output remarks	Ref
Carbobenzoxy-L-asparate, L-phenylalanine methyl ester HCl	Thermolysin	First enzymatic reactions in [Bmim] PF_6 ended with 95% conversion and well maintained enzyme stability	[10]
Transesterfication			
Ethyl butanoate with butanol	Novozym 435	Anhydrous IL gave 81% yield in [Bmim] PF_6	[12]
Rac-1-phenyl ethanol	Lipases and esterase	Esterase failed to maintain enzyme activity in ILs	[13]
Racemic 5-phenyl-1-penten-3-ol	CAL, QL, PS	Enzyme catalyzed enantioselective reaction in a pure IL solvent system	[14]
N-acetyl-L-phenylalanine ethyl ester	α-chy	Increasing the water content enhance the rate of the reaction	[16]
Racemic 5-phenyl-1-penten-3-ol	CAL, PCL, CRL	Transesterification of an allylic alcohol in the [Bmim] PF_6 and [Bmim] BF_4 was successfully explained	[18]
Aryl alcohol	CALB, PCL	Increased E-value showed that [Bmim] PF_6 suitable solvent than conventional solvents	[35]
Butanol	CALB	Increased synthetic activity and 2300 times greater half life time showed in [Bmim] PF_6	[45]
N-acetyl-L-tyrosine ethyl ester and 1-proponal	α-Chy	[Bmim] PF_6 maintained excellent stability than other ILs	[46]
2-hydroxymethyl-1,4-benzodioxane	Lipases	[Bmim] PF_6 reaction medium suitable for reactions with supported and immobilized enzyme	[47]
N-acetyl-L-phenylalanine ethyl ester with n-propanol	PFL	Excellent yield with good E-Value observed in [Bmim] PF_6	[48]
Phenyl ethanol	Esterase	27-fold higher half life observed in [Bmim] PF_6 than hexane	[49]
Phenylethane-1,2-diol	PSL	Good enantioselectivity observed in [Bmim] PF_6	[50]
Phenyl ethanol	CRL, BCL	IL treated enzyme showed higher activity than untreated enzyme	[51]
Rac-secondary alcohol	Subtilisin	[Bmim] PF_6 with 0.5% of water enhanced initial rate 10,000 times	[52]
Methyl butyrate with butyl alcohol	Novozym 435	Nature of IL anions influence activity	[53]
Methyl methacrylate and divinyl adipate	CRL,PPL,CALB, TLL,MML	Hydrophilic ILs with [Bmim] PF_6 co-solvent does not activate the enzyme	[54]
(R,S)-1-chloro-3-(3,4-difluorophenoxy)-2-propanol with vinyl butyrate	PAL	[Bmim] PF_6 proved better co-solvent than [Bmim] BF_4	[58]
Ethyl 3-phenylpropanoate	PSL	Higher yield observed in hydrophobic ILs	[59]
Ethyl butyrate n-butanol	CALB	Lipase preserved enzyme activity in [Bmim] PF_6	[60]
Esterfication			
2-substituted propanoic acids,1-butanol	CRL	Pervaporation used for avoid water by-product	[61]
Rac-ibuprofen	CRL	5% improved resolution and 3 times increased enantioselectivity maintained in [Bmim] PF_6 than isooctane	[62]
β-D-glucose and vinyl ester	CALB	60% conversion observed in [Bmim] PF_6	[63]
β-D-glucose and vinyl ester	PEG-CALB	Compared to [Bmim] BF_4, 5% higher reaction yield observed in [Bmim] PF_6	[64]
Geraniol	CALB	[Bmim] PF_6 maintained enzyme activity around $a_w = 0.6$	[65]
(±)-menthol	CRL	Reusability and stability of enzyme was demonstrated [Bmim] PF_6	[66]
Benzyl alcohol	CRLs	[Bmim] PF_6 and [Bmim] Tf_2N acts as enzyme stabilizer	[67]
Supersaturated glucose with vinyl laurate	Novozym 435	Mixture of ILs showed better conversion efficiency than pure ILs	[68]
Acylation			
5-phenyl-1-penten-3-ol	Novozym 435	Reaction using [Bdmim] PF_6 was failed	[69]
Methyl-6-O-trityl-glucosides	CRL	More favourable structural adaptation of enzyme in [Bmim] PF_6 boost up the regioselectivity	[70]
Rac-secondary alcohol	PSL	Additive triethyl amine enhanced the activity without affecting enzyme enantioselectivity of lipase using [Bmim] PF_6	[71]
1-phenylethylamine and 2-phenyl-1-propylamine	CALB	Initial rate of amide synthesis in [Bmim] PF_6 is much higher than [Emim] BF_4	[34]
Flavonoid	Novozym 435, CRLs	Superior acylation of naringin observed in [Bmim] PF_6	[72]
6-amino-1-hexanol	CALB	Around 100% substrate conversions observed in [Bmim] PF_6	[73]
Sulfonamides	PPL,CCL, PSL	[Bmim] PF_6 and [Bmim] BF_4 showed 100% selectivity for the preparation of valuable intermediates without forming hazardous intermediate	[75]
Hydrolysis			
3,4,6-tri-O-acetyl-D-glucal	PSL	Consider to hydrophilic [Bmim] BF_4 four times higher yield with reduced reaction time demonstrated in [Bmim] PF_6	[76]
Rac-naproxen methyl ester	CRL	In contrast with conventional solvents, [Bmim] PF_6 holds eight times higher yield	[77]
Methyl phenyl acetate, L-phenylglycine methyl ester	PGA	PGA sustained activity even after one week exposure with [Bmim] PF_6	[81]
Penicillin-G	PGA	Compared to [Bmim] BF_4, [Omim] BF_4, [Bmim] PF_6 preserves much higher enzyme stability	[85]
Rac-ibuprofen ester	CALB	Substrate anchored to the OH-appended [Bmim] PF_6 Exhibits 80% optical purity	[86]
p-nitrophenyl butyrate	Lipase	IL pretreated enzyme posses higher activity	[87]
Alcoholysis			
3,4,6-tri-O-acetyl-D-glucal	PSL	Consider to [Bmim] BF_4 four times higher yield with reduced reaction time showed in [Bmim] PF_6	[76]
Vinyl cinnamate	CRL, MJL, PSL	PEG enzyme complex enhance the lipase alcoholysis activity in ILs	[89]
Vinyl acetate	PSL and AK	Addition of water with [Bmim] PF_6 weaken the enzyme activity	[90]

Table 2 (*Continued*)

Substrate	Enzyme	Output remarks	Ref
Oxidation			
Syringaldazine	Laccase C, HRP, SBP	Oxidative enzyme saturated with aqueous buffer showed activity in [Bmim] PF_6	[91]
Guaiacol	Hemin, MP, Cyt-C	Water content in IL does not affect the peroxidase activity	[92]
Ethanol	YADH	Microemulsion maintains YADH activity	[96]
Polymerization			
Diethyl octane-1,8-dicarboxylate	PSL	Polymerization in [Bmim] PF_6 showed significant polydispersity values	[98]
Aniline	HRP	Five consecutive runs with good electrical conductivity observed	[99]
ε-caprolactone	CAL	Enzymatic polyester synthesis was demonstrated in [Bmim] PF_6 and [Bmim] BF_4	[100]
Dynamic kinetic resolution			
1-phenyl ethanol	Lipase–Ru, Subtilisin–Ru	[Bmim] PF_6 enhanced racemization reaction in RT	[102]
Hydroxylation			
Codeinone and neopinone	MDH	One pot chemo-enzymatic reactions catalysis gave 100% yield in [Bmim] PF_6	[106]
Aldol reaction			
Hydroxyacetone with 4- or 3-(trifluoromethyl)-benzaldehyde	Aldolase antibody 38C2	Carbon–carbon bonding reaction was successfully demonstrated in [Bmim] PF_6	[107]
Peptide synthesis			
L-alanine	Subtilisin	Dipeptide and tripeptide formation in [Bmim] PF_6 was proved	[108]

rate than organic solvent. On the other hand, employing butan-1-ol as substrate enhanced reaction efficiency. Schöfer et al. [13] showed transesterfication of rac-1-phenylethanol with vinyl acetate carried out with nine lipases and two esterases in ten different ILs. Reaction medium contain esterases failed to show activity. In contrast, *Candida antarctica* lipase (CAL) and *Pseudomonas* sp. lipase (PSL) displayed better conversion efficiency in ILs. [Bmim] Tf_2N has advantage over other ILs and [Bmim] PF_6 fails to preserve enzyme activity. This might be due to the fact that enzyme activity depends not only on IL, but could also be affected by several other factors such as the diffusion rates of substrates, product, and the conformational rigidity of enzymes.

Itoh et al. [14] achieved asymmetric transesterification of allylic alcohol (racemic 5-phenyl-1-penten-3-ol) using Novozym 435, *Alcaligenes* sp. lipase (ASL), and PSL in [Bmim] PF_6, [Bmim] BF_4, [Bmim] Tf_2N, and [Bmim] hexafluoroantimonate (SbF_6). Novozym 435 anchored in [Bmim] PF_6 preserved its activity for repetitive process. On the contrary, *Candida rugosa* lipase (CRL), *Procine liver* lipase (PLL) were unsuccessful for transesterfication of allylic alchohol. Further, [Bmim] trifluoroacetate (TFA), [Bmim] trifluoromethanesulfonate (TfO) and [Bmim] SbF_6 also exhibited poor reaction rate.

Transesterification of different pharmaceutically important 1,2-diols using PSL (0.5 eq w/w) in [Bmim] PF_6 yields >99% enantioselectivity within a short reaction time. Reusability and excellent transesterification activity of immobilized CALB and native *Pseudomonas cepacia* lipase (PCL) in [Bmim] PF_6 and [Bmim] BF_4 proved the efficiency of hydrophobic IL [15]. Laszlo et al. [16] reported the use of α-chymotrypsin to carry out transesterification reactions. *N*-acetyl-L-phenylalanine ethyl ester or *N*-acetyl-L-tyrosine ethyl ester was transformed into the corresponding propyl esters using 1-octyl-3-methylimidazolium ([Omim]) PF_6 and [Bmim] PF_6. Polarity of the ILs plays vital role to maintain enzyme activity. Similar to polar organic solvents, ILs also require a certain amount of water to maintain enzymatic activity. For both ILs and organic solvents, the reaction rates are of the same order of magnitude.

Independently, Lozano et al. [45,46] reported enzyme catalyzed transesterfication in different ILs. Free CAL strongly stabilized in [Bmim] PF_6 than organic solvents. Reduced enzymatic activity observed in hexane and 1-butanol in comparison with [Bmim] PF_6, 1-ethyl-3-methylimidazolium ([Emim]) BF_4, [Emim] Tf_2N and [Bmim] Tf_2N. Increasing the polarity of the IL enhanced the synthetic activity and selectivity of enzyme catalyzed transesterfication reaction. Specific interaction of substrate and enzyme increases the stability of the lipase 2300 times than lipase incubated without substrate [45]. Likewise, transesterification of 2-hydroxymethyl-1,4-benzidioxane was carried out using vinyl acetate with several lipases in [Bmim] PF_6 and [Bmim] BF_4. As a result, increase in the activity yield of PCL (free) was observed in [Bmim] PF_6, [Bmim] BF_4 and conventional dichloromethane. However, anions such as nitrate, acetate, and trifluoro acetate were more nucelophillic than PF_6 anion and coordinated more substantially to the positive charged sites on lipase causing conformational changes in the enzyme structure which could be the reason for the strengthened enantioselectivity and activity in [Bmim] PF_6 [47]. Similarly, the resolution of the phosphorus substituted primary alcohols in the presence of a *Pseudomonas fluorescens* lipase (PFL) showed a remarkable enantioselectivity in [Bmim] PF_6 than the diisopropyl ether, but was negligible in hydrophilic [Bmim] BF_4 [48].

Bornscheuer and group [49] showed native esterases (esterases from *Bacillus subtilis* and *Bacillus stearothermophilus)* failed to carry out transesterfication reaction in [Bmim] PF_6, [Bmim] BF_4 and [Bmim] bis [(trifluoromethyl) sulfonyl] amide (BTA). On the other hand, enzyme immobilization with celite leads activity in ILs. Specific activities of ILs are comparable to organic solvent MTBE and vinyl acetate. Esterases in [Bmim] PF_6 showed much higher enzyme stability than other ILs and organic solvents. Similarly, the reaction including free CALB with 2% water in [Bmim] PF_6, [Emim] BF_4, [Emim] Tf_2N, and [Bmim] Tf_2N enhanced the synthesis of butyl butyrate from vinyl butyrate and butanol at 50 °C. Enzyme immobilized onto solid support provides excellent activity and/or stability in ILs [50].

Shah et al. [51] confirmed several techniques involving immobilization to activate catalytic efficiency of enzymes in [Bmim] PF_6. Transesterfication of racemic 1-phenylethanol engaged with CRL and *Burkholderia cepacia* lipase (BCL) were taken as model system. Acetone-rinsed enzyme preparation, cross-linked enzyme aggregates and protein-coated micro-crystals gave good conversion rate than free lipase. The application of same methodology to substilisin catalyzed transesterification of N-acetyl-L-phenylalanine ethyl ester. In [Bmim] PF_6, pretreated substilisin (precipitated and rinsed with n-propanol) showed about 10,851 times higher initial rate than pH tuned lyophilized enzyme. In the same manner, 10:1 ratio of the poly(ethylene glycol) (PEG)-lipase complex raised catalytic efficiency in ILs than free lipases [52].

Recently, Zhao et al. [53] briefly studied microwave irriated Novozym 435 activities in twenty-two ILs. Lipase surrounded by

water layer enhanced enzyme activity. Like other studies, polarity scale of the ILs fails to correlate enzyme activity. On the other hand, significance of anions towards enzyme activity also addressed. Likewise, Itoh et al. [14] and Kaar et al. [54] believed that anion nature of the IL might play crucial factor to determine enzyme activity. Free CRL was found only active in [Bmim] $PF_{6\ for}$ the transesterification of methylmethacrylate, but inactive in other ILs including [Bmim] acetate (CH_3COO^-) and [Bmim] nitrate (NO_3). This example showed that the high hydrophilic ILs deactivate enzyme. In addition, immobilization of enzyme also fails to maintain activity in hydrophilic IL. It was possible that the enzyme was destabilized by the three ILs because they are hydrophilic and could strip off the essential water from the enzyme at high salt concentrations. In addition, free CALB displayed 1.5 times more active than hexane [49,55]. Subsequently same group used salt hydrate pairs to control water activity of Chirazyme L-2 C2 in [Bmim] PF_6. Conversion rate in hexane is higher than IL at low water activity. Only sodium iodide (NaI) hydrate pair control water activity. Other salt hydrate pairs fail to control activity. ILs possess high salt solubilities; however, a certain degree salt should remain undissolved to control water activity [56]. Salt activation strategy fails to give excellent performance in ILs. On the other hand, this approach demonstrates good results in organic solvents [57].

Recent finding of Singh et al. [58] proved the co-solvent efficiency of [Bmim] PF_6. *Pseudomonas aeruginosa* lipase (PAL) catalyzed transesterfication in hexane with [Bmim] PF_6 co-solvent successes to produce (R)-1-chloro-3(3,4-difluorophenoxy)-2-propanol. Repetitive use of enzyme with excellent stability also confirmed the co-solvent effect. Likewise, transesterfication of ethyl 3-phenylpropanoate catalyzed with PCL using hydrophobic ILs [Bmim] PF_6, [Emim] Tf_2N showed better performance than [Bmim] BF_4 [59]. On the contrary, CALB catalyzed transesterification of ethylbutanoate in [Bmim] BF_4 offered higher efficiency than [Bmim] PF_6 [60]. A feasible explanation could be that the IL might be interacting with charged groups of enzyme and substrate, which may play key role to determine enzyme activity in ILs.

2.2. Esterfication

Without significant lose in enzyme activity and enantioselectivity, esterfication of 2-substituted-propanoic acids was demonstrated in hydrophobic ILs with PF_6 anion [61]. Esterfication enantioselectivity and stability of CRL in [Bmim] PF_6 is superior to six ILs and isooctane. However, esterfication activity in isooctane and [Bmim] PF_6 were comparable [62]. Bornscheuer et al. [63] reported regioselective transesterfication of D-glucose with fatty acid vinyl ester in [Bmim] PF_6 and [Bmim] BF_4 −40% t-butanol biphasic system. Only commercial CALB enzyme attained 59% conversion rate. All other lipases showed much lower conversion efficiency. PEG-modified CALB, however, could increase the conversion to 90% in the same system [64]. Barahona et al. [65] presented influence of water activity on Novozym 435 catalyzed direct esterfication of geraniol in [Bmim] PF_6. At optimum water level ($a_w = 0.6$), decreased reaction rate was observed in IL than hexane. Esterfication of (±) methanol with propionic anhydride using CRL showed good yield and better enantioselectivity in [Bmim] PF_6 than hexane. Besides, incubation of enzyme in IL enhanced biocatalytic activity. Normally incubation of lipase in organic solvent reduced its activity [66].

The one pot reaction of pharmaceutically important precursor hydroxydihydrocodeinone was achieved by the combination of enzyme and chemical catalysis using [Bmim] PF_6. Integrating hydrogen bonding functional moieties such as hydroxyl group into [Bmim] PF_6 structure facilitates the homogeneous biocatalysis in IL. The capability of the solvent proved to be simultaneously dissolving the substrate, enzyme and the co-factor. The product codeinone is found to be considerably more stable in IL than in aqueous solution [67]. Novozym 435 catalyzed synthesis of glucose fatty acid ester using [Bmim] PF_6, [Bmim] Tf_2N, and [Bmim] TfO were also demonstrated. Supersaturated glucose solution with vinyl laurate showed higher conversion rate in mixture of ILs than pure ILs [68].

2.3. Acylation

ILs possessing BF_4 and PF_6 anions evidenced for higher acylation rate than other ILs. Lipase catalyzed acylation reaction was failed in 1-butyl-2,3-dimethylimidazolium ([Bdmim]) PF_6. On the other hand, acylation reaction was successful in [Bmim] PF_6 and [Bmim] BF_4. 2-Methyl group of the imidazolium ring can also play important role to maintain enzyme activity [69]. Same trend was observed with more rapid yield in [Bmim] PF_6 and 1-methoxyethyl-3-methylimidazolium ([Moemim]) PF_6 with CRL catalyzed acylation of methyl-6-O-trityl glucosides [70].

Salunkhe et al. [71] showed the role of organic base as an additive to improve the rate of enzymatic reactions in [Bmim] PF_6. Kinetic resolution of secondary racemic alcohol in [Bmim] PF_6 using PCL supported celite facilitates biphasic separation of the product with good enantioselectivity. Organic base restores PCL activity via removal acidic by-product. Enzymatic acylation of racemic secondary alcohols reaction was reutilized without significant loss in conversion efficiency. Irimescu et al. [34] confirmed CALB catalyzed acylation of two different amines in twelve ILs. Enzymatic reaction of 2-phenyl-1-propylamine showed superior reaction rate in [Bmim] PF_6 than other ILs. On the other hand, acylation of 1-phenylethylamine in [Bdmim] trifluoromethanesulfonate (Tfms) confirmed better result than [Bmim] PF_6. Theses outcomes justified that the IL suitable for the enzymatic reaction depends upon substrate–solvent–enzyme interactions and other factors.

Furthermore, one step enzymatic acylation of flavonoid glycosides such as naringin and rutin in [Bmim] PF_6 and [Bmim] BF_4 used for the preparation of lipophillic derivatives yielded higher amount of flavonoid. In addition, acylation showed higher regioselectivity than the conventional organic media [72]. N, O-enzymatic acylation 6-amino-1-hexanol was achieved with 97% substrate conversion in [Bmim] PF_6. On the other hand, efficiency is much lower in the case of [Bmim] dicyanamide ($N(CN)_2$). Hydrogen bond formation between substrate and [Bmim] cation and lower nucleophilicity boosts initial rate of product synthesis and shorten the reaction time in [Bmim] PF_6 than organic solvents [73]. Enzymatic acylation of (R,S)-1-phenylethanol with vinyl acetate was carried out in [Bmim] PF_6, [Bmim] BF_4 and [Emim] Tf_2N. Enzyme conformation was modified due to Tf_2N anion, which lowers efficiency and enantioselectivity of lipase. On the other hand, 50% conversion rate with enantiomeric excess >99% demonstrated the achievement of kinetic resolution reaction in [Bmim] PF_6 [74]. *Porcine pancreatic* lipase (PPL), *Candida cylindracea* lipase (CCL) catalyzed acylation of benzenesulfonamide derivatives were carried out in [Bmim] PF_6, [Bmim] Tf_2N and [Bmim] BF_4 with substrate to acylating agent ratio 3:1. Both of the two enzymes demonstrated complete conversion efficiency in ILs. Compared to aqueous system monoacylated sulfonamide showed 100% selectivity in IL [75].

2.4. Hydrolysis

Enzymatic hydrolysis of 3,4,6-tri-O-acetyl-D-glucal in [Bmim] PF_6, [Bmim] BF_4 and organic solvents have been reported. Hydrophobic IL showed 8 times higher yield than hydrophilic IL. Further, [Bmim] PF_6 exhibited much higher selectivity (98%) than [Bmim] BF_4 (59%) [76]. Likewise, hydrolysis of (R,S)-naproxen methyl ester in [Bmim] PF_6 offered 96% optical purity of (S)-Naproxen. Furthermore, yield in [Bmim] PF_6 is proficient than in isooctane, [Bmim] BF_4 and [Omim] PF_6 [77].

Thermodynamic water activity (a_w) is the useful parameter to analyze solvent influence towards biocatalytic reactions [78,79]. It is known that enzyme activity is determined by the water bound to the enzyme ('essential' water, a few monolayers of water), rather than the bulk water content in the system [80–83]. Ebert et al. [84] showed water activity is a predominant factor to carry out enzymatic reactions in ILs. Immobilized penicillin G amidase (PGA) is more stable when suspended in ILs with [Bmim] PF_6 and [Bmim] BF_4 even after 7 days of exposure. Higher initial reaction rate was observed in [Bmim] PF_6 (1.027–3 h) than [Bmim] BF_4 (0.813–24 h). However, gradual decrease in enzyme activity with the increase in the alkyl chain length of the imidazolium ring appeared in the case of [Omim] PF_6. Further, PGA is stable in [Bmim] PF_6 as compared to conventional aqueous solutions that are proved as a good media for hydrolysis of penicillin G. [Bmim] PF_6 and [Omim] PF_6 hydrophobic solvents require only 1% water to maintain water activity (a_w) of immobilized PGA, while 10, 5, 20 and 20% is required for hydrophilic [Bmim] BF_4, [Omim] BF_4, [Bmim] methylsulfate (CH_3SO_4), and [Omim] CH_3SO_4, respectively [85].

Kinetic resolution still has a prevailing role in the synthesis of enantiomerically enriched compounds without the addition of any co-factors. For example, the kinetic resolution of *R, S*-ibuprofen with 1-propanol using with CRL showed a 3-fold higher enantioselectivity and improved stability in [Bmim] PF_6 than conventional isooctane [86]. Furthermore, increased activity and stability of *Mucor javanias* lipase (MJL) pretreated with [Bmim] BF_6, [Bmim] BF_4, [Emim] BF_4, and [Emim] Tf_2N for the hydrolysis of p-nitrophenyl butyrate in aqueous medium was also observed [87]. Likewise, direct comparison of the lipase resolution revealed a 2-fold increase in initial rate when the reaction was performed in the [Bmim] PF_6 as compared with t-butanol [88].

2.5. Alcoholysis

Lipase-polymer complex catalyzed alcoholysis in [Bmim] PF_6 showed less initial rate than [Omim] PF_6 and 1-hexyl-3-methylimidazolium ([Hmim]) PF_6 [89]. PCL catalyzed alcoholysis of 3,4,6-tri-O-acetyl-D-glucal was reported using IL. [Bmim] PF_6 showed higher production rate than hydrophilic IL. Further, extend of product formation and selectivity in [Bmim] BF_4 also comparatively inferior to [Bmim] PF_6. Enzyme supported on celite enhanced the reusability of the lipase [76]. IL environment stabilized the enzyme–substrate complex, which might be the reason enzymatic efficiency in [Bmim] PF_6 is higher than organic solvent [90]. On the contrary, Kaar et al. [54] demonstrated that covalently linked PEG-lipase failed to improve lipase activity.

2.6. Oxidation

[Bmim] PF_6 and 4-methyl-N-butylpyridinium ([4-Mbp]) BF_4 used for oxidation reactions catalyzed with oxidative enzymes such as laccase C, horseradish peroxidase (HRP) and soybean peroxidase. In the case of anhydrous [Bmim] PF_6, IL fails to show appreciable enzyme activity. On the other hand, IL saturate with aqueous buffer displayed enzymatic activity towards oxidation of syringaldazine [91]. Laszlo and group [92] explained the possibilities of oxidation 2-methoxyphenol using hemin, cytochrome C, microperoxidase in [Bmim] Tf_2N, [Bmim] PF_6 and [Omim] PF_6. Addition of electron acceptor to medium enhanced the peroxidase activity. Compared to menthol and, DMSO solvents ILs showed superior catalytic activity of hemin and microperoxidase. In addition, glucose oxidase and peroxidase catalyzed oxidation of thioanisoles were also demonstrated in [Bmim] PF_6 [93].

Swatloski et al. [94,95] and Najdanovic-Visak et al. [96] demonstrated that [Bmim] PF_6 can be entirely soluble in the combination of IL, ethanol, and water. However, combinations of IL/water or IL/ethanol only form biphasic system. This aqueous ethanol (0.5–0.9 mole fraction of ethanol) system applied for elimination of [Bmim] PF_6 from products and vessel. Above 0.9 mole fraction, both ethanol and water form biphasic system, which can be used to purify IL. Water in [Bmim] PF_6 microemulsion can split yeast alcohol dehydrogenase (YADH) from IL, which increase the YADH activity. On the other hand, homogenous solution formed by the combination of [Bmim] PF_6, H_2O and C_2H_5OH decrease the YADH oxidation efficiency [97].

2.7. Polymerization

Only few reports showed IL application towards enzymatic polymerization. Polyester has been synthesized at room temperature and 60 °C with the help of PCL supports on celite in IL. Polycondensation of diethylene octane-1,8-dicarboxylate and 1,4-butanediol at room temperature gave only low molecular weight polymer. The molecular weight could be increased at 60 °C under vacuum. In addition, enzymatic preparation of polymer materials in IL exhibits prominent polydispersed molecular weight [98]. Conducting polymer, polyaniline has been synthesized using HRP enzyme immobilized in [Bmim] PF_6 [99]. Marcilla et al. [100] reported enzymatic synthesis of polyesters in [Bmim] PF_6, [Bmim] Tf_2N and [Bmim] BF_4. Ring-opening polymerization and polycondensation reactions were carried out using Novozym 435 [100,101]. Compared to previous studies [98], molecular weight and polydispersity of the polymer was improved. Reaction carried out with [Bmim] Tf_2N IL showed 92% conversion. In contrast, [Bmim] BF_4 and [Bmim] PF_6 formed polymer layer during ring opening polymerization. This polymer layer forms heterogeneous reaction mixture, which reduce the monomer conversion and polymer yields. Polycondensation of 1,4-butanediol with dimethyl adipate and dimethyl sebacate carried out in open and closed vessel under vacuum at 70 °C. All the ILs formed homogenous reaction mixtures yield higher molecular weight within 24 h. In addition, yields of polycondensation of 1,4-butanediol in an open system are similar to closed system. Further improvement in hydrophobic ILs can be employed to execute enzymatic polymer synthesis in an open pot.

2.8. Other important reactions

One of the easiest approaches to perform dynamic kinetic resolution (DKR) is the combination of the traditional enzymatic kinetic resolution with an *in situ* racemization of the substrate using a transition metal catalyst. This approach has been particularly demonstrated in the DKR of different secondary alcohols by combining a lipase catalyzed transesterification with a ruthenium catalyzed racemization with [Bmim] PF_6 and [Bmim] BF_4. In chemoenzymatic DKR in [Bmim] PF_6 media is more active than hydrophilic [Bmim] BF_4 [61,102–105]. Likewise, challenging 2 stages preparation of oxycodone from codeine have been demonstrated in [Bmim] PF_6 and functionalized [Bmim] PF_6 [106]. Carbon–carbon bond forming aldol reaction catalyzed by aldolase antibody 38C2 carried out in [Bmim] PF_6. Recover and reuse of the aldose-IL offers higher yield. However, solvents such as acetone, methyl ethyl ketone, methoxyacetone, fluoroacetone, and chloroacetone failed to maintain activity of aldolase antibody 38C2 [107]. Xerogel encapsulated substilisin catalyzed synthesis of di- and tri-peptides of alanine in [Bmim] PF_6 gave greater yield than in DMF-ACN mixture [108]. Most of the enzymes suspended as a powder rather than dissolved in [Bmim] PF_6 might be the reason for the significant higher activity. On the other hand, lower hydrogen bond bascity of PF_6 anion has less influence with the internal hydrogen bonds of catalytic site in the enzyme structure. Hydrophobic nature of [Bmim] PF_6 preserves the essential water molecules responsible for enzyme stability. In addition, fluorinated anion in [Bmim] PF_6

provide good environment against organic solvents and oxidizing agents. The enzyme compatible PF_6 anion spreading its negative charge over 6 fluorine atoms reveals lower hydrogen bond basicity and lower nucleophilicity which reduces its intervention with the internal hydrogen bonds of an enzyme. Eventually, the strong ion–ion interaction in ILs directs to ordered 3-D supermolecular networks, creating a complex and/or totally different medium environment than conventional solvents.

3. *In situ* by-products removal under reduced pressure and pervaporation

Non-volatile nature of ILs broadens the application of enzymatic reactions under reduced pressure condition. Strip off volatile reaction products such as water and alcohol under reduced pressure shifts the equilibrium towards desired reaction product. Itoh et al. [20] applied this method to remove methanol from enzyme catalyzed transesterfication, which overcame the inhibitory effect of an acetaldehyde oligomer [14,20]. Lipase (CALB) catalyzed transesterfication of 5-phenyl-1-penten-3-ol with nine different acyl donor exhibited in [Bmim] PF_6 under reduced pressure. Innovative finding suggests that methyl esters act as better acyl donor for lipase catalyzed transesterfication reactions. Particularly, reaction medium with methyl phenylthioacetate under reduced pressure gave excellent results as well as rate of enzyme transesterfication was well maintained throughout three consecutive runs. Further, Uyama et al. [21] proposed CAL catalyzed ring opening polymerization of ε-caprolactone and polycondensation of dicarboxylic acid diesters with 1,4-butanediol in [Bmim] PF_6 and [Bmim] BF_4 under reduced pressure. Lactone polymerization gave higher molecular weight polyester in hydrophilic IL at 60 °C. On the other hand, polycondensation reaction at ambient pressure showed lower molecular weight of the product. Reduced pressure atmosphere facilitates conversion efficiency and molecular weight of the polyester in both ILs. Likewise, Irimescu et al. [32] employed reduced pressure scheme to remove the water from enzymatic amide synthesis. CALB catalyzed acylation of 1-phenylethylamine and 2-phenyl-1-propylamine with carboxylic acid was confirmed in [Bmim] PF_6 and [Emim] BF_4. Both ILs exhibit superior enantioselectivity than non-solvent system. Dodecanoic acid was proved as a good acyl donor for ILs. On the other hand, 4-pendenoic acid showed poor activity for the acylation of 1-phenylethylamine. Likewise, initial acylation rate of 1-phenylethylamine with dodecanoic acid was diminished to half in [Emim] BF_4. Continuous removal of water by-product under reduced pressure improves reaction efficiency in [Bmim] PF_6.

Liquid–liquid extraction with organic solvents or $scCO_2$ was used to recover products of enzyme catalyst reaction in [Bmim] PF_6. As mentioned before, employing organic solvents for product recovery will be beyond green chemistry regulations. Pervaporation method used effectively to remove water from reaction system. In addition, pervaporation is a highly selective and versatile energy efficient process with a combination of membrane permeation and evaporation able to recover quantitatively the volatile solutes from ILs under mild operating conditions. Poly(octylmethylsiloxane), polyether block amide and poly(vinyl alcohol) proved excellent polymeric membranes for >99.2% recovery of water, ethyl hexanoate, chlorobutane and naphthalene from [Bmim] PF_6 [109]. Bélafi-Bakó et al. [110] also proved the success of pervaporation technique using [Bmim] PF_6, l-methyl-3-nonylimidazolium ([Nmim]) PF_6, [Bmim] BF_4 and [Hmim] BF_4. This technique successful removed water in the CRL catalyzed esterfication of 2-chloropropionic acid with butanol. Notably, conversion efficiency of lipase is higher in [Bmim] PF_6 than other ILs. Similarly, CRL catalyzed enantioselective esterfication of (*R, S*)-2-chloropropanoic acid with butanol was shown in [Bmim] PF_6, [Nmim] PF_6, [Bmim] BF_4, and organic solvents. Lipase activity in [Bmim] PF_6 was comparable to hexane and superior to other ILs, toluene, and THF. These examples clearly show the success of using pervaporation integrated enzymatic reactions in hydrophobic ILs [61,111].

4. Whole cell biocatalysis in [Bmim] PF_6

Whole cell biocatalysts reactions possessed significant advantages such as simple catalyst preparation and no need for co-factor addition [112]. Compared to enzyme catalysis considerably less reports on whole cell catalysis in [Bmim] PF_6 have been published. Cull et al. [10] reported the pioneering work of biotransformation in ILs using *Rhodococus R312*. 1,3-Dicyanobenzene was converted to 3-cyanobenzamide in a [Bmim] PF_6-water biphasic system. [Bmim] PF_6 was utilized in a biphasic system as substrate reservoir and/or for *in situ* removal of the product, thereby increasing the productivity of the whole cell reaction. Although the substrate is poorly water soluble, the desired product 3-cyanobenzamide can be further hydrated by the amidase yield the corresponding acid. Howarth et al. [113] compared yeast mediated ketone reduction in [Bmim] PF_6 with organic solvent and observed that the reactivity in IL varied considerably with the substrate structure. Further, whole cells in IL biphasic system exhibited less aggregation than water-toluene system [10,113,114]. *Geotrichum candidum* cell immobilized on water absorbing polymer demonstrated excellent activity towards asymmetric reduction of ketones [114]. Water immiscible ILs are suitable solvents for gram positive *Lactobacillus kefir*, gram negative bacterium *E. coli* as well as yeast *S. cerevisia* to carry out biotransformation. Whole cell biocatalytic reaction in a biphasic system as the cells stay intact and co-factor leaching can be prevented. Normally, density of the [Bmim] PF_6 is above 1.2 kg L^{-1} and viscosity is below 400 mm^2 s^{-1}, which secures effective phase separation and avoids mass transfer limitations [115–117]. Asymmetric synthesis of S-4-chloro-3-hydroxy butananote with yeast in [Bmim] PF_6 biphasic system increase the optical purity from 61.7% to 83.9% and chemical yield from 44.4% to 80.2% compared to n-butyl acetate [118].

The increase in initial activity up to 44% and higher partition coefficient in [Bmim] PF_6/buffer biphasic system than in n-hexane/buffer suggests that IL biphasic system effectively delivers the substrate to the aqueous phase. Eight continuous batch reactions immobilized cells yielded 81% product in [Bmim] PF_6 biphasic system, 63% in [Bmim] BF_4/buffer co-solvent, 51% in pure Tri–HCl buffer and 48% in n-hexane/buffer. This proved that [Bmim] PF_6 is a good media with operational stability and lower toxicity for the microbial cells than the organic solvent used [119].

Furthermore, Lenourry et al. [120] showed the applicability of [Bmim] PF_6 on a whole cell biocatalysis in the absence of mass transfer barrier (water soluble substrate and product) for the reduction of sodium caffeate to hydrocaffeate. Likewise, immobilized baker's yeast utilized for the reduction of ketones in [Bmim] PF_6 and water mixture (10%) was carried out without the addition of coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) [18]. Compared to [Bmim] PF_6, 1-butanol and N, N-dimethyl methanamide possess similar polarity but are notoriously toxic to the whole cells, which proves IL as an efficient medium than organic solvent with equivalent polarity.

Lou et al. [121] succeeded in the preparation of chiral, enantiopure organosilyl alcohol ((S)-1trimethylsilylethanol) via asymmetric reduction of acetyltrimethylsilane with immobilized *Saccharomyces cerevisiae* yeast cell using [Bmim] PF_6 and [Bmim] BF_4. The reaction in IL was more efficient than hexane and buffer solution. Additionally, the biphasic system can efficiently overcome the limitation of substrate and the product inhibition often observed during the reduction of carbonyl compounds in a monophasic system [122]. Asymmetric reduction of 4-methoxyacetophenone catalyzed

by recently isolated *Rhodotorula* sp. AS2.2241 cells was demonstrated in [$C_{2,\ 4,\ 6\text{-}7}$ mim] PF_6 and [$C_{2,4}$ mim] Tf_2N. Among these ILs [Bmim] PF_6 biphasic system showed good biocompatibility with cells, excellent initial rate and maximum substrate conversion. Likewise, operational stability of whole cells is well maintained in [Bmim] PF_6 [123]. IL might interact with the charged groups on substrate and cell membrane, causing changes in the ionic state of cell membrane, and making it easier for the substrate to permeate through the cell membrane [120].

5. Enzymatic reactions in IL/$scCO_2$

Separation or extraction of reaction product from the reactants and/or [Bmim] PF_6 is crucial situation in switching laboratory success into commercial process. Non-volatile nature of [Bmim] PF_6 has the advantage to recover volatile water and alcohol from IL using vacuum. However, aqueous solvents are not suitable for non-volatile or thermo-sensitive product recovery from ILs. The method employing organic solvents successfully recovered the challenging products from IL, but on the other hand it fails to fulfil the green enzymatic credentials. To retrieve the product through efficient green manner, the $scCO_2$ could be engaged for integrated biocatalysis and separation. Extraction/separation of products using $scCO_2$ was demonstrated in several commercial applications such as decaffeination, removal of lipids from bone, extraction of nicotine, hops, aromas, spices, red pepper, and extraction of pharmaceuticals from botanicals [124].

In addition, scCO2 is highly soluble in [Bmim] PF_6 [333.15 K and 10 MPa]. However, the solubility of the IL in scCO2 is very low. The $scCO_2$ forms two-phase systems with ILs such as [Bmim] PF_6, methyktrioctylammonium ([MTOA]) TFA, and [Omim] BF_4, which allows genuinely continuous process. Further, the electrostatic force between cation and anion in ILs avoids the solubility in $scCO_2$ [125,126]. Until 400 bar IL/$scCO_2$ biphasic system is maintained. As a result, this potential advantage can be used to extract products without IL contamination. High pressure phase behaviour of the [Bmim] PF_6-CO_2 system has been studied by many groups [127–133]. Further, scCO2 acts as co-solvent and/or antisolvent to extract soluble and thermally liable products from IL [134]. The combination of the IL and $scCO_2$ might withdraw their individual restrictions for commercially applicable non-aqueous biocatalytic process. The product recovery from IL may turn into more complex, rely on the solubility of the product in [Bmim] PF_6 and boiling point of the product. However, the product isolation from $scCO_2$ is much easier. Pioneering effort was demonstrated by Blanchard et al. [125] for extraction of naphthalene, aromatic, and aliphatic compounds from [Bmim] PF_6 using $scCO_2$. Following the same route three-phase system containing [Bmim] PF_6-$scCO_2$-orgainc solvent was also demonstrated [126,135]. Since 1985, use of supercritical fluids as non-aqueous solvents for biocatalytic reactions was well known [136,137]. Tuneable nature is the key characteristic of the $scCO_2$. Slight change in pressure and temperature directs significant alteration in density of $scCO_2$, which can affect solvent property.

Until recently, little effort was furnished for enzymatic reaction using [Bmim] PF_6/$scCO_2$. Laszlo et al. [16] proposed α-chymotrypsin catalyzed transesterfication of N-acetyl-L-phenylalanine ethyl ester with 1-propanol was demonstrated using dry $scCO_2$ and biphasic system. [Bmim] PF_6/$scCO_2$ system achieved higher yield and faster turnover than [Omim] PF_6/$scCO_2$ system. Addition of 1% water doubled the enzyme efficiency and transesterfication rate in [Bmim] PF_6. In addition, without contamination of the IL N-acetyl-L-phenylalanine propylester was successfully recovered from reaction system. An interesting new approach for enzymatic kinetic resolution of enantiomer separation of racemic phenyl secondary alcohol using [Bmim] PF_6 medium offered superior enantiomer separation, high enantioselectivity and pronounced long term stability. Likewise, the kinetic resolution of rac-1-phenyl ethanol in IL/scCO2 and IL/hexane demonstrated with 12 different kinds of silica supported lipases. Lipase coated with Tf_2N based ILs decreased the enzyme activity in kinetic resolution of rac-1-phenylethanol [138]. Racemization reaction was doubled in [Bmim] PF_6 as compared with other ILs and organic solvents. Under the condition of DKR at 40 °C and 100 MPa of $scCO_2$ enantiomeric selective synthesis provided an excellent yield of 78% R-1-phenylethyl propionate with 97.4% optical purity. Studies have shown that [Bmim] PF_6/$scCO_2$ were the excellent methodology for racemization of the undesired enantiomer into target product. ILs coated lipase reactions system overcome the mass transfer limitation for substrates and products in $scCO_2$ [139]. Higher turnover and product yield chymotrypsin catalyzed transesterfication demonstrates potential of [Bmim] PF_6/$scCO_2$ system.

Reetz et al. [24] showed a process to carry out enantiomer separation using lipase catalyzed esterfication of chiral secondary alcohol followed by supercritical extraction using [Bmim] PF_6/$scCO_2$. This continuous process offered excellent results. Extraction of both chiral alcohol and ester was achieved with a combination of continuous flow and kinetic resolution of racemic secondary alcohol using biphasic system. Extraction was achieved with more than 99% of product purity and with 97% ee of alcohol and ester [24].

Aqueous solution of CALB dissolved in ILs showed excellent transesterfication efficiency in $scCO_2$. Moreover, enzyme dissolved in [Emim] Tf_2N and [Bmim] Tf_2N with $scCO_2$ system showed good stability and reactivity even at higher temperature. Outcome of these studies revealed that ILs with lipophillic non-coordinating anions is the best choice of enzymatic catalysis with $scCO_2$. As [Bmim] PF_6 provides an excellent environment for enzymatic reactions and the IL immobilized support acts a protective shield against denaturation of enzyme from $scCO_2$ [140].

Lipase catalyzed acylation of octan-1-ol by vinyl acetate in [Bmim] BTA/$scCO_2$ biphasic system has also been reported by Reetz et al. [141]. Excellent activity and product purity were observed in batch and continuous enzymatic biphasic process. In the same manner, kinetic resolution of rac-1-phenylethanol with vinyl propionate in IL/$scCO_2$ using lipase showed excellent outputs in continuous biocatalytic process. Even at higher temperature enzyme activity and selectivity are well preserved [142].

ILs such as [Emim] BTA, [Bmim] BTA, [Emim] Tf_2N and, [Bmim] Tf_2N with $scCO_2$ system have also been recently proposed to carry out biocatalytic transesterification reactions and kinetic resolution of 1-phenylethanol under extreme deactivating conditions. IL-$scCO_2$ biphasic system increases the selectivity of CALB immobilized with α-alumina membrane into >99.5%. The immobilization support may be due to multipoint enzyme–IL interactions [140–143]. Hernández et al. [144] proposed butyl propionate synthesis from vinyl propionate and 1-butanol in $scCO_2$ and IL/$scCO_2$ biphasic system using dynamic membrane with immobilized CALB. Butyl propionate formation was showed 95% selectivity and good enzyme activity in $scCO_2$ (50 °C and 80 bar). On the other hand, same reaction with $scCO_2$/ILs (hydrophobic ILs) biphasic system showed reduced enzyme activity. However, selectivity of butyl propionate in IL/$scCO_2$ biphasic model is higher than $scCO_2$. Restriction in the mass-transfer phenomenon across the IL-layer around the lipase might be the reason for lower activity in biphasic system.

Synthesis of glycidyl esters was successfully carried out in IL/$scCO_2$ system. Lipases (CALA and CALB) showed higher activity in [Bmim] PF_6, [Emim] Tf_2N, and [Bmim] Tf_2N. Biphasic system contains trioctylmethylammonium ([Troma]) IL slightly reduced biocatalytic activity. On the other hand, enantioselectivity of enzyme was well maintained in IL/$scCO_2$ [145]. Infra-red spectroscopy data of [Bmim] PF_6/CO_2 indicate that IL forms weak Lewis acid–base complex which might increase the conductivity

[126]. Even though [Bmim] PF_6 is a low molecular weight IL, its phase behaviour with CO_2 is similar to that of a cross-linked polymer solvent system [126]. In brief, $scCO_2$ is a superb solvent for extract/recover hydrophobic compounds from [Bmim] PF_6. The dissolution of CO_2 in [Bmim] PF_6 decreases viscosity of the solution, which facilitates reaction. Appreciable amount of water dissolved in [Bmim] PF_6 decreases CO_2 solubility in IL. In addition, water impurities react with CO_2 forms carbonic acid, which may be influence reaction efficiency. Ultimately, enzyme-IL-$scCO_2$ reaction activity and specificity depends on the substrate solubility, nature of the enzyme, impurity, and water content in the IL. This must be the reason for changed and/or diminished enzyme activity [142,146].

6. [Bmim] PF_6 as an immobilization support

Enzymes suspended in ILs gave comparatively low activity. Likewise, enzyme dissolved in ILs with small amount of water influence conformational state. However, enzyme immobilized onto supports has favoured advantageous and repetitive use over free enzyme. For example, PEG modified enzymes such as lipase showed higher activity and enantioselectivity in [Bmim] PF_6 [147]. Various approaches are reported to enhance enzyme activity in an alternative solvent system. Instead of using existing immobilization techniques, native enzyme suspended in [Bmim] PF_6 offered novel immobilized or anchored enzyme with excellent activity and stability [148,149]. Interestingly, 7-day exposure of MJL in [Bmim] PF_6 showing 85% activity over untreated lipase proved the efficiency of IL pre-treated enzyme. Traditionally, biocatalyst efficiency can be regulated through additives [150]. Itoh et al. [151,152] proved that functionalized imidazolium based ILs with PF_6 and BF_4 anions utilized as additives for well known lipase catalyzed transesterification reaction using diisopropyl ether. The flexibility of the lipase in additive medium improved the enantioselectivity. Studies also evidenced the suitability of this new immobilization approach. Medium containing small quantities of [Bmim] PF_6, [Omim] PF_6, [Emim] BF_4, [Omim] BF_4, [Emim] Tf_2N, and [Omim] Tf_2N were used as additives, which gave excellent stability and higher activity than lipase immobilized without ILs [67]. From this observation it is evident that [Bmim] PF_6 behaves as stabilizer to protect the enzyme. Hydrophobic nature of [Bmim] PF_6 acts as an effective co-solvent and recycle medium for lipase catalyzed hydrolysis of butyl 2-(4-chlorophenoxy) propionate in aqueous buffer [153]. In a similar manner, imidazolium cation holds long alkyl chains strengthened the stability of immobilized enzyme in hydrolysis and esterfication reactions [154]. Furthermore, lipase sol–gel immobilized HRP using IL as an additive showed a 30-fold increase in activity. In the same manner, Lee et al. [155] proposed a PF_6 anion based IL coated lipase-catalyzed transesterification reaction. IL coated enzyme showed enhanced enantioselectivity without losing any activity after five consecutive runs as compared with reaction in toluene. Waller et al. [156] demonstrated that [Bmim] PF_6 is less effective additive than functionalized hydrophilic ILs in pig liver esterase catalyzed hydrolysis of diethyl 2-phenyl-2-methyl malamute. This finding is contrary to the other enzymatic reactions carried out in the [Bmim] PF_6 as a co-solvent or additives.

7. Extraction of biological compounds using [Bmim] PF_6

[Bmim] PF_6 biphasic systems have been used as to separate many biologically important molecules such as carbohydrates [157], amino acid [158] organic acids, butyl alcohol, and polypeptide antibiotic erythromycin [11]. Extraction of metal ions, including alkali, alkaline earth, heavy, and radioactive metals by [Bmim] PF_6 are also well studied [159,160]. Back-distillation was used as a convenient technique to recover the extracted volatile products from IL [161]. Direct extraction of double stranded DNA by using [Bmim] PF_6 has also been reported by Wang et al. [162]. [Bmim] PF_6 also proved as the suitable solvent for the extraction of antibiotics such as erythromycin. Study showed that an equal partition coefficient for antibiotic as in butyl acetate at pH 5–9. Reduced partition coefficient at higher pH (pH > 9) helps to recover the macrolide antibiotic [11]. ILs have been demonstrated as potential extractants in the recovery of butyl alcohols from fermentation broth [163]. Likewise, a unique three-phase extraction system of oil (top), H_2O_2 plus water (middle), and [Bmim] PF_6 (bottom) was developed to remove sulfur containing compound from light oils with a combination of chemical oxidation and solvent extraction. This approach leads to a much higher desulfurization rate [164]. Water/sodium bis (2-ethylhexyl) sulfosuccinate/[Bmim] PF_6 reverse microemulsion selectively extract hemoglobin from human whole blood. Applying stripping agent (urea), 73% isolated hemoglogin was back extracted into aqueous phase [165].

8. Factors affecting enzyme activity in [Bmim] PF_6

Generally kosmotropes are order makers made up of small or multiple charged anion or cation with high charge density whereas chaotropes are disorder makers. For example, PF_6 and BF_4 anions are known as 2 of the most lyotropic and chaotropic anions that disrupt the overall water structure. As per the kosmotropes–chaotropes principles, PF_6 anion is more chaotropic than BF_4 anion and, it could destabilize the enzyme. However, under several circumstances proteases and other enzymes are deactivated directly by IL containing BF_4 anion where as enzymes maintain the excellent activity in IL with PF_6 anion [13,15]. Thermodynamic and viscosity studies revealed that [Bmim] cation might be a stronger kosmotrope than [Emim] cation. Results of B-coefficient measurement show [Emim] cation (0.491 $dm^3\ mol^{-1}$) as a weak chaotrope and [Bmim] cation (0.610 $dm^3\ mol^{-1}$) as a borderline ion or kosmotrope [166–169]. Moreover, synthesis of [Bmim] Cl is much easier than [Emim] Cl. However, other study pointed out that higher cation size of 1,3-alkylimidazolium, giving a higher kosmotropicity of cations [170]. It indicates that [Bmim] cations have stronger interaction with water molecules. However, numerous studies show the strong kosmotropic IL cation interaction with enzyme enhanced its efficiency. Anions possessing higher limiting Walden products such as PF_6, BF_4, I and Br anions are entitled chaotropes, while Cl anion is borderline ion. The PF_6 anions and BF_4 anions are belongs to lyotropic and chaotropic anions [171–173]. Notorious chlorine ion belongs to the border line in kosmotropic series, which has less power to destabilize the enzyme [174,175]. Zhao et al. [36,176] proposed that small size chaotropic cations might stabilize the protease, while large size kosmotropic anions might destabilize enzyme. Effect of ions on enzyme in aqueous solution is entirely opposite to this result. Finally, it can be concluded that the balance between kosmotropic and chaotropic ions, nature of the enzyme, and water content will be important factors determines the enzyme active in particular ILs.

The preparation of the [Bmim] PF_6 involves the formation of [Bmim] cation and anion exchange. The un-exchanged halides ions (Cl/Br/I) can slow down and/or hinder the enzyme activity [18,26]. Particularly, chlorine will be the most influential anion to affect the enzyme even when present in minute quantity in [Bmim] PF_6. High amount of chloride anion in IL affect enzyme like high concentrated salt and it might affect the integrity of the secondary structure of enzyme [177]. To overcome these difficulties, Park et al. [18] used silica gel to remove the chloride ions. Instead of using commercial [Bmim] PF_6, most of the research groups preparing their own IL. This diverse methodology to prepare [Bmim] PF_6 might be the main reason for the fluctuation in performance of different enzymatic reactions [16,45,178,179].

Water solubility in solvent is a practical variable for the selection of particular solvent system [180,181]. The water saturation limit of [Bmim] PF_6 is relatively high at 23,000 ppm [182,183]. Water solubility in [Bmim] PF_6 depends upon electron pair donating and accepting ability. In addition, water is crucial for enzymes in the solid as well as in the solution form because it influences the enzyme structure via non-covalent bonding and disruption of hydrogen bonds [184]. Similarly presence of polar co-solvent such as water with IL decreased the synthetic activity very rapidly [185]. The amount of water needed is specific to each solvent–substrate–enzyme system. Excess water in reaction system favours towards hydrolysis. However, insufficient water inactivates and/or destabilize enzyme. In many cases a monolayer of water on the enzyme surface is often sufficient to support the activity and prevent denaturation of the enzyme [186,187]. It is worth noting that water-immiscible ILs are hygroscopic and dissolve up to 1% of water. Moreover, the solubility of water in [Bmim] PF_6 will be unclear. Till date, there is no agreeable way to predict the enzyme activity and stability in [Bmim] PF_6. However, earlier studies and reviews specified a collection of cases that enzyme could preserve its activity and stability in [Bmim] PF_6. Hydrophobic solvents have a lesser tendency to remove the essential water from the enzyme surface [188–190]. Moreover, extremely polar and hydrophilic ILs interacts with the amount of water which is indispensable for maintenance of the catalytic conformation of the enzyme [47]. Even though [Bmim] PF_6 possessing high polarity does not trigger off enzyme activity like those conventional polar organic solvents with similar polarity. Enzymes can retain an essential shell of water to remain active in ILs. Sometimes the presence of trace amount of water and chloride produce deteriorating effect on the enzyme activity. Most of synthesis procedures start from [Bmim] chloride or bromide, in which enzymes are inactive. If halide exchange with the PF_6 anion is incomplete, the remaining Cl/Br anions may diminish or eliminate enzymatic activity.

Halling and group [79,180,181,191,192] recommend the employing a_w parameter as a replacement of water content to give an account of the nature of water in a non-aqueous solvent. For example, very low a_w is sufficient for some lipases, which can maintain excellent enzymatic activity even in extreme conditions. On the other hand, role of water activity parameter towards biocatalyst enantioselectivity was also unsteady [193–195]. Influence of this parameter in [Bmim] PF_6 was investigated only in few reports. Consequently further examinations must complete to describe impact of a_w in biocatalytic reactions in ILs. In addition, presence of water may support the mobility or flexibility or structural changes of biocatalyst. Obviously, these small changes play important role in enzyme activity [191,196]. Likewise, viscosity changes may affect enzyme conformation, which can influence the activity [197].

As a new research area, a lot off efforts are required to understand how to choose the suitable IL for specific biotransformation reactions. In addition, enantioselectivity is solely depending on the specific solvent–enzyme interaction rather than by physicochemical properties of the solvent [198]. In non-aqueous media, enzymes are heterogeneous with respect to the solvent. Due to this property, enzymatic reaction is influenced by external mass transfer and internal mass transfer. The rate of mass transfer depends on factors such as physical properties of solvent and enzyme powder morphology. Although polar organic solvents inactivate enzymes, polar ILs does not interfere like conventional solvents. However, the influence of impurity in reactivity and selectivity will vary between different enzymes. DuPont and group [199,200] demonstrated that 1,3-dialkylimidazolium ILs such as [Bmim] PF_6, [Bmim] BF_4 and [Bmim] Tf_2N are hydrogen bonded supramolecules, which could form nano-structure with solid, liquid and solution states. These nano-structure arrangements might play important role to preserve essential water of enzyme. To summarize, existence of the water in an [Bmim] PF_6 will be troublesome for certain biocatalytic reactions, but not for other reactions.

Overall, ILs are still lagging behind than that of the conventional aqueous and organic media. The only replacement of organic solvents with [Bmim] PF_6 and/or $scCO_2$ might not create the excellent green route for the preparation of enantio pure compounds. The familiarity of non-aqueous medium holding [Bmim] PF_6 is still under early stage and not well explored like organic solvents. The entire progression ought to be bear in mind to decide the beneficial scheme, because prediction could be diverge with the nature of ILs counter-ion. Furthermore, parameters such as reaction temperature, water content, and immobilization of the enzyme, operational stability and co-solvent will also influence the selectivity and productivity of the desired reaction in the [Bmim] PF_6. Minimum water content requiring for optimum enzyme activity in [Bmim] PF_6 depends upon enzyme, support and reaction type as well.

9. Challenges and future outlook

Recent findings claim the drawbacks of using ILs with fluorinated anions. Instability of PF_6 anion of [Bmim] PF_6 forms hydrolysis products in the presence of water or contact with moisture. In addition, hydrolysis of fluorine based ILs can release HF and POF_3, which might be harmful for biological and/or biocatalytic process [201–203]. In the same manner, white fume produced during IL preparation also toxic and corrosive nature [204,205]. Likewise, unreacted precursor [Bmim] X might increase the cytotoxicity [206,207]. These findings raised doubt whether PF_6 anion based ILs are really alternative and/or green solvents. Further, [Bmim] PF_6 is relatively manyfold expensive than commonly used volatile organic solvents. Extensive understanding of the physico-chemical properties of [Bmim] PF_6 requires to emphasis the nature of IL, which might be useful to establish large scale biocatalytic applications. Another notable disadvantage is disposal of PF_6 anion based ILs. To overcome, either IL supplier might get back the used [Bmim] PF_6 or proper economic feasible recycle methods should be established. Industry scale use of ILs demands sufficient biodegradability and toxicity data. To solve these problems require lengthy and costly life cycle analysis of IL [208]. To fulfil the solvent chemistry scope requires the usage of non-toxic pharmaceutically acceptable ions, which can develop environmental friendly potential IL. In addition, Klembt et al. [209] summarized the seven significant guidelines for biocatalytic reactions in ILs. These key concepts have to be analyzed thoroughly to use biocatalysts in IL. Familiarity of these reaction parameters and numerous improvements in other ILs will direct to fertile future for the preparation of a desired enantiomerically pure commercial product in an environmentally safe and economically viable manner.

Abbreviations

Enzymes—BCL: *Burkholderia cepacia* lipase; CAL: *Candida antarctica* lipase; CCL: *Candida cylindracea* lipase; CRL: *Candida rugosa* lipase; Cyt-C: Cytochrome C; HRP: Horseradish peroxidase; MDH: Morphine dehydrogenase; MJL: *Mucor javanicus* lipase, MML: *Mucor miehei* lipase; MP: Microperoxidase; PAL: *Pseudomonas aeruginosa* lipase; PCL: *Pseudomonas cepacia* lipase; PFL: *Pseudomonas fluorescens* lipase; PGA: Penicillin G acylase; PPL: *Porcine pancreatic* lipase; PSL: *Pseudomonas* sp. lipase; ROL: *Rhizopus oryzae* lipase; SBP: Soybean peroxidise, TLL: *Thermomyces lanuginosa* lipase; α-Chy: α-Chymotrypsin.

Ionic liquids—BF4: tetrafluoroborate; PF6: hexafluorophosphate; Tf2N: bis (trifluoromethanesulfonyl) imide; [Bdmim]: 1-butyl-2,3-dimethylimidazolium; [Bmim]: 1-butyl-3-methylimidazolium; [Emim]: 1-ethyl-3-methylimidazolium; [Omim]: 1-octyl-3-methylimidazolium.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2009.03.008.

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