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Effect of ionic liquid properties on lipase stabilization under microwave irradiation

Hua Zhao [a,*], Gary A. Baker [b,*], Zhiyan Song [a], Olarongbe Olubajo [a], Lavezza Zanders [a], Sophia M. Campbell [a]

[a] Chemistry Program, Savannah State University, Savannah, GA 31404, USA
[b] Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

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ABSTRACT

Ionic liquids (ILs) as neoteric solvents and microwave irradiation as alternative energy source are becoming two important tools for many enzymatic reactions. However, it is not well understood what properties of ILs govern the enzyme stabilization, and whether the microwave irradiation could activate enzymes in ILs. To tackle these two important issues, the synthetic activities of immobilized *Candida antarctica* lipase B (Novozyme 435) were examined in more than twenty ILs through microwave heating. Under microwave irradiation, enhanced enzyme activities were observed when the enzyme was surrounded by a layer of water molecules. However, such enhancement diminished when the reaction system was dried. To understand the effect of IL properties, the enzyme activities under microwave irradiation were correlated with the viscosity, polarity and hydrophobicity (log *P*) of ILs, respectively. The initial reaction rates bear no direct relationship with the viscosity and polarity (in terms of dielectric constant and E_T^N) of ILs, but have a loose correlation (a bell curve) with log *P* values. The enzyme stabilization by ILs was explained from aspects of hydrogen-bond basicity of anions, dissolution of the enzyme, ionic association strength of anions, and substrate ground-state stabilization by ILs.

1. Introduction

The use of non-aqueous media has offered many advantages to the biocatalysis field, including better substrate dissolution, improved enzyme's thermal stability, enhanced enzyme selectivity, and more synthetic strategies [1–3]. However, the volatility and toxicity of many conventional organic solvents are obvious drawbacks of this approach. To substitute these organic solvents, a new type of non-volatile solvents known as ionic liquids (ILs), has recently gained serious attention in biocatalysis [4], mainly due to their extremely low vapor pressures and designable physical/chemical properties. Although many researchers have reported high enzyme activity and stability in ILs, it is still not quite clear what properties of ILs affect the catalytic behaviors of enzymes.

In addition to factors such as water activity, pH, excipients and impurities [5], several solvent properties of ILs are often related to the enzyme's destabilization, such as polarity [6–8], hydrogen-bond basicity [9–11], anion nucleophilicity [12], IL network [13,14], ion's kosmotropicity [15–23], and viscosity [8,24]. However, none of these properties are solely responsible for enzyme functions in ILs; in many cases, multiple factors have to be considered. To further understand the solvent effects on enzyme stabilization, the *first objective* of this study is to investigate the general properties governing the enzyme's activity in ILs.

The *second objective* of this study is to examine whether microwave irradiation (*vs.* conventional conductive heating) induces enzyme activation in ILs. Microwave irradiation has become a routine heating device employed in various chemical reactions, and is an energy-efficient heating method for sealed-vessel processes [25]. However, its applications as an energy source for enzymatic synthesis are rather limited [26,27]. The biggest controversy in the field of microwave-promoted reactions is whether the rate acceleration is caused by the non-thermal effect of microwaves. This controversy is partly due to the fact that many earlier reactions were simply cooked in household microwave ovens without accurate control of temperatures. Many investigators argued that the reaction-rate increase in response to microwave irradiation is caused by the superheating of solvents beyond their normal boiling-points [28,29]. Other investigators, however, proposed the existence of non-thermal effect based on

* Corresponding authors. Tel.: +1 912 353 5290.
E-mail addresses: zhaoh@savstate.edu, huazhao98@gmail.com (H. Zhao), bakerga1@ornl.gov (G.A. Baker).

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Table 1
Initial lipase activities in organic solvents and ILs at 40 °C under microwave irradiation, and correlations with physical properties of solvents[a]

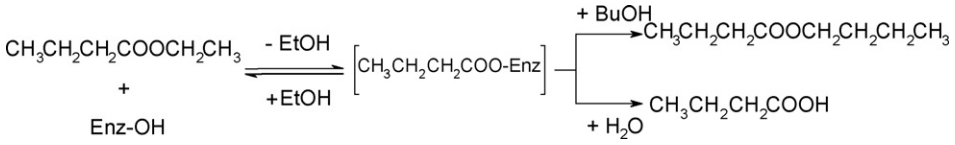
	Solvent	Halide[a] (ppm)	Initial activity (μmol/min/mg *E*)	Dielectric constant ε_r (*T* in °C)	E_T^N (25 °C)	H-bond basicity (β) (25 °C)	Viscosity (cP) (*T* in °C)	C_p (J/g K) (*T* in °C)	log *P*
1	*t*-BuOH	None	1.65	12.47 (25)[b]	0.395[h]	0.95[t]	4.312 (25)[b]	2.97 (25)[b]	0.35[n]
2	1-BuOH	None	0.60	17.84 (20)[b]		0.85[t]	2.544 (25)[b]	2.39 (25)[b]	0.84[n] (0.80[k])
3	DCM (CH_2Cl_2)	None	0.56	8.93 (25)[b]	0.309[f]	−0.014[f]	0.413 (25)[b]	1.19 (25)[b]	1.25[t1,n] (0.633[l])
4	THF	None	0.98	7.52 (22)[b]	0.207[h]	0.523[t]	0.456 (25)[b]	1.72 (25)[b]	0.46[n] (0.49[k])
5	Acetonitrile	None	0.44	36.64 (20)[b]	0.460,[f] 0.451[h]	0.370[f], 0.31[r]	0.369 (25)[b]	2.23 (25)[b]	−0.34[n] (−0.33[k])
6	[EMIM][Tf_2N]	600 Br^-	0.86	12.3 (25)[d,e]	0.676[i]		34 (25)[o]		−1.18[y] (−1.05 to −0.96[w])
7	[BMIM][Tf_2N]	260 Br^-	0.76	11.7 (25)[d]	0.642[g]	0.243[f]	52 (20)[o]	1.05 (25)[q]	0.11±0.01 (−0.96 to −0.21[w], 0.33[y])
8	[HMIM][Tf_2N]	140 Cl^-	0.30		0.654[i]		79.5 (70)[s1]		0.64±0.01 (0.15 to 0.22[w], 0.65[y])
9	[BMIM][PF_6]	160 Cl^{-x}	0.64	11.4 (25)[c]	0.667[g]	0.207[f], 0.21 (20)[s]	207 (25)[o]	1.14 (25)[q]	−1.66[w] (−2.39±0.27,[m,y] −2.38 ± 0.25[r1],−2.06[y])
10	[EMIM][OAc]	350 Br^-	0.12		0.814[v]		162 (20)[o]		−2.53 ± 0.02
11	[EMIM][TFA]	2830 Br^-	0.00				35 (20)[o]		−2.75 ± 0.28
12	[HMIM][TFA]	510 Cl^-	0.14						−2.30 ± 0.21
13	[BMIM][dca]	*n/d*	0.12						−2.32 ± 0.02
14	[EMIM][BF_4]	2420 Br^-	0.04	12.8 (25)[c]	0.71[j]		34 (25)[o]	1.28 (100)[p]	−2.57 ± 0.06
15	[BMIM][BF_4]	160 Cl^{-x}	0.21	11.7 (25) [c]	0.673[g]	0.376[f]	119.78 (25)[p]	1.66 (100)[p]	−2.51 ± 0.04 (−2.44 ± 0.23[r1], −2.52[w])
		9160 Br^-	0.10						
16	[OMIM][BF_4]	150 Cl^{-x}	0.53				439 (20)[aa]		−1.34 ± 0.09 (−1.14[y])
		6050 Br^-	0.31						
17	[EtPy][TFA]	620 Br^-	0.08						−2.57 ± 0.11
18	[EtPy][Tf_2N]	480 Br^-	1.14						−0.90 ± 0.06
19	[BuPy][Tf_2N]	590 Cl^-	0.89				76 (25)[z]		−0.26 ± 0.08
20	[BMPyo][Tf_2N]	800 Br^-	0.91						
21	[Amm110]Cl	*n/d*	0.16		0.569[v]		572 (25)[u]		
22	[Amm110][dca]	*n/d*	0.89		0.543[v]		512 (25)[u]		
23	[Bu_3MeN][beti]	870 Br^-	0.29		0.832[v]		687 (25)[u]		
24	[Bu_3MeN][Tf_2N]	600 Br^-	0.35		0.628[v]				
25	[$OctMe_3N$][beti]	460 Br^-	0.15		0.569[v]				
26	[Oct_3MeN][Tf_2N]	300 Br^-	0.32		0.451[v]				
27	[Et_3HexN][Tf_2N]	640 Br^-	0.61		0.569[v]				

[a]All reactions were conducted in 2.0 mL solvent with 50 mg Novozyme 435; the initial rates were based on the mass of immobilized enzyme, not the actual protein content (20%); X^- is the impurity of halides (the type of halide is determined based on the starting materials used; *n/d* means 'not determined') meanings of ILs see section 2.1; [b]data from Ref. [55]; [c]Ref. [114]; [d]Ref. [115]; [e]Ref. [116] (this Ref. also reported [EMIM][Tf_2N] ε_s = 15.76 or 14.01); [f]Ref. [82]; [g]Ref. [117]; [h]calculated from $E_T(30)$ data in Ref. [118]; [i]Ref. [119]; [j]Ref. [7]; [k]Ref. [79,80]; [l]calculated from hydrophobic fragmental constants in Ref. [120,121]; [m]Ref. [12]; [n]Recommended value of Sangster in Ref. [122]; [o]from a collection by Mantz and Trulove in Ref. [123]; [p]Ref. [124]; [q]Ref. [125]; [r]Ref. [126]; [s]Ref. [127]; [t]Ref. [128]; [r1]Ref. [129]; [s1]Ref. [130]; [t1]Ref. [131]; [u]determined by this study using the Cannon-Fenske Routine (CFR) viscometer at 25 °C using benzyl alcohol as the reference (5.474 mPa s [132]) (based on Solvent Innovation, [Amm110]Cl has a viscosity of 495 at 20 °C; the data reported here are for dried ILs); [v]determined by this study; [w]calculated from octanol–water partition coefficient (K_{OW}) in Ref. [133]; [x]the identity of halide is unknown, and the impurity was calculated as Cl^- concentration; [y]ef. [134,135]; [z]Ref. [136]; [aa]Ref. [137].

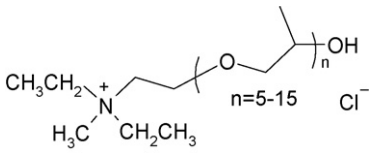
the argument that polar functional groups exhibit higher reactivity with adjacent reactants under microwaves than under conductive heating at the same temperature [30]. Nowadays, specialized microwave reactors equipped with *in situ* temperature and pressure controls are commercially available [31]. With this technology, some researchers still reported higher enzyme activities and selectivities in organic solvents under microwave irradiation [32–34]. Contradictorily, recent studies suggested that the microwave irradiation and thermal heating made no difference on the synthetic activities of *C. antarctica* lipase B (CaLB) at various temperatures (40–100 °C) [35–37].

Despite such a controversy, the utilization of ILs as media for microwave-assisted reactions is advantageous [38–40]. Organic solvents are usually flammable and volatile, which is a safety hazard for high-temperature and closed-vessel applications using microwaves. In contrast, ILs are ideal solvents for microwave reactions because they have high boiling-points, low vapor pressures and high thermal stabilities [41,42]. In addition, typical ILs have moderately high dielectric constants (in the range of 10–15), and relatively low heat capacities (in the range of 1–2) (see Table 1 and Ref. [43]). This unique combination allows ILs to absorb microwaves efficiently and to be heated up easily. Owing to these advantages, ILs have been investigated as solvents in a number of microwave-mediated reactions [38–40,44]. However, the use of microwave irradiation for enzymatic catalysis in ILs is very limited (for example, Ref. [45]).

To achieve the above two objectives, a model reaction chosen for this study is the transesterification between ethyl butyrate and 1-butanol catalyzed by Novozyme 435 (immobilized *C. antarctica* lipase B). Scheme 1 illustrated a fast formation of an acyl-enzyme



Scheme 1. Lipase catalyzed transesterification of ethyl butyrate with 1-butanol.



Scheme 2. Structure of AMMOENG™ 110 ([Amm110]Cl).

intermediate, followed by the competition between esterification and hydrolysis.

2. Materials and methods

2.1. Materials and enzyme

N-ethylpyridinium bromide ([EtPy]Br) and *N*-*n*-butylpyridinium chloride ([BuPy]Cl) were obtained from the Alfa Aesar Company (Ward Hill, MA, USA). 1-Ethyl-3-methylimidazolium hexafluorophosphate ([EMIM][PF_6]) purchased from TCI America (Portland, OR, USA), was washed with water twice to remove soluble halides, followed by an intensive drying before use. 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) (purity 99.9%, water content 0.017%, ≤100 ppm chloride), 1-methyl-3-octylimidazolium tetrafluoroborate ([OMIM][BF_4]) (purity ≥97.0%), 1-ethyl-3-methylimidazolium bromide ([EMIM]Br), 1-butyl-3-methylimidazolium bromide ([BMIM]Br), 1-hexyl-3-methylimidazolium chloride ([HMIM]Cl), 1-butyl-1-methylpyrrolidinium bromide ([BMPyo]Br), silver acetate (Ag[OAc]), silver trifluoroacetate (Ag[TFA]), silver tetrafluoroborate (Ag[BF_4]), Sodium dicyanamide (Na[dca]), bis(trifluoromethane)sulfonimide lithium salt (Li[CF_3SO_2]$_2$N, also known as Li[Tf_2N] or Li[TFSI]), phosphorus pentoxide (P_2O_5), Reichardt's dye, and other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bis(perfluorethylsulfonyl)imide lithium salt (Li[$CF_3CF_2SO_2$]$_2$N, or known as Li[beti]) was obtained from IoLiTec Ionic Liquids Technologies GmbH & Co. (Denzlingen, Germany). AMMOENG™ 110 (short as [Amm110]Cl, Scheme 2) was obtained from Solvent Innovation GmbH (Nattermannallee, Germany) as a colorless liquid (mp <−65 °C, density = 1.03 g/cm^3 at 20 °C, viscosity = 495 mPa s at 20 °C, pH 3.83 and conductivity = 0.090 mS/cm).

Novozyme 435® obtained from Sigma–Aldrich is a thermal stable lipase B from *C. antarctica* (CaLB) immobilized on acrylic resin (0.3–0.9 mm in diameter and 0.430 g/cm^3 in bulk density). Its optimum reaction temperature range is 40–60 °C. The specific activity is 7000 PLU/g. This immobilized enzyme contains about 20 (wt.%) of CaLB [46]. To find the water content of this enzyme, a preweighed sample of enzyme particles was dried in an oven at 100 °C overnight. The weight loss was found to be ~3% (a literature value is 2% H_2O [35]). However, the saturation water content of this enzyme could be as high as 16% [47]. For experiments requiring the dried enzyme, a small amount of enzyme in an open-top vial was placed in a sealed vessel containing the drying agent P_2O_5 for at least 24 h.

2.2. Microwave equipment

The microwave oven (operated at 2.45 GHz) is a commercial monomode unit known as the CEM® Discover Lab-Mate (CEM Corporation in Matthews, NC). This unit is equipped with an infrared temperature probe and pressure monitoring sensor. The microwave oven is controlled by the Discover Applications Software (ChemDriver 3.6) provided by CEM. The temperature, power and pressure profiles were recorded by this software. The reaction was conducted in a 10 mL glass reactor, which was sealed and then clamped on top by the microwave unit. The reaction mixture was stirred by a Teflon-coated magnetic bar in the solution through a rotating magnetic plate beneath the microwave cavity.

2.3. Preparation of ILs

Most hydrophilic ILs were synthesized using the silver-salt metathesis method [7,17]. The hydrophobic ILs (Tf_2N^- and beti$^-$) were synthesized through a precipitation reaction [48]: an aqueous solution of halide based IL (1 molar equiv.) reacted with Li[Tf_2N] (1.1 molar equiv) solution for 2 h. After the phase separation, the IL phase was washed with distilled water several times until no halides could be detected by the silver nitrate test. All final ILs were dried in an oven at 102 °C over 24 h. All prepared ILs are colorless liquids. Dried Acros® 3A molecular sieves were added into ILs to remove residual water. ^{1}H NMR, FT-IR and HPLC spectra confirmed the structures and purities of prepared ILs.

[Amm110][dca] (dca = dicyanamide) was prepared through an anion-exchange method. The procedures are briefly described as the following: about 100 mL of the resin (Amberlite® IRA-400 Cl, 1.4 meq/mL by wetted bed volume, 16–50 mesh) was packed in a glass chromatography column, and washed thoroughly with distilled water and methanol, until the eluent became colorless and no precipitate in the eluent could be detected by 0.1 M $AgNO_3$ solution. The Cl^- ions in the resin were exchanged by dca^- ions through slowly washing the column with an excess amount of sodium dicyanamide solution. The column was then washed with distilled water to remove residues of sodium dicyanamide in the column. [Amm110]Cl (42.0 g) in 200 mL water was further slowly dripping through the column, and the eluting solution was collected and decolorized by activated charcoal. Water was removed from the clear solution through a rotary evaporator under vacuum at 60 °C. The product was further dried in oven at 102 °C over 24 h, yielding 33.4 g colorless and viscous liquid. Dried Acros® 3A molecular sieves were added into the IL during storage.

The halide contents in ILs were determined by the silver chromate method [49]: 1.5 g aqueous solution of K_2CrO_4 (27 mM, as indicator) containing about 0.1 g IL was titrated with 1.4 mM silver nitrate until a red precipitate of silver chromate appeared. The halide content (in ppm) was calculated as the ratio of halide mass over IL mass.

2.4. Enzymatic transesterification

Organic solvents were dried by anhydrous $MgSO_4$ before use. The enzyme was not usually dried over P_2O_5 unless indicated otherwise. A typical reaction procedure is as followings: 2.0 mL solution of organic solvent or ionic liquid containing substrates of 0.1 M ethyl butyrate (26.5 μL) and 0.5 M 1-butyl alcohol (91.5 μL) was placed in a 10 mL capped glass-tube (specifically designed for the microwave oven), followed by the addition of 50 mg Novozyme 435. The reaction mixture was stirred at a constant temperature in a water-bath or under microwave irradiation. When the microwave was used, 1–2 W of energy was sufficient to maintain the desired low temperature (40 °C) for reactions conducted in ILs, and generally less than 10 W energy was needed for organic solvents. Samples of the reaction mixture (50 μL) were periodically withdrawn and diluted with 100 μL of HPLC eluent. The eluent consisted of 65% (v/v) MeOH and 35% (v/v) aqueous acetate buffer (0.05 M, pH 4.5). The flow rate was 1.0 mL min^{-1}. The column is a Schimadzu Premier C18 column (150 mm × 4.6 mm, particle size 5 μm). The LC-10AT Schimadzu HPLC is equipped with a SPD-10Avp UV–vis dual wavelength detector and a Schimadzu RID10 refractive index detector. The UV detection wavelength was 215 nm. The injection volume was 20 μL. The product concentration was determined by comparing the sam-

ple's peak area with the standard curve of butyl butyrate. The initial rate was calculated based on the yield of butyl butyrate at 15 min of reaction time (although the reaction was normally monitored for over 1 h). All experiments were run at least in duplicate. The percent errors were less than 5%.

2.5. Determination of log P

In determining log *P* values of imidazolium ILs, a shake flask method [12,50] was modified. Distilled water saturated with 1-octanol, and 1-octanol saturated with distilled water were used in all measurements. 1-Octanol and water in various volume ratios were added into an 8 mL Wheaton sample vial. The 1-octanol/water volume ratio is 3/2 for all Tf_2N^- based ILs, 6/2 for [BMIM][PF_6], and 7/1 for all water-miscible ILs. A small amount of IL (~5 mg) was added into the vial to ensure that the IL concentration in either phase was less than 10 mM. The sample vial was repeatedly inverted for 5 min at a rate of about 30 inversions per min. The mixture in the vial was completely separated into two clear layers after several hours. There were no or little emulsions observed in this process. The IL concentrations in both phases were analyzed by HPLC: an aliquot of 50 μL was withdrawn from 1-octanol or aqueous layer, and diluted with 100 μL of HPLC eluent. The HPLC eluent was a 70/30 (v/v) MeOH/H_2O mixture. The same HPLC and column described previously were used for the log *P* determination. The detection wavelength was 210 nm since imidazolium ILs showed a strong absorbance near this wavelength [51]. All experiments were run in duplicate. Since the HPLC integrated area of IL is proportional to its concentration, the partition coefficient (*P*) was simply calculated by dividing the IL area of 1-octanol layer over the IL area of aqueous layer.

2.6. Measurement of IL polarity (E_T^N scale)

Reichardt's dye in methanol was added into 1.0 mL solvent, followed by evaporation of methanol by vacuum at 40 °C. The final concentration of Reichardt's dye was 100 μM. The visible spectrum of the dye solution was measured at 25 °C. The E_T^N scale was calculated from the wavelength of maximum absorption λ_{max} (nm) of Reichardt's dye in the solvent by Eq. (1).

$$E_T^N = \frac{28591/\lambda_{max} - 30.7}{32.4} \quad (1)$$

Based on this method, the E_T^N value of methanol was determined as 0.759 (λ_{max} = 517 nm) by this study, which is very close to the literature value (515 nm) [52].

3. Results and discussion

3.1. Effect of IL purity

The purity of ILs is known to affect their physical properties, and further influence the reactions performed in them [53]. Recently, Lee et al. [11] reported that the activity of Novozyme 435 in [OMIM][Tf_2N] decreased linearly with the chloride content, and 1 (wt.%) increase in [OMIM]Cl (~1540 ppm Cl^-) caused 5% decrease in enzyme activity. However, another lipase (from *Rhizomucor miehei*) could tolerate much less Cl^-; its activity in [OMIM][Tf_2N] with 2% [OMIM]Cl was only about 2% of the activity in pure [OMIM][Tf_2N]. Their model reaction was the transesterification of vinyl acetate with benzyl alcohol. The Víllora group [54] also observed higher CaLB activities when the impurities were removed from ILs through washing them with aqueous solutions of $NaHCO_3$ or Na_2CO_3. Our data in Fig. 1 suggested that when 3000 ppm Cl^- or Br^- anions were present in [BMIM][Tf_2N], the initial rates were 0.45 and 0.41 μmol/(min mg enzyme), respectively, which were ~60% of the activity (0.76) in [BMIM][Tf_2N] with little halide (260 ppm Br^-). These data suggest that the degree of halide inhibition depends on specific substrates and enzymes. In our model reaction, when the halide contents of ILs fell below 1000 ppm, there was no direct correlation between the lipase activity and the halide concentration (Table 1). However, it is always important to determine the halide contents of ILs before using them in enzymatic reactions. Halides and other impurities should be removed from ILs as much as possible.

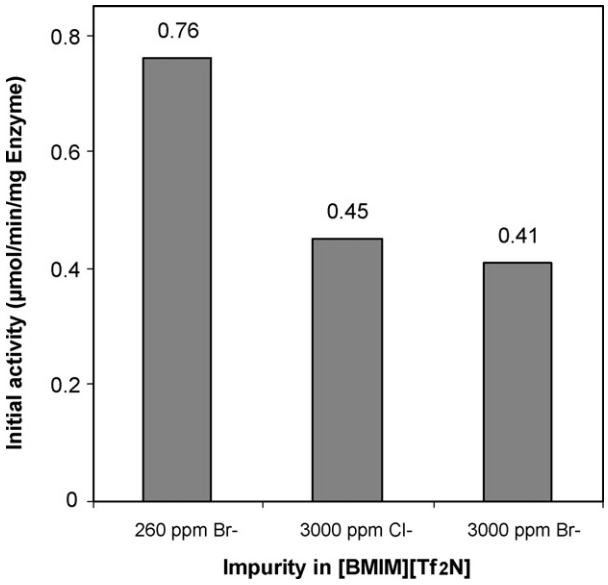


Fig. 1. Effect of halide contents in [BMIM][Tf_2N] on lipase activity (40 °C, microwave irradiation).

3.2. Effect of microwaves on the lipase activity

As shown in Table 1, most ILs have moderately high dielectric constants (usually 10–15), which are close to that of *t*-butanol (12.47 at 25 °C, all dielectric data of organic solvents from Ref. [55]). We conducted a series of experiments (Fig. 2) in order to better understand the microwave effect. When the solvents (dichloromethane or [BMIM][Tf_2N]) and substrates were dried[1] but the lipase was not intensively dried (containing ~3 wt.% water), higher reaction rates were observed under microwaves than those in water-bath. However, when the enzyme was also dried (over P_2O_5), the differences in reaction rates became insignificant. On the other hand, if 1% (v/v) water was added into the bulk solvent ([BMIM][Tf_2N]) and the enzyme was not dried, such a difference also diminished. This interesting behavior has actually been reported by a number of papers. When the substrates and/or enzyme(s) were not completely dried or a small amount of water was added into the reactions (*i.e.* the enzyme is surrounded by at least a layer of water), the reaction rates were generally higher under microwaves than through regular thermal heating [32–34,59-64]. On the other hand, if the enzyme and substrates were intensively dried before reactions, the microwave irradiation

[1] Most ILs are hydroscopic in nature and may absorb water from air. After 24 h exposure to air, the water contents in [BMIM][BF_4], [BMIM][Tf_2N] and [BMIM][PF_6] are 0.320, 0.097 and 0.083 M, respectively [56]. At room temperature, water content at saturation in [BMIM][PF_6] is about 1.8% (v/v) [57], or 2.1 (wt.%) [58], and in [EMIM][Tf_2N] and [BMIM][Tf_2N] is 1.4 (wt.%) [48]. Our ILs were dried in oven and over molecular sieves, and FT-IR spectra did not show the —OH absorption peak.

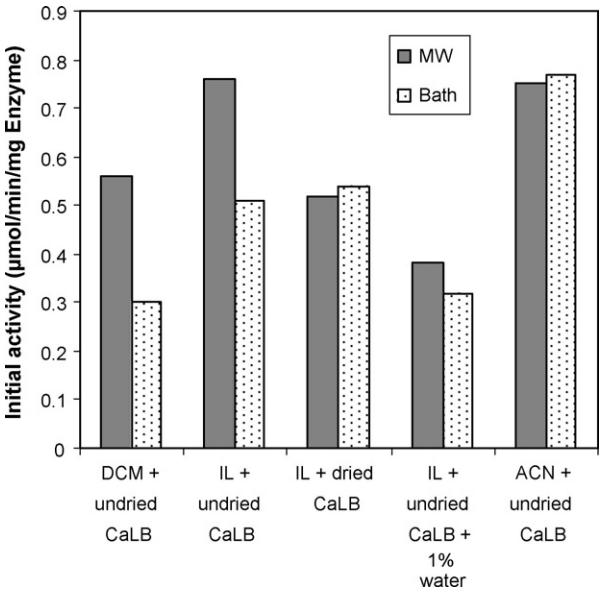


Fig. 2. Influence of microwave (MW) irradiation and water content on enzyme activity (temperature is 40 °C; DCM is dichloromethane; IL is [BMIM][Tf_2N]; ACN is acetonitrile).

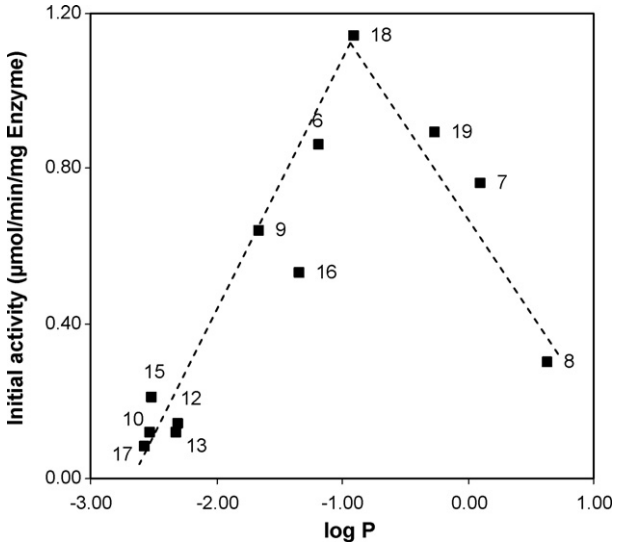


Fig. 4. Relationship between the enzyme activity and solvent's log *P* values (40 °C; Novozyme 435 was used as received without drying with P_2O_5; plot from data in Table 1, and the solvent numbers in the graph are consistent with those in Table 1; the dash-lines are to guide the general trend, not to make correlations).

and conductive heating made no difference on the initial rates and conversion rates of the same reaction catalyzed by free or immobilized CaLB at temperatures ranging from 40 to 100 °C (however, the same group reported that the microwave irradiation caused a slower enzyme inactivation in 1-butanol than the thermal heating did) [35,36].

This fascinating phenomenon might be explained by the superheating of the water layer near the enzyme, rather than a true non-thermal effect. As illustrated in Fig. 3(a), in a fairly dried hydrophobic solvent and substrate environment, the enzyme particle is surrounded by (at least) a layer of water molecules. The solvent is hydrophobic so it does not strip off the water layer. In this microenvironment, the water layer near the enzyme surface has a much higher dielectric constant (80.1 at 20 °C) than surrounding organic solvent or IL (in the range of 7–40, mostly 7–20). The static dielectric constant of dry protein powder is about 4 [65–68]. The immobilization support (acrylic resin) has a ε_r value in the range of 2.7–4.5 [69] (most plastics are in the range of 2–4). Therefore, under microwave irradiation, the enzyme's surface is likely to have a higher temperature than the bulk solvent due to the superheating of the water layer. This could explain the higher reaction rates observed under microwaves when the enzyme was not dried but the solvent and substrates were dried. If the enzyme is also intensively dried, this layer of water is expected to disappear. Dolman et al. [70] observed that during an intensive drying over P_2O_5, lysozyme and subtilisin Carlsberg lost the surface layer of 'essential water', but retained 3–4 and 14–16 buried (or structural) water molecules per enzyme molecule, respectively. In this case, there is no superheating of the enzyme's surface, therefore, the reactions rates under both heating modes are very close. If additional water is added and dispersed (through vigorous stirring) into the bulk solvent (Fig. 3b), the overall dielectric constant of the medium is increased and the superheating effect on the enzyme surface is reduced. In acetonitrile, the hydrophilic solvent tends to strip the water molecules off the enzyme surface and disperses them throughout the reaction system. As a result, no superheating of the enzyme's surface and no enhanced rate were observed under microwave irradiation (Fig. 2). However, it is interesting to mention that in aqueous solutions the non-thermal effect has been observed for thermophilic enzymes [71,72].

3.3. Effect of IL viscosity

Lozano et al. [8] concluded that in addition to the IL polarity, the activity of α-chymotrypsin also depended on the IL viscosity; a higher enzyme activity was observed in [EMIM][Tf_2N] than in [MTOA][Tf_2N] (MTOA = methyl trioctylammonium) because the

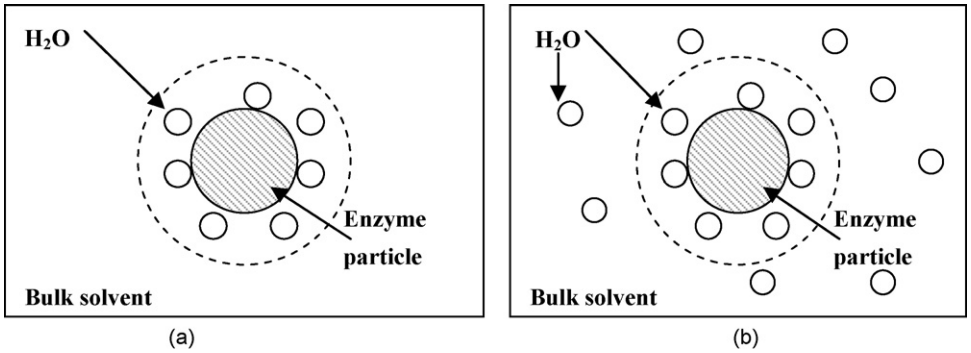


Fig. 3. Illustrations of water-induced superheating: (a) the free or immobilized enzyme particle is surrounded by a layer of water molecules while the bulk hydrophobic solvent is dry; (b) the enzyme particle is surrounded by a layer of water molecules while the bulk solvent contains a small amount of dispersed water.

former IL (34 cP) is less viscous that the latter one (574 cP). van Rantwijk and Sheldon [4] rationalized that the high viscosity of ILs slows down the conformation changes of proteins, allowing enzymes to maintain their native structures and activity. The solvent viscosity could affect the reaction rate in terms of the mass transfer limitation when the reaction is rapid and the IL is relatively viscous. However, this is not always true for all biocatalytic reactions in ILs. For example, Basso et al. [73] suggested that during the amide synthesis through immobilized penicillin G amidase, the viscosities of ILs ([BMIM][PF_6] and [BMIM][BF_4]) did not affect the initial rates despite their much higher viscosities than toluene.

Our data in Table 1 also suggest that IL viscosity is not directly related to the enzyme activity in our model reaction. A high viscosity may reduce the reaction rate, but the nature of IL structures seems to be the determining factor of enzyme stabilization. For example, [Amm110][dca] (**22**) possesses a high viscosity (512 mPa s at 25 °C), but it did not reduce the reaction rate despite the presence of the denaturing anion dca^- [49] (see more discussion of this IL in a later section). In summary, the IL viscosity could influence the enzymatic reaction rates in some cases, but is not the primary factor in controlling enzyme stabilization.

3.4. Effect of IL polarity

It is known that polar organic solvents could remove internally bound (essential) water from the enzyme, causing the enzyme inactivation [2,74]. Therefore, high enzyme activities could be achieved in less polar, hydrophobic organic solvents, especially at high temperatures. However, Narayan and Klibanov [75] found that the solvent polarity and water-miscibility are irrelevant to enzymatic activities of three lipases and one protease in organic solvents. The common polarity parameters include dielectric constants (ε_r), Hildebrandt solubility (δ), dipole moments (μ), and those popular solvatochromic polarity scales (such as E_T^N and Kamlet–Taft scales).

Enzymes are more flexible in solvents with high ε_r (such as water) than in those with low ε_r [76–78]. The higher flexibility may afford a higher enzyme activity, but also gives more freedom to the protein chains for extension and denaturation especially at high temperatures. However, we did not observe any correlation between the initial enzyme activity and the solvent's ε_r (Table 1). Lanne et al. [79–81] also found no direct relationship between the epoxidizing immobilized-cell activities with various solvent polarity indicators including ε_r, Hildebrand solubility parameter (δ), and $E_T(30)$ scale. Park and Kazlauskas [7] observed the trend of lipase (from *Pseudomonas cepacia*) activity increasing with the IL polarity. Lower synthetic activities of α-chymotrypsin were also found in less polar ILs [8]. Our data in Table 1 suggested no correlation between the lipase activity and the solvatochromic polarity scale E_T^N.

As mentioned in Section 1, another parameter that may influence the enzyme activity is the hydrogen-bond (H-bond) basicity. The solvent's H-bond basicity is usually quantified by the Kamlet and Taft scale, which is a multi-parameter system through comparing the UV–vis spectra of closely related dyes in various solvents (Eq. (2)),

$$\nu_{max}(\text{probe}) = \nu_{max,0} + a\alpha + b\beta + s\pi^* \quad (2)$$

where ν_{max}(probe) is the maximum wavelength of UV–vis absorption band of the solvatochromic indicator, α reflects the H-bond acidity (H-bond donating ability, property of IL cation), β reflects the H-bond basicity (H-bond accepting ability, property of IL anion), and π^* reflects dipolarity/polarizability. The H-bond acidities are in a decreasing order of [BMIM][PF_6] (0.634) > [BMIM][BF_4] (0.627) > [BMIM][Tf_2N] (0.617) > acetonitrile (0.350) > dichloromethane (0.042) [82], which is not consistent with the enzyme activities obtained in these solvents (Table 1). Meanwhile, only three ILs in Table 1 have literature values of β factors, which do not afford a full discussion of the dependence on H-bond basicity. However, the H-bond basicity could have a considerable impact on the enzyme stabilization in some ILs, such as denaturing chloride [9,11] or lactate based ones [10]. Anderson et al. [83] reported that [BMIM][BF_4], [BMIM][Tf_2N] and [BMIM][PF_6] have close H-bond basicities, which are much lower than that of [BMIM]Cl. In our work, the enzyme is less active in [Amm110]Cl (**21**) than in [Amm110][dca] (**22**), suggesting the stronger H-bond acceptor, Cl^- ion, is more enzyme-denaturing than dca^-. The role of H-bond basicity will be further discussed in the next section.

3.5. Effect of IL hydrophobicity and enzyme dissolution

'Hydrophobicity' may be considered as a narrower concept of 'polarity'. However, it is practically important to separate 'hydrophobicity' from 'polarity' because the former is often related to the miscibility with water [6]. For example, [BMIM][Tf_2N] and [BMIM][BF_4] have about the same polarity (based on E_T^N values in Table 1), but the former IL is much more hydrophobic than the latter one.

The hydrophobicity of ILs may be quantified by the log *P* scale, a concept derived from the partition coefficient of ILs between 1-octanol and water. Generally, enzymes are more stable in solvents with a larger log *P* (>3) (such as hexane, log *P* = 3.9) than lower log *P* (such as ethanol, log *P* = −0.24) [79,84]. Russell's group [12] measured the log *P* values (<−2.0) of several ILs, and suggested that they are very hydrophilic in nature based on the Laane's scale. They also observed that free lipase (*Candida rugosa*) was only active in hydrophobic IL [BMIM][PF_6] (log *P* = −2.39), but inactive in other hydrophilic ILs including [BMIM][CH_3COO] (log *P* = −2.77), [BMIM][NO_3] (log *P* = −2.90) and [BMIM][CF_3COO] [12]. Nara et al. [85] achieved higher transesterification activities of lipases in [BMIM][PF_6] than in [BMIM][BF_4]. Goto's group also reported higher activities of PEG-modified lipase [86] and subtilisin [87] in more hydrophobic ILs such as [EMIM][Tf_2N]. Zhang et al. [88] obtained low penicillin acylase stabilities in [BMIM][BF_4] and [BMIM][dca]. The Víllora group [89] observed lower stability of penicillin G acylase in [BMIM][BF_4] than in hydrophobic ILs (Tf_2N^- and PF_6^-), particularly in the absence of substrate. Ha et al. [90] also found Novozyme 435 was less active and less stable in [BMIM][BF_4] than in other hydrophobic ILs. These examples implied that the high hydrophobicity (large log *P*) of ILs could be beneficial to the enzyme stabilization. However, some studies also revealed relatively high enzyme activities in hydrophilic ILs (such as [BMIM][BF_4], [EMIM][BF_4], [BMIM][OTf] and [MMIM][$MeSO_4$]) [7,10,91–94].

Our lipase activity data were plotted against log *P* values of some ILs (Fig. 4). The enzyme activities in imidazolium and pyridinium based ILs (**6–19**) suggested a bell-shaped pattern: the enzyme activity increasing with log *P*, reaching the highest activity in [EtPy][Tf_2N] (**18**), and then declining with a further increase in log *P*. However, it is important to be aware that the log *P* scale is not a universal scale for solvent effect due to the complexity of enzyme-solvent interactions; many exceptions to the log *P* rule have been reported [95,96].

The activity in [OMIM][BF_4] (**16**) is lower than the trend, and the lipase was not quite active in other two BF_4^- based ILs (**14** and **15**). However, the enzyme activity still increased with the IL hydrophobicity: **14** < **15** < **16** (Table 1). It is surprising to notice that the lipase was still active in water-miscible [Amm110]Cl (**21**) and [Amm110][dca] (**22**). Although the anions Cl^- and dca^- are enzyme denaturing [9,49,88], their molar concentrations are much lower in **21** and **22** than those in [BMIM]Cl and [BMIM][dca], respectively,

due to the high molar mass of $[Amm110]^+$. The chloride anion is smaller than dicyanamide, which allows the chlorides to have a stronger interaction with the protein, resulting in a lower lipase activity in [Amm110]Cl (**21**) than in [Amm110][dca] (**22**).

As shown in Table 1, moderate to high enzyme activities were also seen in the hydrophobic pyrrolidinium IL (**20**) and several tetraalkylammonium ILs (**23–27**). A relatively low activity (0.15 μmol/min mg enzyme) was obtained in the lipophilic $[OctMe_3N][beti]$ (**25**). Unfortunately, the log *P* values of these ILs are not available for comparative discussion. However, by examining their structures, an increasing order of hydrophobicity can be established as $[OctMe_3N][beti]$ (**25**) < $[Et_3HexN][Tf_2N]$ (**27**) < $[Bu_3MeN][Tf_2N]$ (**24**) < $[Bu_3MeN][beti]$ (**23**). The respective enzyme activities in these ILs are 0.15, 0.61, 0.35, and 0.29 μmol/(min mg enzyme) (Table 1), which is a bell-shape trend as well. $[Oct_3MeN][Tf_2N]$ (**26**) is more hydrophobic than $[Bu_3MeN][Tf_2N]$ (**24**), but it is unknown if it is less hydrophobic than $[Bu_3MeN][beti]$ (**23**). The enzyme activity in **26** is 0.32 μmol/(min mg enzyme).

Reslow et al. [97,98] observed a similar parabolic pattern (maximum enzyme activity at log *P* = ~1.6) for α-chymotrypsin catalyzed esterification of *N*-acetyl-L-phenylalanine with ethanol. Their explanation of lower enzyme activities in very hydrophobic solvents (such as toluene, log *P* = 2.60) was that the immobilized enzyme (on glass beads) might aggregate, causing the mass transfer limitations. Since Novozyme 435 was immobilized on acrylic resin and under agitation, we did not observe any particle aggregation. A very recent study [99] on the alcoholysis of vinyl butyrate and 1-butanol by free CaLB suggested that the lipase activities were generally much lower in water-miscible ILs (such as BF_4^-, dca^-, NO_3^-, OAc^-, etc.) than in water-immiscible ones (PF_6^- and Tf_2N^-), which is consistent with our study. But their enzyme activities [99] increased with the cation's hydrophobicity ($EMIM^+ < BMIM^+ < HMIM^+ < OMIM^+$), which is not quite the same as this study.

Our previous discussion implied that the enzyme is active in hydrophobic solvents (with high log *P*). However, a higher log *P* of the solvent also means a greater thermodynamic ground-state stabilization of substrates [100], resulting in a lower conversion of the substrate. This could explain the decreasing reaction rate in very hydrophobic ILs (Table 1 and Fig. 4).

At present, we have not understood why higher hydrophobicity of ILs may lead to higher enzyme activity (up to the optimum activity as shown in Fig. 4). A common knowledge gained from biocatalysis in organic solvents is that polar solvents strip off the 'essential' water from enzyme molecules, causing them to inactivate [2,79]. However, Fig. 2 suggests that a relatively high enzyme activity could still be maintained in $[BMIM][Tf_2N]$ when substrates, IL, and enzyme were all intensively dried. CaLB is known to be active in organic solvents containing little or no water [35,36,101]. Therefore, the solvent stripping capacity is likely not the main mechanism of Novozyme 435 inactivation by hydrophilic ILs. One possible explanation of the anion effect is based upon nucleophilicity. Anions CH_3COO^-, CF_3COO^- (TFA^-), and NO_3^- are strongly coordinating species and more nucleophilic than PF_6^-, and may interact with the enzyme's surface, causing lipase inactivation [12,102]. However, BF_4^- is weakly coordinating and less nucleophilic, and the enzyme activity in $[BMIM][BF_4]$ is much lower than that in $[BMIM][Tf_2N]$. Not the nucleophilicity can explain this.

An alternative explanation may be derived from the correlation between log P and solvent hydrogen-bond basicity. A general regression equation has been established as the following [103],

$$\log P = c + rR + s\pi^* + a\alpha + b\beta + vV_x \quad (3)$$

where *R* is the excess molar refraction obtained from refractive index measurements, V_x is the McGowan's characteristic volume or simply the intrinsic volume of the solute, other symbols have the same meanings as Eq. (2). The correlations of 613 organic solutes yielded the coefficient values of $c = 0.088$, $r = 0.562$, $s = -1.054$, $a = 0.034$, $b = -3.460$ and $v = 3.814$ (V_x in the unit of $cm^3\ mol^{-1}/100$) [103,104]. The near-zero *a*-coefficient suggests that the hydrogen-bond acidity does not contribute much to the partition. The *s*- and *b*-coefficients are negative because water is more dipolar and is a stronger hydrogen-bond acid than wet octanol [105]. The large and positive *v*-coefficient shows that larger solute molecules are more hydrophobic and tend to partition into the octanol layer. Other correlations using different solutes reached similar conclusions [106–108]. Based on Eq. (3), the higher the hydrogen-bond basicity of an IL anion, the lower the log *P* value, and thus the lower the enzyme activity due to the H-bonding interactions between the anion and enzyme. This explains the trend of increasing enzyme activity with the increasing log *P* up to a critical value. Why then does the lipase activity decrease with a further increase in log *P*? Since the increasing *v*-coefficient is quite positive in Eq. (3), an increase in the cation's size results in increasing log *P*. As discussed previously, the stabilization of substrates could be one reason. But the possibility of hydrophobic interactions between large IL molecules and the enzyme cannot be completely excluded.

On the other hand, cations play an important role in enzyme stabilization as well. CaLB showed higher activities in several ILs with more hydrophobic cations, for examples, [HMIM][TFA] (**12**) *vs.* [EMIM][TFA] (**11**), $[OMIM][BF_4]$ (**16**) *vs.* $[BMIM][BF_4]$ (**15**) and $[EMIM][BF_4]$ (**14**), as shown in Table 1. However, the increase in cation hydrophobicity also increases the overall IL hydrophobicity, which could induce further more substrate ground-state stabilization or hydrophobic interactions. This appears to be the case for $[EMIM][Tf_2N]$ (**6**), $[BMIM][Tf_2N]$ (**7**), and $[HMIM][Tf_2N]$ (**8**) in Table 1, where the IL hydrophobicity increased (as indicated by their log *P* values), but the lipase activities decreased.

The high hydrogen-bonding basicity and overall hydrophilic nature of water-miscible ILs drive the enzyme to dissolve in these media (more or less); on the other hand, enzymes are barely soluble in hydrophobic ILs [10,102]. The dissolution of lipase in most hydrophilic ILs is an indication of strong interactions between the enzyme and solvent molecules. If such interactions are unfavorable for active sites and/or strong enough to disrupt the protein structures (such as CaLB in [BMIM][lactate] and nitrate-based ILs [10,101]), the enzyme loses its catalytic capability in these hydrophilic media. However, if such interactions are not too strong but preferential for maintaining the enzyme's structures, these hydrophilic ILs do not inactivate the enzyme (such as $[Et_3MeN][MeSO_4]$ [10], Amm110-series ILs (Table 1 and Refs. [109,110]), and ether-functionalized ILs [109]). Therefore, hydrophilic ILs have the general tendency of dissolving and inactivating the enzyme, but some of them could stabilize the enzyme. $[BMIM][BF_4]$ is an interesting IL: it is hydrophilic but does not dissolve CaLB [10], and there are contradictory results on the enzyme activity in this solvent. This IL behaves more like a polar and hydrophilic organic solvent, which does not dissolve the enzyme but may denature it.

3.6. Ionic association strength and hydrogen-bonds

The ionic association strength of LiX salts has been investigated in a variety of aprotic solvents including glymes (see a short review in the Supporting Information of Ref. [111]). The approximate ionic

association strength in aprotic solvents is listed below in an increasing order [111,112]:

$$\text{beti}^-, Tf_2N^- < PF_6^- < ClO_4^-, I^- < SCN^- < BF_4^- < CF_3SO_3^- < Br^- < NO_3^- < CF_3COO^- < Cl^-$$

Amazingly, this order is quite consistent with the anion effect on enzyme activities (Table 1). This order represents the strength of an anion in interacting with solvated cations through ionic attraction, or could be implied to represent the strength of interactions between the anion and the changed surface of macromolecules (such as proteins). The exact mechanism of this ion–protein interaction is not fully understood.

Bernson and Lindgren [113] dissolved lithium salts LiX in poly(propylene glycol) (MW = 3000) with hydroxy end-groups. Using IR spectroscopy, they observed that the shifts of —OH stretching band depend on the strength of hydrogen bond formed between the —OH group and the anion, as well as the coordination of cations with the —OH group. The strength of anion coordination is further dependent on the hydrogen-bond basicity of the anion, and is summarized from the IR band shifts as (an increasing order),

$$PF_6^- < BF_4^- < ClO_4^- < CF_3SO_3^- < I^- < Br^- < Cl^-$$

In general, this series is consistent with the above ionic association order observed in aprotic solvents, with some variations.

From experimental data of IR and ESI-MS, Dupont [14] suggested the strength of hydrogen-bond basicity in the similarly increasing order of

$$BPh_4^- < PF_6^- < BF_4^- < CF_3COO^-$$

These sequences confirm that the enzyme activity is likely related to the hydrogen-bond acceptor strength of the anion: anions with low hydrogen-bond basicity are enzyme stabilizing. But the role of cations and overall IL properties on enzyme stabilization cannot be neglected.

4. Conclusions

Higher enzyme activity was observed in ILs under microwave heating when the lipase was surrounded by a water layer. The effect of microwave irradiation on the enzyme activation was explained due to the superheating of the water layer near the enzyme's surface. Several physical properties of ILs were correlated with the Novozyme 435 activities in ILs under microwave irradiation. High viscosity may reduce the reaction rate, but likely is not the determining factor. IL polarity scales (in terms of dielectric constant and E_T^N) were not found to have a direct relationship with the lipase activity. However, IL hydrophobicity ($\log P$) was observed to have a loose correlation with enzyme activity. The lipase activity increased with the $\log P$ value to a maximum, and then decreased with a further increase in $\log P$. The increasing trend was explained have as being the anion's H-bond basicity, enzyme dissolution, anion ionic association ability, and cation hydrophobicity, while the decreasing trend is likely caused by substrate ground-state stabilization or hydrophobic interactions.

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