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Review

Enzyme Stability and Activity in Non-Aqueous Reaction Systems: A Mini Review

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Abstract: Enormous interest in biocatalysis in non-aqueous phase has recently been triggered due to the merits of good enantioselectivity, reverse thermodynamic equilibrium, and no water-dependent side reactions. It has been demonstrated that enzyme has high activity and stability in non-aqueous media, and the variation of enzyme activity is attributed to its conformational modifications. This review comprehensively addresses the stability and activity of the intact enzymes in various non-aqueous systems, such as organic solvents, ionic liquids, sub-/super-critical fluids and their combined mixtures. It has been revealed that critical factors such as Log *P*, functional groups and the molecular structures of the solvents define the microenvironment surrounding the enzyme molecule and affect enzyme tertiary and secondary structure, influencing enzyme catalytic properties. Therefore, it is of high importance for biocatalysis in non-aqueous media to elucidate the links between the microenvironment surrounding enzyme surface and its stability and activity. In fact, a better understanding of the correlation between different non-aqueous environments and enzyme structure, stability and activity can contribute to identifying the most suitable reaction medium for a given biotransformation.

Keywords: enzyme; conformation; activity; applications; non-aqueous media

1. Introduction

Since the 1980s, biocatalysis in non-aqueous media has undergone a tremendous development and numerous reactions have been proposed and optimized for synthetic applications. In comparison with conventional aqueous enzymology, biocatalysis in non-aqueous phase offers unique merits, such as the possibility of altering enzyme regio- and enantio-selectivity, to reverse thermodynamic equilibrium toward synthesis (e.g., in the case of reactions catalyzed by hydrolases), to avoid water-dependent side reactions, and bacterial contamination [1,2]. Organic solvents are the most commonly used non-aqueous media for biocatalysis. Researchers have investigated that log *P* of organic solvents impacts enzyme's activity [3–5]. In our previous work, we demonstrated that, apart from log *P*, functional groups and molecular structure of organic solvents would also exert significant influences on enzymes activity [6]. Recently, we have also found that some enzymes show high catalytic activity, enantioselectivity, and stability in ionic liquids (ILs) and sub-/super-critical fluids media, especially

in their mixture solvents [7,8]. The satisfactory activity of enzymes in non-aqueous media has allowed many synthetic applications. However, in most cases, enzyme activity in non-aqueous media is lower than in water (up to several orders of magnitude). Different factors, such as diffusion limitation, high saturating substrate concentration, restricted protein flexibility, low stabilization of the enzyme-substrate intermediate, and non-optimal hydration of the biocatalyst, have been suggested to be responsible for the lower catalytic activity of enzymes in non-aqueous media [9]. It is universally accepted that conformational changes also play a very important role in the decrease of enzyme activity in non-aqueous media.

However, Gupta *et al.* [10] demonstrated that, after incubated in acetonitrile at 70 °C for 3 h, six enzymes (proteinase K, wheat germ acid phosphatase, α-amylase, β-glucosidase, chymotrypsin and trypsin) show much higher activity than that of the untreated enzyme. The authors claimed that the probable reason was attributed to the unchanged stable three-dimensional structure. Taking the above factors into consideration, it is essential to emphasize that enzyme denaturation is not only due to the interactions of the enzyme molecules with the components of the non-aqueous media [11], but also to the (freeze)-drying process used to prepare the enzyme in a suitable form for biocatalysis in these media [12–14]. So it is crucial to have a deep insight into the conformation variance and activity shift of enzyme in non-aqueous media, which is helpful for the selection of the suitable reaction medium for biotransformation. Therefore, this review aims to highlight the mechanisms of enzyme's activity variance according to enzyme structure changes in four different common non-aqueous media, *i.e.*, organic solvents, ILs, sub-/super-critical fluids, and their combination mixture systems. Furthermore, some major factors affecting the microenvironment surrounding enzyme in non-aqueous media, such as log *P*, solvent type, functional group, molecular structure, cation and anion type, pressure and temperature, are comprehensively discussed in this review. Besides, some specific applications of biocatalysis in non-aqueous phases are also addressed in the corresponding section.

2. Structure and Activity of Enzymes in Organic Solvents

2.1. Effect of Log P Value of Organic Solvent

Log *P*, the partition coefficient, is a measure of hydrophobicity of organic solvent. Generally, hydrophilic (water miscible) solvents exhibit log *P* values less than 1.0, while hydrophobic (water immiscible) solvents exhibit log *P* values more than 4.0. The higher the log *P* value, the more hydrophobic the solvent is. It has been proved that enzyme activity is higher in hydrophobic solvents than hydrophilic solvents [6,15,16]. The reasonable explanation was that hydrophilic solvents have a greater tendency to "strip" tightly bound water (which is essential for enzyme catalytic activity) from the enzyme molecules surface, leading to the decrease of the enzyme activity. Although the log *P* value of organic solvent shows no obvious effect on the backbone structure of enzyme, organic solvent will impact the water content urrounding the enzyme surface and active site region, which can been observed through the molecular dynamics (MD) simulation. Water stripping is always accompanied by the penetration of organic solvent molecules into crevices on the enzyme surface, especially the active site [17,18]. Water activity (a_w) is indicative of water content around enzymes, and it is defined as the partial vapor pressure of water in a substance divided by the standard state partial vapor pressure of water. a_w is an important consideration for enzyme activity in non-aqueous media because enzymes require a certain level of water in their structures (bound water) to maintain their natural conformation, allowing them to deliver their full functionality [19]. Bovara *et al.* [3] reported that water activity ranging from <0.1 to 0.53 does not influence the enantioselectivity of enzymes in organic solvents using lipase PS and lipoprotein lipase as models. Miroliaei and Nemat-Gorgani [20] reported that the thermophilic alcohol dehydrogenase from *Thermoanaerobacter brockii* remains approx. 80% of its original activity at 90 °C in *n*-octane (log *P* = 4.183).

The Log *P* value of organic solvents also affects the flexibility of enzyme when biocatalysis is occurred in the organic solvents media. For instance, Trodler *et al.* [17] reported that the flexibility of

lipase B from *Candida Antarctica* (CALB) decreases with increasing log *P* values of organic solvents. Fasoli *et al.* [21] also revealed that the flexibility of subtilisin from *Bacillus licheniformis* in octane (log *P* = 4.183) was lower than in acetonitrile (log *P* = −0.334). As discussing the flexibility of the enzyme, it was worthily noted that enyzme flexibility was usually determined from MD simulations and it is measured by the relative calculated B-factors [22].

As well known, non-aqueous enzymology is a bell-shaped mechanism in dependence on hydration [23]. Therefore, the water content and hydrophobicity (Log *P*) of organic solvent have a dramatic influence on the properties of enzymes. It was revealed that there was an optimum water content (~10% *w*/*w*) for enzyme properties, at which the enzyme properties are similar to the ones found in pure water [24]. At lower water content, the enzyme is very rigid; while at higher water content the enzyme starts to unfold. If the reaction medium is too dry, the enzyme lacks flexibility resulting in un-efficient catalysis. When the water content increases, the enzyme becomes more flexible and its activity increases. Beyond the optimum water concentration, the protein starts to unfold and its activity decreases again.

2.2. Effect of the Functional Groups of Organic Solvent

The functional groups of organic solvents are also critical factors affecting enzyme activity [6]. Alkanes (such as hexane, cyclohexane, octane, and dodecane *etc.*), just have hydrophobic interactions with enzyme, so they do not significantly change the global structure and active site of enzyme [25,26]. For the biocatalysis in these organic solvents, the solvent molecules are located near the active site and/or close to the hydrophobic regions of the enzyme (e.g., CALB, *r*-chymotrypsin, subtilisin, cutinase, triosephosphate isomerase, *etc.*), resulting in the re-orientation of the side chains of some amino acids, obtained from the MD simulation or X-ray crystal structure analysis [23,27]. Although these re-orientations of side chains do not necessarily alter the active site of the enzyme, they affect the enzyme activity by changing the substrate affinity and specificity, as well as the hydration of enzyme. For example, Pramod *et al.* [28] reported that although octane almost had no influence on the secondary and tertiary structures of subtilisin BPN', the catalytic efficiency k_{cat}/K_m of the enzyme in octane was only 10.6% of that in aqueous solution. Nevertheless, the stability of subtilisin BPN' in octane was 645 fold of that in aqueous solution, owing to the absence of autolysis in octane. Therefore, over its active lifetime the productivity of the soluble enzyme should be higher in octane than in water. Similarly, Burke *et al.* [29] also stated that octane had little impacts on the secondary structure and active site of the α-lytic protease, rather it reduced the activity through the alteration of binding affinities of substrates. Guinn *et al.* [30] found that the activity of horse liver alcohol dehydrogenase (HLAD) dramatically increased from 0% to 370% with the increase of water from 0% to 10% in the hexane aqueous solution, although the structure of the enzyme was nearly identical to the native enzyme. These results indicate that the water activity is essential for the activity [30]. Several studies have corroborated that alkanes (especially hexane and octane) enhance the rigidity and stability of enzymes, such as *Rhizomucor miehei* lipase (RML), *r*-chymotrypsin, subtilisin, cutinase, and horseradish peroxidase, and the level of increase is positively related to the chain length of the alkane, and thus to its hydrophobicity [23,26,31].

In many situations, alcohols are used as media for biocatalysis. The introduction of OH group increases the hydrophilicity of the organic solvent and therefore enhances the interactions between the solvent and enzyme. MD simulation has been extensively used to investigate the molecular interactions between the alcohols and enzymes. For instance, it was reported that *tert*-butanol molecules could directly bind to the active site of CALB and alter its tertiary structure [32]. Furthermore, ethanol molecules were found to bind to the surface of the cutinase and strip water molecules from the hydration layer surrounding the enzyme, which was considered to be an essential factor affecting the enzyme activity [33]. Using lipase as a model enzyme, Kamal *et al.* [34] demonstrated that methanol and isopropanol made lipase structure less rigid and more prone to unfolding, which increased the instability of the enzyme. The changes of enzyme structures substantially alter enzyme activities. For

instance, the *Rhizomucor miehei* lipase (ROL) activity decreases from 100% to 0% with the increase of alcohol concentration, and the decreasing rate increases with the increase of carbon chain length from methanol to butanol [24]. The activity inhibition of ROL might stemmed from the fact that the OH group acted as a product inhibitor, competing with that of the substrate in the case of the hydrolytic reaction [24]. Similarly, the papain showed 60% and 20% of its original activity in 90% and 99% v/v methanol aqueous solution. Since no global conformational change and minor secondary structure rearrangements were detected, it was suggested that the active site of the papain was somehow altered by the methanol molecules [35]. The activities of α-chymotrypsin and trypsin first decreased and then increased with increasing ethanol concentration from 0% to 100%, in corresponding with the changes of the secondary structure elements (α-helix and β-sheet) [36]. Moreover, the apparent K_m values of the two enzymes decreasing as the low ethanol concentrations were elevated, but then increased in the presence of higher ethanol concentrations, indicating the substrate affinity of the two enzymes first increased and then decreased with increasing ethanol concentrations [36].

Similar phenomena were observed in organic solvents with C=O (e.g., acetone and *N*,*N*-dimethylformamide), C≡N (e.g., acetonitrile), and cyclic molecules (e.g., benzene, dioxane and tetrahydrofuran) as in alcohols [37,38]. Through MD simulation, acetonitrile molecules were found to penetrate into the active site of lipase, leading to structure variation of the active site and therefore the drop in the enzymatic activity in acetonitrile aqueous solution [38]; acetone, acetonitrile, and 1,4-dioxane could bind to the active site of subtilis and disturb its structure [21,34]. Gupta *et al.* [39] observed that the activity of polyphenol oxidase and trypsin reduced to different extents by 50% of tetrahydrofuran, dioxane, acetone, and acetonitrile. Liu *et al.* [6] demonstrated that three commercial lipases, Novozym 435, lipase PS, and Lipozyme TLIM, showed highest esterification activities when pretreated (the term "pretreatment" means enzymes were preincubated in organic solvent, and then the enzymes were filtered and dried to remove the organic solvent. The resulting enzymes were dissolved in aqueous solution for activity assay) with organic solvents containing C=O and C≡N groups. Instead, the activity was lower if pretreated with alkanes, and even less, with solvents with OH and aromatic groups.

However, the effects of organic solvents with S=O group (e.g., dimethyl sulfoxide, DMSO) on enzyme activity and structure are quite different from the above-mentioned ones. Roy *et al.* [40], through MD simulation, reported that about 5% (v/v) DMSO could markedly suppress the flexibility of lysozyme, caused by the preferential solvation of exposed hydrophobic residues by the methyl groups of DMSO. DMSO with the concentration of 15%–20% (v/v) could partially unfold lysozyme, accompanied with an increase of both fluctuation and exposure of protein surface area. At 15%–20% (v/v) DMSO, conformational fluctuation and solvent accessible protein surface area suddenly decrease to form an intermediate collapse state. This structural transformation was attributed to the cluster of the methyl groups of DMSO on the enzyme surface. When the content of DMSO was higher than 20% (v/v), the enzyme became denaturation and lost its activity completely.

In summary, hydrophobic functional groups, such as alkaline, could maintain the intact structure of enzymes so as to dramatically prolong their stability. The enzyme activity usually achieved maximum in these solvents containing *ca.* 10% water content, stemmed from the native structure and water activity. As a result, a great deal of industrial reactions has been successfully applied in alkaline systems such as hexane, octane, and isooctane [41–43]. Hydrophilic functional groups (e.g., OH, C=O, C≡N, S=O, *etc.*) changed the enzyme structure to different extents, as well as "stripped" the essential water from enzymes, and thereby reduced the enzyme activity.

2.3. Effect of Molecular Structure of Organic Solvent

Several experimental studies have shown that the molecular structure of organic solvent has a dramatic influence on the properties of enzymes. Generally, the organic solvent with its functional group in the terminal carbon atoms shows higher inhibitory effect on the enzyme activity than that in the internal carbon atoms. For example, the α-chymotrypsin activity decreased with the

increase of 1-propanol (log P = 0.34) and 2-propanol (log P = −0.77) concentrations, and the threshold concentrations (defined as the values at which half inactivation of the enzyme is observed) for 1-propanol and 2-propanol were 27% and 33%, respectively, indicating that 1-propanol had higher inhibitory effect on the enzyme activity [44]; trypsin showed 97% and 100% of its original activity in 50% 1-propanol and 2-propanol, respectively [39]; lipase PS from *Pseudomonas cepacia* presented much higher activity after pretreatment with isopropanol than with *n*-butanol, although both the solvents have the same log P of 0.8 [6]; the activity of *Candida rugosa* lipase in isooctane was much higher than in octane, although the two solvents are of the same log P (log P = 4.5) [45]. A possible mechanism might be that organic solvents with functional groups in internal carbon atoms had higher steric effects than those in terminal carbon atoms. The higher steric effects hinder the effective interactions between these functional groups and enzyme, the lower inhibitory effect on enzyme activity is caused.

3. Structure and Activity of Enzymes in Ionic Liquids (ILs)

Ionic liquids (ILs), also called molten salts, are organic salts melting below 100 °C. ILs possesses high thermal stability, negligible vapor pressure, and moderate polarity. Moreover, the physiochemical properties of ILs (e.g., viscosity, melting point, polarity, and hydrogen bond basicity) can be altered by simply changing anions or cations. Due to these advantages, ILs has been becoming attractive alternatives to volatile and unstable organic solvents nowadays [46,47].

3.1. Effect of Hydrophobicity of Ionic Liquid

The activity and stability of enzymes in ILs system can be significantly affected by the hydrophobicity of ILs [48]. Researchers observed that the enzyme could achieve higher activity and stability in more hydrophobic ILs. Nakashima *et al.* [49,50] studied the properties of PEG-modified lipase and subtilisin in three different ILs, 1-ethyl-3-methylimidazolium bis (trifluoromethanesulfonyl) imide ([Emim][Tf_2N]), and its ether ([C_2OC_1mim][Tf_2N]) and hydroxyl ([C_2OHmim][Tf_2N]) analogues. They found that the activities and stabilities of the two enzymes increased with increasing hydrophobicity of the three ILs (the hydrophobicity decreases in the order of [Emim][Tf_2N] > [C_2OC_1mim][Tf_2N] > [C_2OHmim][Tf_2N]). Specifically, the transesterification activity of PEG-modified subtilisin was [Emim][Tf_2N] (65% compared to that in aqueous solution; the same below) > [C_2OC_1mim][Tf_2N] (55%) > [C_2OHmim][Tf_2N] (28%). This enzyme also showed good stability for a long period in [Emim][Tf_2N]. Namely, it maintained 80% of its initial activity after 60-h incubation. For PEG-modified lipase, the initial rate exhibited [Emim][Tf_2N] (29 mmol/h· g) > [C_2OC_1mim][Tf_2N] (25 mmol/h· g, which is 86% of [Emim][Tf_2N]) > [C_2OHmim][Tf_2N] (23 mmol/h· g, which is 79% of [Emim][Tf_2N]). PEG-modified lipase was exceedingly stable in [Emim][Tf_2N], maintaining its original activity for 144 h [50]. Zhang *et al.* [51] reported that the stability of penicillin acylase was higher in more hydrophobic IL of 1-butyl-3-methylimidazolim hexafluorophosphate ([Bmim][PF_6]) than in 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) and 1-butyl-3-methylimidazolim dicyanamide ([Bmim][dca]) after 48-h incubation. Similarly, De Los Ríos *et al.* [52] observed that the synthetic activity of CALB was only 0.079 U/mg in IL of 1-butyl-3-methylimidazolium octylsulfate ([Bmim][$OcSO_4$]). However, the activity reached 116.097 U/mg in more hydrophobic IL of 1-octyl-3-methylimidazolium hexafluorophosphate ([Omim][PF_6]). Lozano *et al.* [53] evaluated the stability of α-chymotrypsin in propanol and four ILs by testing the half-life time ($t_{1/2}$) and deactivation rate (k_d) at 50 °C. The α-chymotrypsin exhibited an important activity loss in aqueous solution at temperatures beyond the melting point (43.9 °C). In propanol, the activity falled quickly with the $t_{1/2}$ of 0.15 h and the k_d of 4.45 h^{-1}. In ILs, the $t_{1/2}$ values increased and the k_d values decreased dramatically as compared in propanol, indicating that the enzyme stability was greatly enhanced. The increase in $t_{1/2}$ values and decrease in k_d values were practically in agreement with the increase in hydrophobicity of the ionic liquid. Namely, the hydrophobicity of the ILs decreases in the order of [MTOA][Tf_2N] > [Bmim][BF_4] > [Bmim][PF_6] > [Emim][Tf_2N]. Correspondingly, their $t_{1/2}$ values decreased in the order of 2.63 h > 1.93 h > 1.58 h > 1.08 h, and the k_d values increased in

the order of 0.26 h^{-1} > 0.36 h^{-1} > 0.44 h^{-1} > 0.64 h^{-1}. The observed correlation between hydrophobicity of ILs and the enzyme activity and stability might be explained by the fact that the increase in hydrophobicity of the ILs could increase the preservation of the essential water layer around the protein molecule, reducing the direct protein-ion interactions and then enhancing the enzyme stability towards denaturative conditions [53].

In our previous work, biodiesel synthesis and conformation of lipase from *Burlkholderia cepacia* (BCL) in 19 different ILs were comprehensively evaluated. Among them, *N*-octyl-3-pyridine tetrafluoroborate ([OmPy][BF_4]) was screened as the best reaction medium for biodiesel synthesis with the yield of 82.2% ± 1.2% (yield = mass of actual yield/mass of theoretical yield × 100%) after 12-h reaction [54]. The high yield of biodiesel achieved by [OmPy][BF_4] might be explained by the fact that [OmPy][BF_4] readily dissolved methanol and byproduct glycerol as a storage phase, which prevented direct exposure of the lipase to excess methanol and glycerol. Table 1 shows some examples of biodiesel synthesis by lipases in different IL media.

Table 1. Comparisons of biodiesel yield catalyzed by lipase in ionic liquids (ILs).

ILs	Lipase *	Biodiesel Yield (%)	Refernce
[OmPy][BF_4]	BCL	82.2 ± 1.2	[55]
[Bmim][PF_6]	CALB	38 ± 1.5	[56]
[Bmim][PF_4]	CALB	2.3 ± 0.2	[57]
[Bmim][Tf_2N]	PS-D Amano I	24.7	[58]
[Bmim][Tf_2N]	PS-C Amano I	73.9	[58]
[Bmim][Tf_2N]	PS	21.2	[58]

* CALB: Candida Antarctica lipase B; PS: Pseudomonas cepacia lipase.

Hydrophobicity of ILs could alter the selectivity of enzymes. It was claimed that enzymes showed different selectivity in the water-immiscible and water-miscible IL systems, the fact might be attributed to water activity (a_w) around the enzyme microenvironment was altered in IL media. Shen *et al.* [59] reported that Amano lipase PS from *Pseudomonas cepacia* showed higher enantioselectivity (ee_p = 80%) in hydrophobic [Omim][PF_6] than in hydrophilic 1-hexyl-3-methylimidazolium tetrafluoroborate ([Hmim][BF_4]) and 1-ethyl-3-methylimidazolium chloride ([Hmim]Cl) (ee_p < 5%) for resolution of racemic cyanohydrins. Lou *et al.* [60] reported that the enantioselective acylation of (*R*,*S*)-1-trimethylsilylethanol with vinyl acetate catalyzed by Novozym 435 increased with the increase of hydrophobicity of ILs by the order of [Bmim][PF_6] (ee_s = 90.7%) > [Omim][BF_4] (ee_s = 86.3%) > [C_7mim][BF_4] (ee_s = 83.7%) > [Hmim][BF_4] (ee_s = 76.2%) > [C_5mim][BF_4] (ee_s = 70.5%) > [Bmim][BF_4] (ee_s = 62.6%). Hernández-Fernández *et al.* [61] declaimed that the transesterification activity of CALB could be reached up to 99.99% in water-immiscible IL systems.

3.2. Effects of Cation and Anion Types of Ionic Liquids

The types of cation and anion of ILs show great influence on the enzyme activity and stability. Because of a more localized charge and stronger internal polarization of compact anion, the hydrogen bonding between enzyme and anion is much stronger than the weak van der Waals force between enzyme and cation [62]. In order to maintain the activity of IL-dissolved enzymes, a balance of mild hydrogen bond-accepting and donating property is required [63]. Therefore, anions are universally believed to exert more powerful impact on the catalytic activity and stability of enzyme than cations. This conclusion can be supported by the study by Liu *et al.* [54], who comprehensively investigated the transesterification activity and conformation of BCL in 19 ILs with 6 different cations and 7 different anions (the 19 ILs were [Omim][Cl], [Emim][TfO], [Bmim][Cl], [Omim][BF_4], [Bmim][CH_3SO_3], [Emim][Cl], [NMP][CH_3SO_3], [Hmim][TfO], [Hmim][CH_3SO_3], [Bmim][PF_6], [Bmim][OH], [Emim][PF_6], [Hmim][Cl], [Bmim][Tf_2N], [Bmim][BF_4], [Omim][PF6], [Emim][BF_4],

[Hmim][PF_6], and [OmPy][BF_4]). By comparing the BCL activity in ILs with same cations or anions, it was concluded that anions had much greater influence on the BCL activity than cations.

Hofmeister series of cations and anions are also widely used to predict the behaviors of enzyme in ILs. Generally, kosmotropic anions and chaotropic cations of ILs are deemed as good stabilizers of enzyme proteins [64]. However, the interactions between ILs and enzyme are complicated in practice experiments. It was speculated that the shift of enzyme activity in ILs stemmed from the secondary structure variance of enzyme, especially the alteration of α-helix and β-sheet elements [65]. ILs, in particular anions, which form strong hydrogen bonding may dissociate the hydrogen bonding that maintains the structural integrity of the α-helices and β-sheets, causing the protein to unfold wholly or partially [66]. Dabirmanesh *et al.* [67] demonstrated that imidazolium based ILs could affect kinetics, structure and stability of the alcohol dehydrogenase from thermophilic *Thermoanaerobacter brockii* (TBADH). Ajloo *et al.* [64] found that ILs could change the tertiary structure of adenosine deaminase (ADA) after studying the interactions between two ILs (1-allyl 3-methyl-imidazolium chlorides ([Amim]Cl) and 1-octhyl-3-methyl-imidozolium chlorides ([Omim]Cl)) and ADA. [Amim]Cl has higher salt properties and then electrostatic interactions dominate, so it denatures ADA by dissociate the essential hydrogen bonding. While [Omim]Cl has surfactant-like properties and hydrophobic interaction is dominate. Therefore, the denaturing mechanisms of [Omim]Cl is similar to that of surfactants.

3.3. Biocatalysis in Mixture Solvents of Organic Solvent and Ionic Liquid

Nowadays, more and more attention has been focussed on the biocatalysis in mixture solvents of organic solvent and ILs [47,68]. Table 2 shows the typical applications of biotransformation by enzymes in the mixtures of organic solvent and ILs.

Table 2. Activity of enzyme in mixture solvents of organic solvent and ionic liquids.

Enzyme *	Mixture Solvents	Applications	Reference
BCL	[Bmim][PF_6], [Bmim][Tf_2N], [Hmim][PF_6], and [Bmim][NO_3]/hexane, benzene, and *tert*-butanol	Esterification of lauric acid with dodecanol	[5]
PAL	[Bmim][PF_6]/hexane	Transesterification of 1-phenyl ethanol	[69]
CALB	[Btma][Tf_2N] and [Toma][Tf_2N]/hexane	Transesterification of *rac*-1-phenylethanol	[70]
PSL	[Bmim][PF_6] and [Bmim][BF_4]/*tert*-butanol, and chloroform	Aminolysis of (*R*,*S*)-methyl mandelate	[71]
CALB	[Bmim][PF_6]/*tert*-amyl alcohol and hexane	Acylation of alaninol, 4-amino-1-pentanol and 6-amino-1-hexanol with myristic acid	[72]
	[Bmim][PF_6]/pyridine	Acylation of 1-β-D-arabinofuranosylcytosine with vinyl benzoate	[73]
	[Bmim][SCN], [Bmim]Cl [Bmim][BF_4], and [Bmim][PF_6]/various organic solvents	oxidation of *N*-benzyliden-2-ethylhexylamine to form E- and Z-isomers of oxaziridines	[74]
	[Hmim][PF_6], [Omim][PF_6]/isooctane)	Esterification of ferulic acid to form oleyl alcohol ester	[75]

Table 2. *Cont.*

Enzyme *	Mixture Solvents	Applications	Reference
CRL	[Bmim][PF_6]/Tween 20 or Triton X-100	Esterification of natural fatty acids with various aliphatic alcohols	[76]
	[Bmim][PF_6]/AOT and Triton X-100	Hydrolysis of 4-nitrophenyl butyrate (*p*-NPB)	[77]
RML	[Bmim][PF_6]/benzene, toluene, ethylbenzene, hexane, heptane, octane, and nonane	Esterification of amyl caprylate	[78]
BCL	[MOPMIM][PF_6]/*t*BuOMe, THF, $CHCl_3$	Transesterification of various bulky secondary alcohols	[79]
	[Emim][Tf_2N], [Emim][BF_4], and [Bmim][PF_6]/several organic solvents	Acylations of secondary alcohols	[79]
PSL	[Bmim]Cl and [Hmim]Cl/methanol and 2-propanol	Hydrolysis of *p*-NPB	[80]

* CALB: Candida Antarctica Lipase B; PAL: Pseudomonas aeruginosa lipase; CRL: Candida rugosa lipase; RML: Rhizomucor mielei lipase; BCL: Burkholderia cepacia lipase; PSL: Pseudomonas sp. Lipase.

It has been found that the catalytic activity, stability and enantioselectivity of enzymes are obviously improved in mixture solvents of organic solvent and IL comparing to the corresponding single organic solvent or ILs. These observations probably stemmed from the fact that the viscosity of ILs was largely reduced by adding organic solvents, which largely eliminated the mass transfer limitation of ILs and enhanced the biocatalysis reaction rate [69]. For example, Singh *et al.* [81] comprehensively compared the transesterification of (*R*,*S*)-1-chloro-3-(3,4-difluorophenoxy)-2-propanol (*rac*-CDPP) with vinyl butyrate by lipases in hexane, [Bmim][PF_6], [Bmim][BF_4], and IL/hexane co-solvents systems. Results showed that the maximum conversion (>49%) and enantiomeric excess (*ee* > 99.9%) of *rac*-CDPP were achieved after 6-h incubation at 30 °C in [Bmim][PF_6]/hexane co-solvents system, where the tertiary structure of lipase was supposed to be well stabilized [82]. Ganske and Bornscheuer [83] reported that lipase from *Candida antarctica* showed little activity in the synthesis of sugar esters in pure [Bmim][PF_6] and [Bmim][BF_4] media. However, the reaction became feasible in IL/butanol co-solvents system containing 60% of IL ([Bmim][PF_6] or [Bmim][BF_4]) and 40% of butanol. Tan *et al.* [84] applied a mixture of [Bmim][PF_6] and pyridine (80:20, *v*/*v*) for acylation of 1-β-D-arabinofuranosyl-cytosine using CALB as a biocatalyst, and the results showed that the conversion was dramatically increased to 99.4% compared with other solvent systems.

In IL/organic solvent mixture systems, the proportion of organic solvent is an important factor that affects enzyme activity. For instance, Contesini and Oliveira [85] studied the effect of organic solvent proportion on the kinetic resolution of (*R*,*S*)-Ibuprofen catalyzed by lipases in isooctance/[Bmim][PF_6] co-solvents mixture. The enantioselectivity of lipase decreased in the order of 50% [Bmim][PF_6] (*E*-value = 4.6) > 70% [Bmim][PF_6] (*E*-value = 4.1) > 30% [Bmim][PF_6] (*E*-value = 3.2) > 100% [Bmim][PF_6] (*E*-value = 3.1) > 0% [Bmim][PF_6] (*E*-value = 2.1) [85].

However, the inappropriate mix of organic solvent and IL may cause negative influence on enzyme activity. For instance, enzyme showed higher activity in single ILs benzyltrimethylamine chloride bis (trifluoromethylsulfonyl)-imide ([Btma][Tf_2N]) and 1-ethyl-3-methylpyridinium bis(trifluoromethylsulfonyl)-imide ([EMpy][Tf_2N]) or hexane than in their mixture solvents. The reasons were that [Btma][Tf_2N] and [EMpy][Tf_2N] are not soluble in hexane, so mass-transfer limitations were introduced in the liquid/liquid biphasic system of their mixture. Moreover, the authors stated that the homogeneous distribution of the enzyme onto a support with preferential

enzyme-surface interactions and at an optimal hydration level were crucial for the enzyme activity, indicating that a suitable water content in the enzyme microenvironment was essential for the retaining of the native structure of the enzyme and therefore its activity [70,71,86].

In summary, ILs hhaveas proven themselves as excellent media for the enzyme catalyzed reactions in many instances. Enzymes can not only be stabilized in certain ILs, but also is irreversibly activated once incubated in ILs. Moreover, ILs may retain adequate microenvironment water content stabilizing the structure of enzyme active site and therefore elevating the activity. The utilization of enzymes in ILs also has limitations, including the unease of purification, high cost and mass transfer limitations. However, ILs could provide numerous advantages in biocatalysis reactions due to their great diversity, and this field surely marks a milestone on the path to future research [87].

4. Structure and Activity of Enzymes in Sub-/Super-Critical Fluids

4.1. Effects of Pressure and Temperature on the Structure and Activity of Enzyme

Supercritical fluids are materials above their critical temperature and critical pressure. Sub-critical fluids refer to liquid at temperatures between their atmospheric boiling point and critical temperature. The physical properties of sub-/super-critical (SC) fluids, such as density, polarity, diffusivities and viscosities, are sensitive to the pressure and temperature. Since these properties of solvents exert great impacts on the structure, stability, enantioselectivity and mass transfer rate of enzyme, biocatalyzed reactions with specific requirements (especially high enzyme activity and enantioselectivity) can be achieved by tuning the temperature and pressure of the SC fluids.

In SC fluids, enzyme activity usually firstly increases with increasing temperature, and then decreases with the further increase of temperature due to thermal deactivation. For instance, Knez *et al.* [88] studied the activity of lipase in SC-CO_2 in the temperature range of 40–80 °C and pressure range of 80–450 bar. They found that, at various pressures, the lipase activity showed maximal activity within 50–60 °C. Similarly, the subtilisin and *Aspergillus* proteases had highest activity at 50 °C in supercritical fluids [89]. Kamat *et al.* [89] studied the effect of pressure on the lipase activity in SC-fluoroform, and found that the activity reached maximum value near the critical point of fluoroform, and then gradually approached zero as pressure increased. In our previous work, we evaluated the effects of SC-CO_2 pretreatment, including pressure (6 and 10 MPa), exposure time (20, 30, and 150 min) and temperature (35 and 40 °C), on the conformation (e.g., secondary and tertiary structures) and catalytic properties (e.g., residual activity, kinetics constants (K_m and V_{max}), activation energies (E_α), thermo-stability, and organic solvent tolerance) of two commercial enzymes CALB and lipase PS in their solution forms. Results showed that the catalytic activities and kinetic constants of both lipases were markedly altered by SC-CO_2 pretreatment due to the changes of α-helix content in the secondary structure as well as tertiary structure of the enzymes [8]. In particular, for the biocatalysis in SC-CO_2, pressure variance could significantly alter the interactions between CO_2 and enzyme through the formation of carbamates by CO_2 and the free amine groups of the enzyme. These interactions might gradually change the conformation and activity of the enzyme in response to pressure [8,90–92].

The stability of enzyme is usually assessed by measuring residual activity after incubation with sub-/super-critical fluids. Hu *et al.* [93] reported that the residual activity of tyrosinase showed a significant reduction of about 25%–30% after the pretreatment of SC-CO_2 under the condition of 8–12 MPa, 35 °C, and 20 min pretreatment time. At 8 MPa and 55 °C, the residual activity decreased by 40% after 20-min pretreatment of SC-CO_2. However, Liu *et al.* [94] observed that after high pressure SC-CO_2 pretreatment (100 MPa and 25 °C), the activity of mushroom polyphenoloxidase enhanced by 11% compared with the native enzyme (0.1 MPa and 25 °C). Kamat *et al.* [89] reported that the lipase stability increased with increasing temperature in SC-CO_2 since high temperature could inhibit carbamate formation.

Natalia *et al.* [95] studied the selectivity of benzaldehyde lyase (BAL) in four supercritical fluids (carbon dioxide, fluoroform, ethane, and sulphur hexafluoride), and found that the enzyme

enantioselectivity was almost racemic with the highest enantiomeric excess for fluoroform (40%). However, when excess water was added to the supercritical fluids, the enantiomeric excess increased up to more than 90% for fluoroform, ethane, and sulphur hexafluoride, indicating that water activity was a main factor in the selectivity. Ottosson *et al.* [96] demonstrated that there was a correlation between enzyme enantioselectivity and the molecular volume of the solvent when CALB was used as a catalyst for the transesterification of *sec*-alcohol in eight liquid organic solvents and SC-CO_2. The correlation was explained by the fact that a solvent with large molecular volume would lose translational entropy of fewer solvent molecules than that with smaller molecular volume when restricted in the active site, resulting in higher enantioselectivity.

4.2. Biocatalysis in Mixture Solvents of Organic Solvent and Supercritical Fluid

Randolph *et al.* [97] investigated for the first time the catalytic performance of cholesterol oxidase from *G. Cirysocreas* in SC-CO_2 mixed with six organic solvents. Results indicated that reaction rate slightly decreased with increasing methanol content, while progressively increased with increasing contents (*v*/*v*, 0%–2.5%) of acetone, *n*-butanol, ethanol, isobutanol, and *tert*-butanol. The authors asserted that this phenomenon could not be explained by the solubility data of substrate cholesterol in these solvents. However, it was attributed to the conformational change of the enzyme and the aggregation degree of cholesterol in organic solvent. The greater the aggregation of cholesterol caused by organic solvent, the larger increase in the rate of enzymatic oxidation in the mixture solvent.

Liu *et al.* [8] reported that two commercial lipases, CALB and lipase PS, showed high tolerance towards the five tested organic solvents with the relative activity of more than 85% following SC-CO_2 pretreatment in the condition of subcritical CO_2 (6 MPa, 35 °C, 30 min) and supercritical CO_2 (10 MPa, 40 °C , 30 min). CALB showed higher hydrolysis activity in hexane (log *P* = 3.5) and isooctane (log *P* = 4.5), in which CALB performed more than 80 U/g, whereas lipase PS from *Pseudomonas cepacia* showed higher activity in isopropanol (log *P* = 0.28) and acetonitrile (log *P* = −0.33). The residue activity of lipase PS in isopropanol and acetonitrile was 140 and 110 U/g, respectively. It was postulated that log *P* was an important factor that characterizes the catalytic activity of lipases in an organic solvent. This was probably due to a variation of water retained in the microenvironment of the catalytic active site, which is essential for the maintenance of the dynamical properties of the enzyme. However, conformation analysis of enzyme has not been reported in the mixture solvents of organic solvent and supercritical fluid so far.

4.3. Biocatalysis in Combined Mixture Solvents of Ionic Liquid and Supercritical Fluid

Reports have easily been available on the biocatalysis reactions in the mixture solvents of IL and SC-CO_2 fluid [97–100]. Bogel-Łukasik *et al.* [101] applied a ternary system of [Omim][PF_6]/SC-CO_2/products for the acylation of (*R*,*S*)-2-octanol with succinic anhydride catalyzed by lipase. They stated that the recovery of >99.99 mol % was obtained at optimized conditions of 35 °C and 11 MPa. Lozano *et al.* [102] described the utilization of [Emim][Tf_2N]/SC-CO_2 and [Bmim][Tf_2N]/SC-CO_2 systems for the transesterification of vinyl butyratewith 1-butanol and the kinetic resolution of *rac*-1-phenylethanol with vinyl propionate by CALB. In both systems, the enantiomeric excess of the recovered product fraction (ee_p) was above 99.9% for continuous (*R*)-1-phenylethyl propionate synthesis at 100 °C and 15 MPa, and the enzyme showed excellent activity and stability.

Through assaying the property of CALB in five different SC-CO_2/IL systems based on quaternary ammonium cations and Tf_2N anion, it was observed that all of the five ILs acted as enzyme stabilizing agents with respect to hexane, leading to increasing the free energy of deactivation (to 25 kJ/mol protein) and an improvement in the half-life time of the enzyme (2000-fold) [103]. Monhemi *et al.* [100] confirmed it through all-atom MD simulation. It was showed that enzyme and IL molecules formed a supramolecular-like structure in SC-CO_2, where IL molecules function as a coating layer and protect enzyme from denaturing condition in SC-CO_2. The data of root mean square deviation implied that

the enzyme had more native and stable conformation in SC-CO_2/IL system than in SC-CO_2. Moreover, based on the radius of gyration values, it was found that enzyme had a more compact and active conformation in SC-CO_2/IL system than in SC-CO_2.

On the other hand, the combination of SC-CO_2 and IL could also achieve higher reaction rate than IL alone by decreasing the viscosity of IL and enhancing the mass transfer [104]. Therefore, enzymes showed higher activity and stability in the mixture solvents of IL and SC-CO_2 than the corresponding single medium. Interestingly, a homogeneous enzymatic reaction in SC-CO_2/IL system could be achieved by elevating pressure; and a subsequent phase separation would be attained by lowering the pressure, where free or immobilized enzyme dissolved or suspended in the ionic liquid phase (catalytic phase), while substrates and/or products resided largely in the supercritical phase (extractive phase).

In summary, more attention should be focused on developing the bioreactor integrated with high efficiency reaction and easy product separation in SC-CO_2/IL systems in the coming years.

5. Remarks and Prospects

Enzymes in non-aqueous solutions have been largely studied and employed in the areas of food, synthesis, pharmaceuticals, and analysis. The utilization of non-aqueous solutions as reaction media could enable high enzyme activity and stability, alter enzyme selectivity, and facilitate the transformation of substrates that are unstable or poorly soluble in water. To take full advantage of the opportunities afforded by non-aqueous enzymology and develop more feasible industrial processes, this article comprehensively reviewed the structure-activity relationship of enzymes in organic solvents, ILs, sub-/super-critical fluids, and their combined mixtures systems. As for organic solvents and ILs media, molecular interactions between the enzyme and the solvent dramatically affect the advanced structure of the enzyme and therefore its activity and stability. Generally, solvent molecules or functional groups that interact with the enzyme through weak interactions could hold the essential bound water on the enzyme surface, stabilizing the native structure and retaining the activity of the enzyme. Unlike organic solvents, ILs also interacts with the enzyme through electrostatic interaction due to the charged cation and anion. Kosmotropic anions and chaotropic cations of ILs, according to the Hofmeister series, usually act as good stabilizers of enzymes, though anions exert much greater influence on the enzyme properties owing to the strong hydrogen bonding between them. However, due to the complex interactions involved in them, it is difficult to provide a general basis for assessing the impacts of ILs on enzyme conformation and activity. In case of sub-/super-critical fluids medium, the main advantage for enzyme-catalyzed reactions is the tunability of solvent properties. Therefore, the enzyme activity and product separation efficiency are dependent on temperature and pressure. It is commonly suggested that the deactivation of enzyme in sub-/super-critical fluids is caused by carbamate formation or acidification of reaction media. Interestingly, using binary mixture media of organic solvents, ILs, or sub-/super-critical fluid could effectively eliminate the demerits of the single solvent whilst preserving the merits [105].

Despite the advance of mechanisms and applications on non-aqueous enzymology, there is still a great deal of space to be improved on the way of reserach. First of all, more efforts should be made to understand the causes of reduced enzyme activity in non-aqueous media and how to prevent it. The synergies of solvent engineering and protein engineering could be a potential strategy to enhance enzyme catalytic properties in non-aqueous media. Second, it is urgent to test more enzymes in non-aqueous media, especially complex enzymes. Finally, it is required to illustrate the mechanisms in deeply and to screen more solvent-tolerant bacteria and fungal strains producing enymes. The advances in the understanding of biocatalysis in non-aqueous systems will open a new pathway to elucidate the mechanism between structure and activity of enzymes, which will facilitate the screening of a suitable reaction medium for biotransformation.

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