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Review Paper

Enzymatic Bioconversion in Non-conventional Media

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Abstract

One strategy for optimizing biocatalysis for the production of flavors compounds, oleochemicals and drug intermediates in pharmaceutical industries is to use non-conventional media, such as non-aqueous heterogeneous systems. In this article, we highlight some of the current trends in biocatalysis in systems, focusing on organic solvent systems, reverse micelles and supercritical fluids. This review also summarizes recent applications of ionic liquids (ILs) as 'green' solvents in biocatalytic transformations of commercially important compounds, extractions of a variety of substances, including metal ions, organic and bio-molecules, organosulfur from fuels and gases. For more effective separation of products from ILs, they could also be used along with another 'green' technology, supercritical fluid extraction. In addition to their environmentally benign characteristics, ILs have other favourable properties such as hydrophobicity, polarity and selectivity over organic solvents.

Keywords: Ionic liquid, supercritical fluid, reverse micelle, green solvent, lipase.

Introduction

Enzymes need a small amount of water to retain their active three-dimensional conformational state, even when the enzyme is covalently bound to a support. Water contributes to the structural integrity, active site polarity and protein stability. It provides hydrophobic interactions with polar residues on the enzyme molecule, which would otherwise be interacting with each other, creating an incorrect conformational structure. Water can also limit the solubility of hydrophobic substrates around the enzyme. In reactions where the substrates are poorly soluble in water or water is formed as a product, the reaction yields in aqueous system are generally low.

The search for new reaction systems with favorable conditions led to enzyme catalysis in non-conventional media. In biocatalysis, the term non-conventional media refers to systems that are solvents other than water. Non-conventional media can be used for biocatalysis with enzymes. In non-aqueous media, the structures of enzymes are more rigid which increased the stability of the enzyme[1-3]. The use of enzymes in organic solvents can offer advantages such as improved substrate specificity, stereoselectivity, recoverability and low solubility of enzymes[4-6]. Non-conventional media are of special interest for hydrolases since low water contents can be used in order to favour synthesis reactions, as in case of lipases a large-scale use of solvents able to dissolve hydrophobic solvents to obtain high productivity[7]. In organic media, hydrophobic enzymes such as lipases can be usefully employed to carry out synthetic reactions such as esterification and transesterification reactions since the equilibrium position of the reaction is shifted sufficiently to give a high yield of the synthesis product[8].

Organic solvents may also help to keep a low thermodynamic water activity, which then decrease the thermodynamic barrier for reactions such as the esterification or hydrolysis reaction[9]. Higher conversion yields in non-aqueous systems simplify product recovery. The low solubility of enzymes in organic solvents decreases the amount of enzyme loss via desorption from support material. This enables the use of simple enzyme immobilization techniques such as adsorption which lowers the cost of enzyme preparation[10].

Strategy for Biocatalysis in Non-Conventional Media

Dispersed System: Enzymes used in organic media require some water to achieve good catalytic activity[11]. In esterification reactions, the initial activity of enzyme exhibits an optimum value at certain water content in the reaction media[12]. The enzymes do not dissolve in most of the commonly used organic solvents and hence catalysis in general takes place in dispersed media. Successful biocatalysis in non-conventional media has been reported in the main application for ester and peptide synthesis, the resolution of chiral building blocks. Despite the advantages of biocatalysis in organic solvent based system, enzyme stability is lower in hydrophilic solvents (-2.5 <logP<0), such as acetone and ethers than in hydrophobic solvent (2<logP<4) such as hexane, heptane or haloalkanes. Hydrophobic solvents do not strip off the essential water layer that stabilizes the biocatalyst while hydrophilic solvents distort this water from the enzyme surface, leading to unfolding of the molecule [13-16]. On the contrary, solvent toxicity is a problem for many applications. However, for ester synthesis, the major interest is still in organic liquid solvent systems[17-37] represented in table-2

Table-1
Nomenclature

S.No.	Formula	Nomenclature
1.	[BMIM][BF_4]	1-butyl-3-methylimidazolium tetrafluoroborate
2.	[BMIM][BF_6]	1-butyl-3-methylimidazolium hexafluoroborate
3.	[BMIM] [PF_6]	1-butyl-3-methylimidazolium hexafluorophosphate
4.	[OMIM][PF_6]	1-octyl-3-methylimidazolium tetrafluorophosphate
5.	[BMIM][CF_3SO_3]	1-butyl-3-methylimidazolium triflate
6.	MTBE	methyl *tert*-butylether
7.	[BMIM][NTf_2]	1-butyl-3-methylimidazolium bis (trifluoromethyl) - sulfonyl) amide
8.	[BMIM][dca]	1-butyl-3-methylimidazolium dicyanamide
9.	[BDMIM][BF_4]	1-butyl-2,3-dimethylimidazolium tetrafluoroborate
10.	[MOEMIM][BF_6]	1-methoxyethyl-3-methylimidazolium hexafluoroborate
11.	[HMIM][BF_4]	1-hexyl-3-methylimidazolium tetrafluoroborate
12.	[BMIM][$OCSO_4$]	1-butyl-3-methylimidazolium 3-octylsulphate
13.	[EMIM][TfO]	1-ethyl-3-methylimidazolium
14.	CALB	*Candida antarctica* lipase B
15.	CCL	*Candida cylindracea* lipase
16.	PPL	*Porcine pancreas* lipase
17.	PCL	*Pseudomonas cepacia* lipase
18.	CRL	*Candida rugosa* lipase
19.	MML	*Mucor miehei* lipase
20.	RML	*Rhizomucor miehei* lipase
21.	CLEA	Cross-linked enzyme aggregate
22.	PFL	*Pseudomonas fluorescens* lipase
23.	CSL	*Candida species* lipase
24.	RDL	*Rhizopus delemer* lipase
25.	MJL	*Mucor javanicus* lipase
26.	TLL	*Thermomyces lanuginose* lipase
27.	AOT	Sodium bis(2-ethylhexyl)sulfosuccinate
28.	BHDC	Benzyl-n-hexadecyldimethyl ammonium chloride

Supercritical Fluids (SCF): The use of enzymes in non-conventional media such as supercritical fluids (SCF) has been proposed as a means of improving the activity and utility of such enzymes in anhydrous environments[38]. Supercritical fluids are mainly carbon dioxide, freons, hydrocarbons or inorganic compound such as SF_6, N_2O etc. The most commonly used system is supercritical carbon dioxide ($SCCO_2$) which is because of its critical point of 73.8 and 31.1^{0}C makes equipment design and reaction set-up relatively simple[39]. The high diffusivities and low viscosities in SCFs greatly enhance mass transfer of substrates to the enzymes. Solubilization of substrates is generally better compared to that in organic liquid solvents. With the absence of organic solvents, higher purity products can be achieved. Since many ester products are used in food, the favourite choice of supercritical fluid for ester production is carbon dioxide.

In these systems, some enzyme activity may be lost during the depressurization of the reaction mixture. The use of high pressure vessels complicates continuous production and scale up. Studies on ester synthesis reactions in supercritical carbon addition, the use of supercritical carbon dioxide can have dioxide have resulted in mixed opinions on the economic viability of such systems compared to organic liquid systems[40]. Separation of products from reactants can be greatly facilitated by the ease with which the solvent power of the SCFs can be adjusted.

In supercritical fluids, a small change in pressure or temperature near the critical point may result in great change in its viscosity and of the diffusivitiy and solubility of compounds dissolved in it. This may leds to control of the rate and enantioselectivity of enzyme catalysed reactions. In enzymatic reactions, in a very limited pressure range near the critical point, interaction between carbon dioxide and enzyme molecules greatly increased with consequent conformational changes in the enzymes, causing active sites to emerge to catalyse stereoselective synthesis. The main advantage of use of supercritical fluids such as carbon dioxide, it is non-toxic, chemically inert and can be removed easily after the reaction. Further, in SCFs due to high diffusion rates, it facilitates the transport phenomena and can increase the bioconversion rate. The high diffusion rate can also facilitate product separation. In adverse effects on enzymes by decreasing the pH of the

.**Table-2**
A literature survey of enzyme catalysed reactions in organic solvent media

Enzyme	Solvent	Reaction	Ref.
PCL	Various organic solvents	Transesterification reaction between 2-*O*-benzyl alcohol and vinyl acetate	17
CRL	cyclohexane	Resolution of (±) 2,6-dimethyl-1,7-heptandioic acid with n-butanol	18
CALB	*n*-hexane	Esterification of lauric acid and isopropyl alcohol	19
CALB	none	Transesterification reaction between ethyl lactate and butanol	20
CALB	*n*-hexane	Reaction between acetic anhydride and isoamyl alcohol	21
CRL and MML	number of organic solvents	Transesterification reaction between *sec*-phenetyhl alcohol and vinyl butyrate	22
Immobilzed CRL	*n*-hexane	Esterification reaction between isobutyric acid and n-butanol	23
CAL, P. species, CRL	*n*-heptane	Esterification reaction between tetrahydrofurfurl alcohol and butyric acid	24
Immobilized RML	*n*-hexane	Esterification reaction between 9,10-dihydroxy steric acid and 1 -octanol	25
Novozym-435	*n*-heptane	Transesterification reaction between vinyl acetate and *n*-octanol	26
Novozym 435	Various organic solvent	Esterification of lactic acid and glycoside	27
CLEA-Subtilisin Carlsberg	Several organic solvents	Reaction between *N*-acetyl-L-tyrosine ethyl ester and 1-propanol	28
PPL	Various organic solvents	Esterification of lauryl alcohol with lauric acid	29
MML	-do-	Esterification of iso-amyl alcohol with acetic acid	30
Novozym-435	Several organic solvents	Synthesis of lauryl palmitate	31
Novozym-435	acetronitrile	Synthesis of erythrobyl laurate	32
Novozym-435	Solvent free system	Transesterification between ethyl acetate and cinnamoyl alcohol	33
Novozym-435	toluene	Synthesis of cinnamyl acetate	34
Rhizopus Oryzae	Bi-phasic solvent system	Esterification of tuna fish oil fatty acids with butanol	35
Immobilized CRL	Bi-phasic solvent system	Esterification of lauric acid with butanol	36
CAL	Solvent free system	Transesterification of isoamyl alcohol with acetate anhydride	37

microenvironment of the enzyme and by the formation of carbamates due to covalent modification of free amino groups at the surface of the protein[40,41]. Hence, supercritical carbon dioxide medium in the near critical region should trigger the activation of the enzyme by causing movement of its surface groups and creating active sites[42]. Furthermore, supercritical carbon dioxide is an excellent solvent for non-polar organics. The main limitation of use of carbon dioxide as an analytical extraction solvent is that its polarity is too low to obtain efficient extraction of products, because the analytes lack sufficient solubility or the extractant has a poor ability to displace the analytes from active matrix sites. The use of this type media have been mainly observed for reactions catalyzed by hydrolases, especially lipases[43-52] given in table-3.

Reverse Micelles: The low water content necessary to favour the synthesis reactions in organic solvents by enzymes can be achieved by micro-encapsulation of the biocatalyst within reverse micelles. In the presence of a certain surfactant in a suitable concentration, reverse micelles form ordered structure readily upon the addition of a small amount of water to a water immiscible hydrophobic solvent[53]. Reverse miceller systems offer a very large aqueous/ organic solvent interface of approximately 100 m^2mL^{-1} of micro emulsion. Due to dynamic structures, the micelles can exchange components between each other and also with bulk organic solvent. This system is highly applicable for biocatalysis because it mimics the natural environment that many enzymes experience within cells.

The AOT (Sodium bis 2-ethyl hexyl) sulfosuccinate/Isooctane system is one of the most suitable systems for enzymatic catalysis[54-56]. Since the reverse micelles formed by this surfactant are very stable over a wide range of concentrations in the absence of co-surfactants. The reversed micellar AOT system is particularly interesting for hydrolases such as lipases, which provide a high interfacial area of contact with the enzyme anchoring at the aqueous side of the AOT interface. Further, lipase catalysed reaction enables the use of hydrophobic substrates, which are readily soluble in the bulk organic phase[57]

Table-3
Reactions catalyzed by enzymes in supercritical fluids

Enzyme	Reaction	Remarks	Ref
Free and immobilized RML, PFL	Synthesis of ethyloleate	Economy of the process is given in this paper	43
Immobilized MML	Hydrolysis of blackcurrant oil	Simultaneous extraction and hydrolysis of the oil	44
Immobilized CALB	Synthesis of butyl butyrate	Combines SCF with membrane technology	42
PCL	Transesterification reaction between 1-phenylethanol and vinylacetate	Selective towards one isomer of 1-phenyl ethanol, higher reaction rate	45
Immobilized CALB,RML	Synthesis of isoamyl acetate	Higher initial rate in $SCCO_2$	46
Immobilized CALB	Synthesis of butyl laurate	Productivity dependent on the substrate amount, catalyst remain active after five cycles	47
CLEA-CALB	Esterification of isoamyl alcohol with acetic acid	Initial activity of the enzyme decreased with increase in pressure, highly stable in $SCCO_2$	48
Immobilized RML	Esterification of oleic acid with 1-octanol	Obtained about 93% yield in a continuously operating bioreactor, higher than those obtained In batch mode	49
CRL	Regioselective acylation of methyl-6-*O*-Trityl β-*D*-glucopyranoside with vinylacetate	Obtained 91.4% final conversion	50
Novozym-435	Synthesis of butyl butyrate	Reaction followed Ping-Pong Bi-Bi mechanism	51
Novozym-435	Kinetic modeling of decyl acetate	Enhanced initial rate at 35^0C and 100 bar	52

In reverse miceller system, the enzyme may interact with the micellar membrane for which changes in the micellar concentration affect the catalytic activity as the extent of inhibitory interactions between the enzyme and the micelles changes. On the contrary, the enzyme may be dissolved in the aqueous interior pool of the micelles. In that case, the activity of enzyme is independent of micelle concentration. The concentration of micelles in a system is the concentration of surfactant at a constant water-to-surfactant ratio (Wo). With the increase of surfactant concentration at a constant Wo, increases the surface area[58]. As a result, the number of micelles interact, with a lipase molecule increases undergoing the changes in the secondary structure of lipase upon incorporation in to reverse micellar system from an aqueous system causing decrease in lipase activity.

In ionic AOT reverse micellar system, the activity and stability of enzymes are adversely affected due to strong electrostatic interactions between AOT and enzymes[59]. However, enzymes in reverse micelles formed by some nonionic surfactants have a high activity and stability due to weak interactions between enzyme and the nonionic surfactants[60]. Nevertheless, the addition of some co-surfactants is still necessary for formation of the non-ionic reverse micelles. A new type of surfactant sodium bis (2-ethylhexyl polyoxyethylene) sulfosuccinate (MAOT), which is structurally designed as a chemically modified AOT, was prepared in the laboratory[61]. The chemically modified AOT (MAOT)- Isooctane reversed micellar system increased substrate conversion due to decreased electrostatic and hydrophobic interactions between enzyme and MAOT molecules[62].

At large scale, due to the presence of surfactant and other components such as enzyme and water, it is difficult to recover the product from the reverse micellar system. This problem was overcome by use of membrane reactors in which by continuous operation through ultrafiltration membrane retains the micelle and hence the enzymes while the substrate and product molecules pass freely. Such enzyme bioreactors have been used for ester synthesis[63]. Table 4 reports some reactions, with potential applications in the food, pharmaceutical and chemical industries.

Table-4
Recent examples of enzyme catalysed reactions in reverse micellar system

Enzyme	Surfactant	Organic solvent	Reaction	Ref
CCL, CSL	AOT	decane	Esterification of (±) 2-octanol and hexanoic acid	64
RDL	AOT	Isooctane	Esterification of oleic acid and octyl alcohol	65
CRL	AOT	Isooctane	Hydrolysis of Olive oil	62
MJL	AOT	Isooctane	Acylation of doxorubicin	66
TLL	AOT	isooctane	Synthesis of ethyl laurate	67
α-chymotripsin	AOT	*n*-heptane	Hydrolysis of 2-naphthyl acetate	68
α-chymotripsin	BHDC	benzene	Hydrolysis of 2-naphthyl acetate	69
α-chymotripsin	AOT	n-heptane	Hydrolysis of 2-naphthyl acetate	70

Ionic Liquids (ILs): An active area of current research in biotechnology, biocatalysis in non-conventional media, is the use of ionic liquids to improve activity, stability and selectivity of enzymes. Ionic liquids are organic salts, which are liquid over a broad range of temperature and good solvents for wide range of organic, inorganic and polymeric compounds. The first ionic liquid ($EtNH_3$ x NO_3) was reported in 1914. However, nowadays the most common ionic liquids for biocatalysis are imidazolium based ionic liquids such as [BMIM][BF_4] (1-butyl-3-methylimidazolium tetrafluoroborate), [BMIM][BF_6] (1-butyl-3-methylimidazolium hexafluoroborate) etc. Ionic liquids have low melting point and composed entirely of ions and considered to be highly polar solvents. They possess negligible vapour pressure that can be taken advantage of for the separation of volatile products. Ionic liquids, because of their negligible vapour pressures, have been generally recognized as 'green' solvents. Enzymatic reactions based on ionic liquids appear highly promising alternatives for developing 'green' chemical processes because of their physical and chemical characteristics.

Ionic liquids can selectively dissolve a gas, which makes them potential solvents for gas separations[71]. Carbon dioxide has relatively high solubility in imidazolium based ILs[72,73]. The solubility of gases, e.g. H_2, CO, O_2, is generally good which makes the ionic liquids as attractive solvents for catalytic hydrogenations, carbonylations, hydroformylations and aerobic oxidations. They are immiscible with some organic solvents, such as alkanes, and hence can be used in two-phase systems. The unique properties of ionic liquids are such as they are non-volatile, non-flammable and have excellent chemical and thermal stability. It is possible also to conventionally synthesize them in preparatory scale. The non-volatile nature of ILs provides environmental advantages and is beneficial for plant design due to reduced pressure build-up.

One obvious advantage of using ionic liquids over the use of normal organic solvents is that the physical and chemical properties of ionic liquids, including their polarity, hydrophobicity, viscosity and solvent miscibility, can be finely tuned by alternating the organic cation, inorganic anion and attached substituents. Hence, ionic liquids have been referred to as 'designer' solvent. This is important because by manipulating the solvent properties, one is allowed to design an ionic liquid for specific reaction conditions, such as to increase the substrate solubility, to modify the enzyme selectivity or to tailor the reaction rate. ILs may provide an ideal solvent for engineering media for biocatalytic reactions because of their advantages. The recent research results indicated that the enantioselectivity and activity of enzymes in ionic liquids were better or comparable to them in organic solvents in some reactions[74,75]. ILs can also be used as 'green' solvents in extraction of variety of substances, including metal ions, organic and biomolecules, organosulfur from fuels and gases[76-80].

ILs was also used to form two-phase systems with many solvents. The investigations of new biphasic reactions using ILs are of special interest because of the possibility to adjust solubility properties using different cation/anion combinations[77]. This allows for systematic optimization of a biphasic reaction with regard to product recovery. Ionic liquid biphasic systems were used to separate many biologically important molecules such as carbohydrates and organic acids. Moreover, recently two group of researchers have been employed ionic liquids to whole-cell in situ fermentation and showed its great potential in whole-cell biocatalytic processing due to their low toxicity to microorganisms[81,82]. In both cases [BMIM][PF_6] was used in a two phase system as substrate reservoir and / or for in situ removal of the product formed, thereby increasing the productivity of the catalyst. This is useful for systems where organic solvents in combination with an aqueous phase either do not dissolve enough substrate or lead to increased enzyme deactivation[83]. Table 5 represents some recently reported examples of processes that are carried out in ionic liquids using enzymes.

Table-5
Recent examples of reactions catalysed by enzymes in Ionic liquids (ILs)

Enzyme	ILs	Reaction	Comments	Ref
Immobilized esterases, Bacillus Stearothermophilies, Bacilus subtilis	Various ILs	Transesterification of 1-phenylethanol	Higher stability of enzyme compared to organic solvents	84
CRL	[BMIM][BF_6], [MOEMIM][BF_6]	Acylation of glycosides	Higher reaction rates and selectivity than in conventional organic solvents	85
Immobilized CALB	[BDMIM][BF_4]	Transesterification using vinylacetate	Lipase was recycled for 10 times without losing enantioselectivity and reactivity	86
Immobilized CALB, α-chymotripsin	Several ILs	Transesterification reaction	Improved the thermal stability of both the enzymes	87
Free Epoxide Hydrolases	Several ILs	Stereoselective hydrolysis of epoxides	comparable reaction rate and ster selectivity than those in buffer	88
Immobilized PCL	[BMIM][BF_6]	Resolution of racemic alcohols	Addition of triethyl amine to ILs enhanced the rate of reaction	89
CRL	[BMIM][BF_4] [HMIM][BF_4] [BMIM][BF_6]	Enantioselective hydrolysis	ILs as co-solvent markedly enhanced enantioselectivity	90
Immobilized CALB	Several ILs	Enantioselective acylation	Increased reaction rate, decreased enantioselectivity	91
CRL	[BMIM][BF_6], [ONIM][PF_6]	Esterification of 2-substituted propanoic acids and 1-butanol	Higher enantioselectivity than in *n*-hexane	92
Immobilized PCL	[BMIM][BF_4], [BMIM][BF_6]	Hydrolysis and alcoholysis of 3,4,6- tri-*O*-acetyl-*D*-glucal	High regioselectivity	93
CALB	Several ILs	Transesterification of ethylbutanoate with 1-butanol	Higher activity in [BMIM][BF_4], [Et_3MeN][$MeSO_4$]	94
Mandelate racemase, Pseudomonas putida	[MMIM][$MeSO_4$] [BMIM][$OCSO_4$] [BMIM][OCSO [BMIM][O]	Kinetic resolution of mendalic acid	Reaction rate strongly influenced by aw	95
CAL	[BMIM][NTf_2]	Kinetic resolution of *rac*-2-pentanol vinyl propionate	Higher activity than in hexane, greater enantioselectivity greater en enantioselectivi	96
Protease: Papain Alcalase Lipase: Novozym-435	[BMIM][BF_4]	Hydrolysis of amino acid esters	Higher enantioselectivity in papain, varied enzyme activity and enantioselectivity with substrate and enzyme used	97
CALB	Several ILs	Transesterification of ethyl butanoate with 1-butanol	Activity of CLEA-CALB was twice both in [BMIM][dca] and *tert*-butyl alcohol than free lipase	98
Lipase: Burkholderia Cepacia	Several ILs	Acetylation of secondary alcohols by vinyl acetate	Increased enantioselectivity and reaction rate in imidazolium-PEG-Alkyl sulfate	99
PPL, CRL	[BMIM][BF_4] [BMIM][PF_6]	Resolution of racemic secondary alcohols by vinyl acetate	Higher enantioselectivity and reaction rate	100
CLEA-CRL, Burkholderia cepacia	[BMIM][PF_6]	Kinetic resolution of (±)1-phenyl ethanol	Higher enantioselectivity	101
CALB	Several ILs	Transesterification of vinyl butyrate and 1-butanol	Higher activity of CALB in ILs containing anions of lower nucleophilicity in water immiscible ILs	102
Bacillus thermocate-Nulatus lipase	[BMIM][PF_6] [BMIM][BF_4]	Esterification and amidation	Best selectivity towards the formation of monoester over the di-ester	103

Enzyme	ILs	Reaction	Comments	Ref
Immobilized CALB	Several ILs	Kinetic resolution *rac*-2-pentanol	A membrane bioreactor containing supported liquid Membrane based on ionic liquid was employed	104
Novozym-435	[BMIM][PF_6]	Regioselective acylation of 1-β-D-Arabinofuranosyl cytosine with vinyl acetate	Remarkable enhancement of substrate conversion with a co-solvent mixture of [BMIM][PF6] and pyridine compared to other organic solvents	105
Novozym-435	[BMIM][PF_6] [EMIM][PF_6], [BMIM][BF_4]	Methanolysis of Sunflower oil	Both IL and lipase were recycled for four successive reaction cycles without any significant loss of activity	106
Immobilized CALB	[BMIM][PF_6]	Production of isoamyl acetate	The mixture of IL-enzyme could be recycled for 7 repeated cycles in IL-isoamyl alcohol biphasic System	107
CALB	[BMIM][TfO]	Synthesis of Palmitoylglucose ester	Highest yield of ester at 50^0C and enzyme Concentration of 50mg/ml	108
Novozym 435	[EMIM][Tf_2N]	Synthesis of caffeic acid phenyl ester	High conversion at 70^0C	109
Novozym 435	[BMIM][TfO]	Synthesis of mannosyl myristate	Full factorial design was used and was found Optimal temperature 80^0C and substrate molar Ratio of 1/10	110
CALB	[BMPyrr][Dca]	Synthesis of isoamyl acetate	More than 80% of productivity was preserved	111
CALB	[BMIM][$FeCl_4$]	Oleic acid to biodiesel	Maximum yield 83.4%, molar ratio 22:1	112

Erbeldinger *et al*. reported first the use of enzymes in ILs. They used protease thermolysin for the synthesis of the dipeptide Z-aspartame[113]. The enzyme stability was increased in IL, but the rate of reaction was comparable with those found in conventional organic solvents. The protease α-chymotrypsin was also used to carry out some transesterification reactions of *N*-acetyl-L-phenylalanine ethyl ester or *N*-acetyl-L-tyrosine ethyl ester to transform into their corresponding propyl esters[114-116]. To carry out these reactions, Laszlo and Compton[114] used [OMIM][PF_6] (1-octyl-3-methylimidazolium tetrafluoro phosphate) and [BMIM][PF_6] and compared the results with those obtained in other organic solvents. They found that as in polar organic solvents, a certain amount of water is necessary to maintain enzymatic activity. The reaction rates were also comparable in both ILs and organic solvents. Iborra and coworkers[115] compared stability of *α-chymotrypsin* in different ILs through the transesterification of *N*-acetyl-L-tyrosine ethyl ester with 1-propanol. In the ionic liquids tested, the stability of enzyme increased which led to higher final product concentration.

The majority of enzymes reported to be active in ILs belong to the class of lipases. Sheldon and co-workers first demonstrate the potential use of ILs for lipase catalysis[116]. They compared the reactivity of CALB in ILs, such as [BMIM][PF_6] and [BMIM][BF_4] with conventional organic solvents. Lipases and esterases are usually used for the kinetic resolution of racemates by esterification, transesterification or hydrolysis reactions. Schofer *et al*. studied the kinetic resolution of 1-phenylethanol for a set of eight different lipases and two esterases in ten different ILs with methyl-*tert*-butylether (MTBE) as reference using vinylacetate as acyl donor[117]. For the lipases from *Pseudomonas* and *Alcaligenes* species, an improved enantioselectivity was observed in [BMIM][NTf_2] as compared with MTBE as solvent. The best results were obtained in CALB in [BMIM][CF_3SO_3], [BMIM] [NTf_2] and [OMIM][PF_6]. Other groups investigating the same system reported good activities in these ILs[118-120]. Park and Kazlauskas demonstrated the influence of additional washing steps upon the enzyme activity[118]. Persson and Bornscheuer investigated the same system catalysed by esterases from *Bacillus subtilis* and *Bacillus stearothermophilus* and two lipases (CALB and *Pseudomonas sp*.), reported no activity of the lyophilized powder of the esterases in ILs[84]. When immobilized onto Celite, higher specific activity and enantioselectivity was obtained which is comparable to those in conventional organic solvents such as n-hexane, MTBE and vinyl acetate and for the two lipases. The stability of esterase from *B. stearothermophilus* at 40^0C was considerably increased in the ionic liquids [BMIM][BF_4] and [BMIM][PF_6] as compared to *n*-hexane and MTBE. The lipase catalysed enantioselective transesterification of racemic alcohols was also studied for three lipases using vinyl acetate as acyl donor in two ILs and hexane as reference[100]. In the presence of catalytic amounts of organic bases such as triethylamine or pyridine, both the rate and enantioselectivity of the reaction was increased by CALB compared to hexane. No reaction was observed in PPL or CRL.

Earlier studies of the lipase from *Pseudomonas sp.* revealed that the water content of the reaction medium had a strong influence

on enzyme activity[121]. To compare the enzyme activity and selectivity in solvents of different polarities independently of the water content, it is necessary to evaluate the water activity (a_w) in these solvents. Eckstein *et al*. used the method of water activity equilibration over saturated salt solutions and found that the enantioselectivity of the lipase is less influenced by the water content or temperature when the reaction is performed in [BMIM] [NTf_2] [122]. Barahona *et al*. studied the effect of water activity on the immobilized *candida antarctica* lipase B catalyzed esterification of geraniol in [BMIM][PF_6] [95]. The reaction rate was lower than in hexane. The concentration based equilibrium constant for the reaction in [BMIM][PF_6] was dependent on the water activity in the system, which is about 20 times lower than in hexane. Iborra *et al.* reported that for the resolution of *rac*-2-pentanol using CALB at 2% (v/v) water content, the use of ionic liquid, [BMIM][NTf_2] is more effective than hexane[74]. Both the synthetic activity and selectivity of the process is dependent on the water activity, although it has no affect on the enantioselectivity of the enzyme.

Sheldon and coworkers investigated the structure and activity of CALB on seven different ILs and *tert*-butyl alcohol as reference through a simple transesterification reaction of ethyl butanoate with1-butanol[94]. They reported that in [BMIM][BF_4] and [BMIM][PF_6], the reaction rate was comparable with that in *tert*-butyl alcohol. However, in ionic liquids containing alkylsulfate, nitrate and lactate anions, which dissolved CALB, the reaction rate was about ten times slower than in [BMIM][BF_4]. An exception was the [Et_3MeN][$MeSO_4$], as dissolved CALB maintains its activity in this solvent. The denaturation of CALB was observed upon dissolution in ILs in which the activity was low, whereas the conformation of enzyme dissolved in [Et_3MeN][$MeSO_4$] closely resembled the native one. The CLEA of CALB was twice as active in [BMIM][dca] that deactivated the free enzyme[98]. The stability and activity of CALB in ILs was also studied through transesterification of 1-butanol with vinyl butyrate[102]. They studied the reaction in nineteen different 1,3-dialkylimidazolium based ionic liquids and in hexane as reference solvent. They reported that in water-immiscible ILs, the enzymatic activity and selectivity were higher than that obtained in hexane. However, in water-miscible ILs, the activity was lower than in the reference solvent. CALB exhibited greater stability in water-immiscible ILs than in water-miscible ILs.

One of the main advantages of ILs that makes them 'green' is the low volatility, which creates challenges for product separation and recovery. If the product is volatile, back distillation may be used to remove the product from IL[123]. If the product is of hydrophilic nature in a hydrophobic IL, water may be used to remove the product from IL[124]. However, one of the main drawbacks of IL technique is the difficulty of recovery of non-volatile or low volatility products. In such cases, to overcome this limitation, another type of 'green' solvent, supercritical fluids (SCFs), has been adapted for product recovery from ILs[125]. Among SCFs, $SCCO_2$ is volatile and non-polar, hence easily forms two-phase systems with non-volatile and polar ILs. The principle of product recovery by these biphasic systems is based on the solubility of $SCCO_2$ in the ILs to transfer organic products to the $SCCO_2$-rich phase and the insolubility of the IL in $SCCO_2$.

More recently, the biphasic IL / $SCCO_2$ system have been used in metal catalysed and enzyme catalysed reactions. This biphasic system offers advantages that enzyme catalyst are soluble and stable in ILs, but have low solubility in $SCCO_2$. Moreover, many organic compounds are soluble in $SCCO_2$, offering easy separation of products from ILs and the process can be designed as batchwise or continuous operations[126-131].

A number of biocatalytic reactions were achieved successfully in IL/$SCCO_2$ biphasic system[114,132-133]. The kinetic resolution of rac-1-phenylethanol with vinyl propionate catalysed by free and immobilized CALB in IL/$SCCO_2$ biphasic system was studied at 10 Mpa and at 120 and 150^{0}C[134]. Both free and immobilized CALB were able to catalyze specifically the synthesis of (R)-1-phenylethylpropionate and excellent activity, stability and enantioselectivity were reported in continuous operation. Iborra *et al.*[135] demonstrated the dynamic kinetic resolution of the same system in different IL/$SCCO_2$ biphasic systems by simultaneously using both immobilized CALB and silica modified with benzenosulphonic acid (SCX) catalysts at 40^{0}C and 10Mpa. SCX was reported to be an efficient heterogeneous chemical catalyst for the racemization of (S)-1-phenylethanol in [EMIM][NTf_2], [BTMA][NTf_2] and [BMIM][PF_6]. Coating both CALB and SCX with ILs greatly improved the efficiency of the process in $SCCO_2$, providing good yield of (R)-1-phenylethylpropionate (78%) with excellent enantioselectivity (ee = 91-98%) in continuous operation.

The biphasic IL/alkane system have also been used in some enzyme catalysed reactions[136-138]. A continuously operated ψ-shaped microreactor was used for CALB catalysed synthesis of isoamyl acetate in 1-butyl-3-methyl pyridinium dicyanamide/n-heptane two phase system [136] and 48.4gm-3s-1 of isoamyl acetate was produced, which was almost three fold better as compared to the intensely mixed batch process. The synthesis of ferulic acid oleylalcohol ester was studied in IL/isooctane system [138]. Both considerable bioconversion and volumetric productivity were reported in [HMIM][PF_6] and [OMIM][PF_6] mediated system. Under optimized reaction conditions of 60^{0}C, 150 mg of Novozym 435 and 100 mg molecular sieve, upto 48.50 mg/ml productivity of ester was reported for [HMIM][PF6]/isooctane (0.5ml/1.5ml) system with a substrate concentration of ferulic acid of 0.08mmol/ml and oleyl alcohol of 0.32 mmol, while an optimum volumetric productivity of 26.92 mg/ml was obtained.

Conclusion

This review summarizes some of the current advances in the field of non-aqueous biocatalysis in heterogeneous solvent systems, including ionic liquids (ILs) which offer new possibilities for the application of solvent engineering to

biocatalytic reactions. Biocatalysis in ionic liquids is an exciting area of research which holds considerable potential for industrial applications. In some cases, biocatalyst can have profound effect on activities and selectivities in ionic liquid media. Furthermore, ILs is designer solvents. Hence, for a specific system, ILs can be achieved by a suitable combination of cation and anion.

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