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Both hydrolytic and transesterification activities of *Penicillium expansum* lipase are significantly enhanced in ionic liquid [BMIm][PF_6]

Zhen Yang*, Kai-Pei Zhang, Ying Huang, Zhi Wang

College of Life Sciences, Shenzhen University, Nanshan District, Shenzhen 518060, Guangdong, China

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ABSTRACT

Both hydrolytic and transesterification activities of *Penicillium expansum* lipase (PEL) were investigated in the ionic liquid [BMIm][PF_6] as well as in organic solvents such as hexane. The initial rate of PEL-catalyzed hydrolysis of *p*-nitrophenyl palmitate (pNPP) was 12-fold enhanced in the ionic liquid. The optimal water content required by PEL in [BMIm][PF_6] was 10 times higher relative to that in hexane due to the greater polarity of the ionic liquid. Direct addition of salt hydrates into the two nonaqueous reaction media showed different impacts on the enzyme activity, which could be related to the dual functions of the salt hydrates, i.e., the water buffering effect, and the specific ion effect (Hofmeister effect). The transesterification activity of PEL was reflected by the yield of producing fatty acid methyl esters (FAMEs) in the methanolysis of corn oil for 25 h, which was 69.7% in [BMIm][PF_6], as compared to 19.4%, 14.0%, and 1.0% obtained in *tert*-butanol, solvent-free system, and hexane, respectively. The high production yield of FAMEs obtained in [BMIm][PF_6] demonstrates the potential use of ionic liquids as the reaction media for PEL-catalyzed biodiesel production.

1. Introduction

Lipases are triacylglycerol ester hydrolases (EC 3.1.1.3) that catalyze the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids. Being widely found in nature, many lipases are active in organic solvents where they catalyze a number of useful reactions including hydrolysis, esterification, transesterification, kinetic resolution of racemic acids and alcohols, regioselective acylation of steroids, sugars, and sugar derivatives, and synthesis of peptides and other chemicals [1,2]. Lipases have become industrially important, especially in detergent, food, and pulp and paper industries [3]. More recently, lipases have found an important application in biodiesel production [4].

As a biodegradable, nontoxic, cleaning, and renewable fuel, biodiesel is a mixture of fatty acid methyl esters (FAMEs) which are produced from a broad range of crude oil materials, such as vegetable oil, animal fats, and waste oil, via transesterification of triglycerides with methanol or ethanol. Biodiesel has been regarded as a promising fuel to be able to partly substitute for conventional fossil diesel. Being environmentally friendly, lipase-catalyzed biodiesel production is more advantageous over the alternative chemical approaches due to the mild reaction conditions and easy product recovery [5]. The lipase that is mostly used in biodiesel production is the lipase B from *Candida antarctica* (CALB), which has been commercialized by Novozymes as an immobilized form (Novozym 435).

As compared to those lipases that have been commonly used and widely investigated, the lipase used in this study has not gained sufficient interest from researchers in terms of its catalytic properties in nonaqueous reaction media and its applications in biotransformations. It was produced from a special strain, PF898, of *Penicillium expansum*. This *P. expansum* lipase (PEL) has been crystallized with a molecular mass of 28 kDa and it hydrolyzes acylglycerols at the optimal temperature of 34 °C and the optimal pH of 9.5 [6]. According to the cloning and sequencing results [7], this enzyme has a low protein sequence homology compared with other lipases and a high percentage of hydrophobic residues (51.4%) in the N-terminal region compared with other typical lipases. Its application in catalyzing biodiesel production in organic solvents from corn oil [8] and waste oil [9] has been explored. But investigation regarding its catalytic properties in organic solvents and ionic liquids has not been reported.

Ionic liquids (ILs) are organic salts remaining as liquids under ambient temperatures. Interests in using them as a new type of solvents for biotransformations have been increasing rapidly because they have the ability of presenting excellent enzyme activity, stability, and selectivity [10,11]. So far different types of enzymes have been demonstrated to show catalytic activity in ILs, and it seems evident that enzymes in ILs basically follow the same catalytic mechanism as in organic solvents [12–14]. Therefore, it is

* Corresponding author. Tel.: +86 755 2653 4152; fax: +86 755 2653 4274.
E-mail address: zyang@szu.edu.cn (Z. Yang).

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expected that the fundamental rules governing the enzyme activity in organic solvents may also apply to that in ionic liquids.

It has been well known that water plays an important role in affecting enzyme activity in organic media [15]. An enzyme is usually inactive under completely dry conditions, but addition of a small amount of water to the nonaqueous reaction medium normally accelerates the enzymatic reactions, and the major function of the water is to assure the enzyme of its optimal flexibility. The key determinant of the enzyme activity is the amount of water that is bound to the enzyme rather than the total water content in the reaction system. Depending on the solvent polarity, the reaction system requires different amounts of water for optimizing the enzyme activity. The fact that an enzyme (such as a lipase) usually presents a higher activity in a less polar and more hydrophobic solvent [16] can also be explained by the lower ability of such a solvent to strip off the essential water from the enzyme [15]. It is therefore necessary to control the water level associated with the enzyme, which is characterized by the thermodynamic water activity (a_w) [17]. Two methods are commonly used to achieve a constant water activity: (1) separate pre-equilibration of substrate solution and enzyme preparation with a saturated aqueous solution of a salt, and (2) direct addition of a salt hydrate to the reaction mixture to act as a water buffer. Originally discussed by Halling [18], the latter method provides a convenient means of buffering water activity and has been widely used in the organic solvent system. Although the effects of water, water activity, and salt hydrates have been well demonstrated in conventional organic solvents, it is necessary to assess whether these effects also apply to the enzymes in ionic liquids. A few recent reports have covered this aspect [12,19–22].

In this study, the catalytic activity of PEL in both organic solvents (hexane) and ionic liquids ([BMIm][PF_6]) was investigated (see Scheme 1 for the enzymatic reactions). The hydrolytic activity of PEL was determined by following the formation of the product, *p*-nitrophenol (pNP), from hydrolysis of *p*-nitrophenyl palmitate (pNPP), and the transesterification activity of PEL in these two reaction media was evaluated by conducting the PEL-catalyzed methanolysis of corn oil. The latter reaction was also used to demonstrate the feasibility of utilizing PEL for biodiesel production in ionic liquids. So far there has been only one report in the literature regarding the potential use of ionic liquids as the reaction media for enzymatic approach of biodiesel production, which was demonstrated by using an immobilized *C. antarctica* lipase B (CALB) [23]. Our work also aimed to investigate the effect of water, water activity, and salt hydrates on enzyme activity in ionic liquids as well as in organic solvents, by using PEL as a model enzyme.

2. Experimental

2.1. Materials

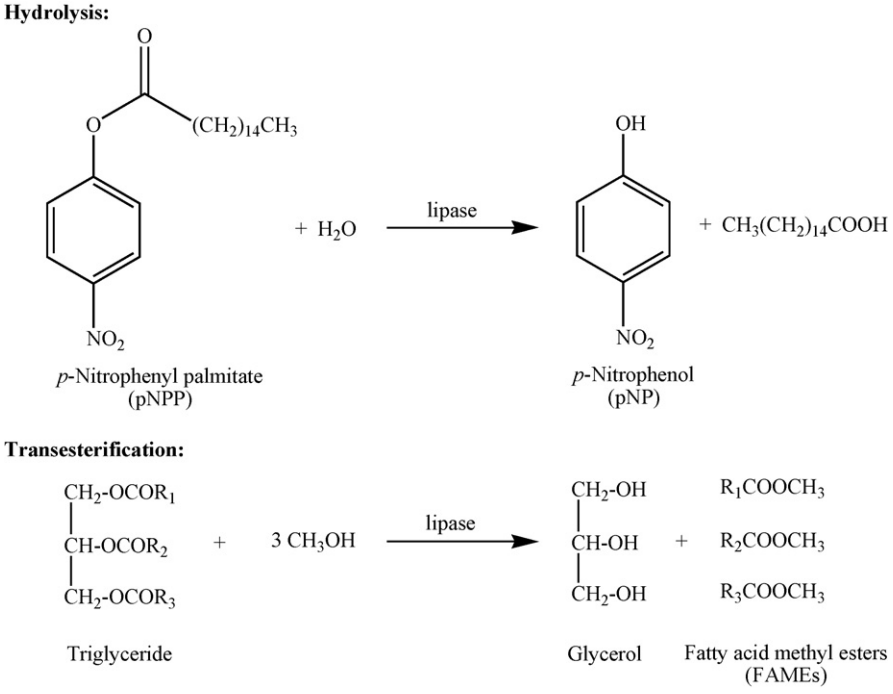
Crude lipase from *P. expansum* (PEL) was kindly donated by Shenzhen Leveking Bio-engineering Co. Ltd., China. *p*-Nitrophenyl palmitate (pNPP), *p*-nitrophenol (pNP), and the ionic liquid [BMIm][PF_6] (≥97%, HPLC) were purchased from Sigma–Aldrich China Inc. Methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate, and methyl heptadecanoate were of chromatographic purity also from Sigma–Aldrich China Inc. Refined corn oil was obtained from a local supermarket in Shenzhen, China. All other reagents used were of analytical grade from local manufacturers in China.

2.2. Determination of molar extinction coefficients for pNP in different buffer solutions

A series of pNP solutions (0–0.01 mM) were prepared in a 50 mM buffer at a particular pH (phosphate buffer, pH 5.0–8.0; Tris–HCl buffer, pH 7.0–9.0; barbiturate–HCl buffer, pH 7.5–10.0), and their absorbances were measured at 410 nm. The molar extinction coefficients of pNP were then determined as slopes derived from the A_{410} vs. [pNP] plots.

2.3. Activity assay in aqueous solution

The hydrolytic activity of PEL in aqueous solution was determined according to the method described in [24], with a slight modification. The PEL solution (~0.23 U/ml, based on the activity



Scheme 1. PEL-catalyzed hydrolysis and transesterification reactions used in this study.

of catalyzing the hydrolysis of pNPP at 37 °C in 50 mM phosphate buffer, pH 6.0) was obtained as the supernatant after dissolution of 1.5 g of the enzyme powders in 10.0 ml phosphate buffer (50 mM, pH 8.0) followed by centrifugation. A substrate mixture was prepared by mixing 1 ml of 15.0 mM pNPP in isopropanol and 9 ml of a 50 mM buffer solution (phosphate buffer, pH 5.0–8.0; Tris–HCl buffer, pH 7.0–9.0; barbiturate–HCl buffer, pH 7.5–10.0) containing 0.1% (w/v) arabic gum and 0.4% (w/v) Triton X-100. A cuvette containing 2.4 ml of this substrate mixture was placed in the Pharmacia Biotech Ultraspec 2000 UV/Vis spectrophotometer, equipped with a thermostated cell, for pre-equilibration at 37 °C, and the reaction was started by the addition of 0.1 ml of the PEL solution. The variation of the absorbance at 410 nm of the assay against a blank without enzyme was monitored for 2–5 min. The reaction rate was calculated from the slope of the absorbance vs. the time curve by using the molar extinction coefficient for pNP, obtained as measured above.

2.4. Activity assay in hexane

According to the method described in [24], a typical hydrolysis reaction was started by the addition of 160 mg PEL to 2.0 ml hexane containing 10 mM pNPP in a 5-ml capped bottle, which was placed in a shaker and incubated at 37 °C with agitation of 200 rpm. Occasionally, a certain amount of water or an inorganic salt hydrate (0.5 g) was added to control the water content in the reaction system. Samples (0.1 ml) were taken at intervals (typically every 10 min) and mixed thoroughly with 2.0 ml of 0.1 M NaOH solution. After centrifugation, the bottom phase was taken for absorbance reading at 410 nm. A blank reaction (without addition of the enzyme) was also followed in parallel. The reaction rate was calculated from the slope of the absorbance (subtracting the one for the blank reaction) vs. the time curve by using the molar extinction coefficient for pNP determined in 0.1 M NaOH as described above. For transesterification of pNPP with methanol, enzyme powders (160 mg) were added to 2.0 ml hexane containing 10 mM pNPP and 10 mM methanol to start the reaction.

2.5. Activity assay in [BMIm][PF_6]

The hydrolytic activity of PEL in this ionic liquid was determined basically following the method described above for the hexane system. But due to the low solubility of pNPP in [BMIm][PF_6], the substrate solution was prepared by first dissolving pNPP in isopropanol in a concentration of 25 mM, which was then diluted with [BMIm][PF_6] to a final concentration of 2.3 mM.

2.6. Methanolysis of corn oil

In a typical experiment, the reaction was carried out in a 25 ml capped flask containing 1 g of the corn oil, two molar equivalent of methanol, 2 ml of a given solvent, and 100 mg of the PEL. The reaction flask was placed in a shaker at 40 °C with agitation of 220 rpm. Prior to use, all the solvents have been dried over molecular sieves, and no additional water control was conducted during the reactions. Periodically, samples of 50 μl were withdrawn and centrifuged at 10,000 rpm for 10 min. Then 5 μl from the supernatant was taken and diluted with 295 μl of hexane, followed by addition of 300 μl of methyl heptadecanoate (1 mg/ml, as an internal standard). This mixture was then ready for gas chromatographic analysis.

2.7. GC analysis

The fatty acyl methyl esters (FAMEs) produced from lipase-catalyzed methanolysis of corn oil was assayed with a Trace2000 gas chromatogarphy (Thermal Co., Germany) equipped with a SUPELCOWAX 10 column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies, USA). The column temperature was hold at 160 °C for 2 min, and raised at 10 °C/min to 240 °C, where it was then maintained for 10 min. Nitrogen was used as the carrier gas at 1.5 ml/min. Splitless mode was selected. The temperatures of the injector and the detector were 250 and 260 °C, respectively.

3. Results and discussion

3.1. Effect of pH and buffer type on PEL activity in aqueous solution

The hydrolytic activity of PEL was determined by spectrophotometrically following the formation of the product pNP based on its strong absorbance at 410 nm. It is necessary to emphasize the need to take account of the variation of pNP's extinction coefficient, used in estimation of the enzyme activity, upon the change in the buffer conditions [25]. In the present study, it was found that the molar extinction coefficient of pNP is much more sensitive to the change in the buffer pH (varying from pH 5.0 to 10.0), showing a sigmoid-curve relation, and less to the change in the buffer type (Fig. 1).

Based on the molar extinction coefficients of pNP determined in different buffer systems, our experiments have demonstrated that the hydrolytic activity of PEL is very much dependent on both the type and pH of the buffer used (Fig. 2). Among the three buffer types tested, phosphate buffer promoted the highest activity, whereas barbiturate buffer the lowest. In both phosphate and Tris buffers, the hydrolytic activity of PEL decreased with an increase in the buffer pH within the pH ranges tested (pH 5–8 for phosphate buffer and pH 7–9 for Tris buffer). In barbiturate buffer, however, there seemed to be an abnormal V-shaped relationship between enzyme activity and the buffer pH within the pH range of 7.5 and 10. The fact that lipase presented different activities in different buffers does not seem to be uncommon. For instance, activities of lipase from *Penicillium aurantiogriseum* have also been reported to be higher in phosphate buffer than in Tris–HCl buffer [26]. In our very recent paper regarding the study of Hofmeister effects on alkaline phosphatase [25], we have also demonstrated that the enzyme presented a variant activity in the same DEA buffer, depending on the type and concentration of the salt present. It can be certain that pH is not the only factor controlling the enzyme activity, but instead, the species present in the buffer solution, whether in the form of ions or not, may also be responsible for regulating the

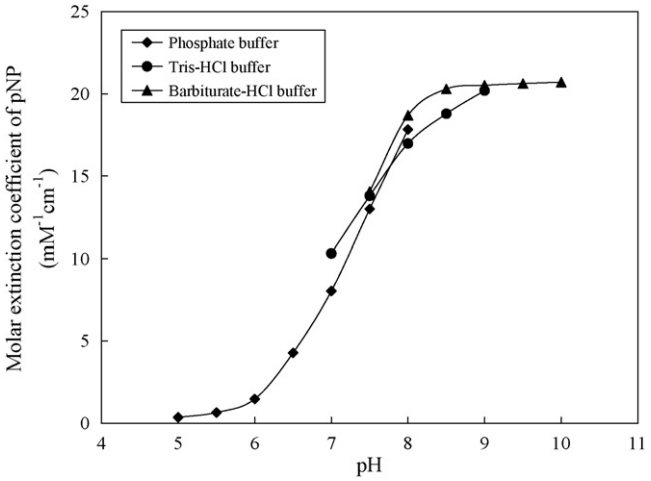


Fig. 1. Dependence of molar extinction coefficient of pNP on both buffer type and buffer pH. Concentrations for all the buffers were 50 mM.

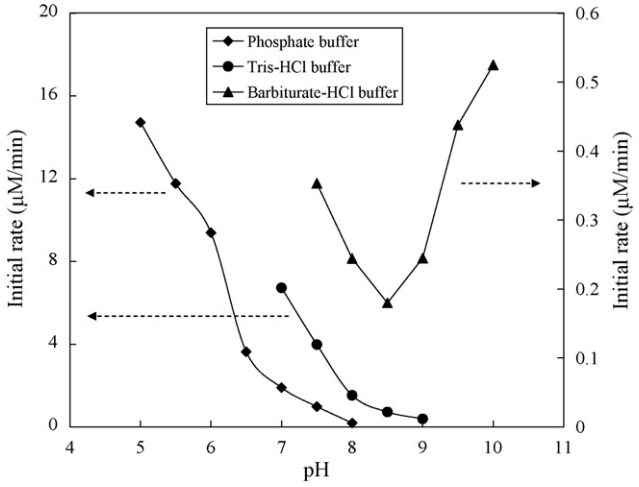


Fig. 2. Effect of pH and buffer type on the hydrolytic activity of PEL in aqueous solution. Concentrations for all the buffers were 50 mM.

enzyme activity, presumably by interacting with either the enzyme or the substrate, thereby affecting the enzyme conformation and the substrate binding.

Although *P. expansum* has been taken as a major source for producing alkaline lipases and the lipase from the strain PF898 of this genus has been shown to hydrolyze acylglycerols at the optimal pH of 9.5 [6], a neutral [27,28] or slightly basic [26] pH optimum for lipases produced from a few other strains of the genus *Penicillium* has been reported. The pH dependence of PEL shown in Fig. 2 seems rather abnormal.

3.2. Comparing the hydrolytic activity of PEL in both hexane and [BMIm][PF_6]

It is interesting that even under a low-water condition, PEL holds a strong power of catalyzing the hydrolysis of pNPP rather than the transesterification of the same substrate with methanol. The enzyme was fairly active when catalyzing the hydrolysis of pNPP in hexane. When the enzyme powders were suspended in hexane containing 10 mM pNPP and 10 mM methanol, the initial rate of pNP formation was 95% of that obtained in the absence of methanol; and this rate was dropped significantly with gradual addition of more methanol, presumably due to the methanol-induced inactivation. Therefore, in our study the hydrolytic activity of PEL was compared in both hexane and [BMIm][PF_6].

Fig. 3 depicts the proportional increase of the initial reaction rates of PEL-catalyzed pNPP hydrolysis upon addition of the enzyme in the two solvent systems. When tested at the same substrate concentration (2.3 mM), the hydrolytic activity of PEL (in the unit of μmol/h/mg) in [BMIm][PF_6] was 12.1 times as high as that in hexane. A low substrate concentration of 2.3 mM was selected simply because of the low solubility of pNPP in [BMIm][PF_6]. A few enzymes have been reported to show higher activity in ionic liquids than in conventional organic solvents [22,29,30], but an enhancement in activity for more than 10-fold is rarely reported.

Lipase is the type of enzymes that has been extensively investigated in ionic liquids and has been reported to present activities in such reaction media that is greater than, or comparable to, those obtained in conventional organic solvents. For instance, *C. antarctica* lipase B (CALB), whether free or immobilized on three different carriers, exhibited improved ascorbyl oleate synthesis in all the six ionic liquids tested, with an initial rate up to 9-fold higher as compared to the reaction conducted in acetone [21]. However, the results are sometimes contradictory. Take the comparison of lipase activity in the ionic liquid, [BMIm][PF_6], and in the organic solvent, hexane, as an example. *Candida rugosa* lipase produced a lower yield in the esterification of (R,S)-2-substituted-propanoic acids with 1-butanol in [BMIm][PF_6] than in hexane [19], whereas the same enzyme in [BMIm][PF_6] catalyzed a transesterification reaction between methyl methacrylate and 2-ethyl-1-hexanol at an initial rate that is 1.5 times faster than the reaction in hexane [31]. For this latter reaction, another lipase from *C. antarctica*, adsorbed onto a macroporous acrylic support (Novozym 435), was active in organic solvents such as hexane and acetonitrile but totally inactive in all the ionic liquids tested including [BMIm][PF_6] [31]; whereas the same CALB, when immobilized on a different acrylic resin, was active in [BMIm][PF_6], but with an initial rate higher or lower than that obtained in hexane depending on the water content in the reaction system [12]. The immobilized CALB (Novozym 435) was also active in catalyzing an esterification of geraniol with acetic acid in [BMIm][PF_6], but the reaction rate was lower than in hexane [20]. Very recently, de Diego et al. [22] have compared the catalytic efficiency of four free lipases from different microbes (i.e., *C. antarctica* lipase A, CALA; *C. antarctica* lipase B, CALB; *Thermomyces lanuginosus* lipase, TLL; and *Rhizomucor miehei* lipase, RML) in five different ionic liquids and in hexane. The transesterification activities of CALB, TLL, and RML were 1.5-, 6.1-, and 1.4-fold increased in [BMIm][PF_6] as compared to that obtained in hexane, respectively, while CALA was less active in the ionic liquid. All these different results can probably be attributed to differences in the enzyme source and preparation, the reaction type, the substrate, and also the water content in the reaction system. Therefore, our experimental result about PEL offers a new type of lipases that exhibits a significantly improved activity in ionic liquids as compared to conventional organic solvents.

3.3. Effect of water content, water activity, and salt hydrates

Fig. 4 confirms that the hydrolytic activity of PEL in [BMIm][PF_6] was sensitive to the amount of water added. For both solvents [BMIm][PF_6] and hexane, there is a similar bell-shaped relationship between enzyme activity and amount of water added to the reaction system, although the reaction rates obtained in the IL are much higher. The enzyme requires a greater optimal water content in the IL system (2.3%, v/v) than that for the hexane system (0.2%, v/v). Water is a co-substrate for the PEL-catalyzed hydrolysis reaction, and this can partly account for the initial enhancement

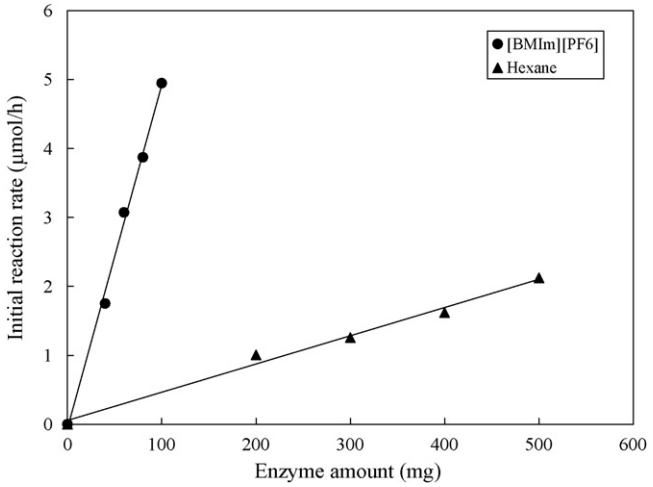


Fig. 3. Comparison of PEL's hydrolytic activity in hexane and in [BMIm][PF_6]. Different amounts of enzyme powders were added to the reaction system containing 2.3 mM pNPP in 2.0 ml of either [BMIm][PF_6] or hexane, and the reactions were carried out at 37 °C with agitation of 240 rpm. Both solvents were used directly as supplied and no additional water was added.

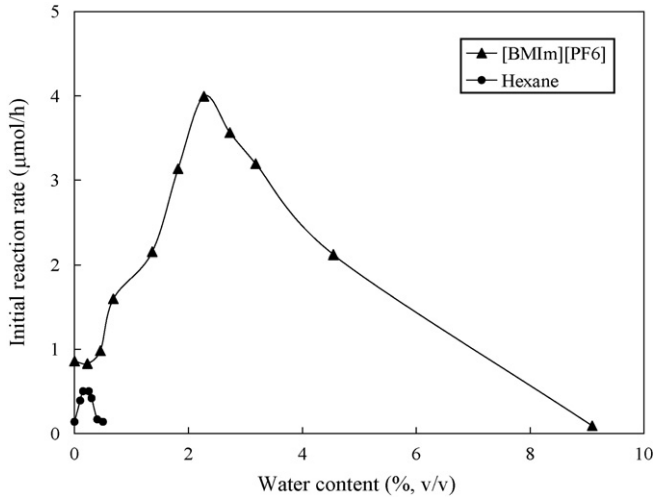


Fig. 4. Dependence of PEL's hydrolytic activity in hexane and in [BMIm][PF_6] on the amount of water added to the reaction system. Different amounts of water were added to the reaction system containing 2.0 ml of 2.3 mM pNPP in the solvent and 160 mg enzyme powders, and the reactions were carried out at 37 °C with agitation of 240 rpm. Both solvents were dried over molecular sieves prior to use.

of the enzyme's hydrolytic activity with an increase in the water content. Generally, water also plays an important role in affecting the enzyme activity in nonaqueous media [15]: essential amount of water may activate the enzyme by increasing the polarity and structural flexibility of the enzyme's active site; but too much water is harmful to the enzyme by facilitating enzyme aggregation, thus diminishing the substrate diffusion and eventually leading to enzyme inactivation. Lumps of enzyme powders have indeed been observed in our experiments, when a high water content was applied in the IL. One may notice that the enzyme activity obtained in Fig. 4 was approximately half of what was obtained in Fig. 3, and this can be attributed to the relatively low storage stability of the enzyme: the two experiments were done with more than half a year apart. Research will be undertaken in our laboratory to improve the enzyme's storage stability.

A previous report by Ulbert et al. [19] has shown that the optimal water content for *C. rugosa* lipase varied according to the solvent used, following the order of [BMIm][BF_4] > [BMIm][PF_6] > [ONIm][PF_6] > toluene > hexane; which is consistent with the decreasing order of solvent polarity (the log*P* values for the above solvents are −2.44, −2.38, −2.19, 2.5, and 3.5, respectively). A more polar solvent has a higher tendency of stripping off the essential water from the enzyme. Ionic liquids are highly polar [32,33], and their greater ability to retain water has been demonstrated by de Diego et al. [22], who investigated the effect of water content on the water activity of the reaction system of lipase-catalyzed butyl propionate synthesis in the ionic liquid [CPMA][MS] at 40 °C: the water activity was slowly increased from 0.2 to about 0.5 when the water content was raised abruptly from 1 to 8% (v/v). It is therefore understandable that the IL system requires a higher water content for an optimal enzyme activity.

Recently, Russell's group has conducted a successful demonstration of using salt hydrate pairs to control water activity for enzyme catalysis in ionic liquids [12]. In our experiment, 13 salt hydrates with different water activity values at 30 °C ranging from 0.041 to 0.85 were selected for the study of their effect on controlling the water activity as well as the hydrolytic activity of PEL in hexane and in [BMIm][PF_6]. Considering the extremely low activity of PEL in hexane, a higher substrate concentration was used in this reaction medium (10 mM, instead of 2.3 mM used in [BMIm][PF_6]). The enzyme in hexane was most active at very low-water activity (i.e., in the presence of $CaCl_2{\cdot}2H_2O$ and $MgCl_2{\cdot}6H_2O$, with a water activity of 0.043 and 0.041 at 30 °C, respectively), and then its activity showed a general trend, although somewhat scattering, of decreasing upon an increase in the water activity (Fig. 5B). This trend is clearly confirmed by a comparison of the activity data obtained in hexane with addition of three different hydrate forms of a same salt, Na_2HPO_4 (i.e., $Na_2HPO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}7H_2O$, $Na_2HPO_4{\cdot}12H_2O$, see the points b, c, and d on Fig. 5B). In fact, it is not uncommon for a lipase to be active at a low a_w. *R. miehei* lipase has been reported to remain highly active at water activity below 0.0001 [34]. However, the activity of PEL in the IL [BMIm][PF_6] did not show an obvious

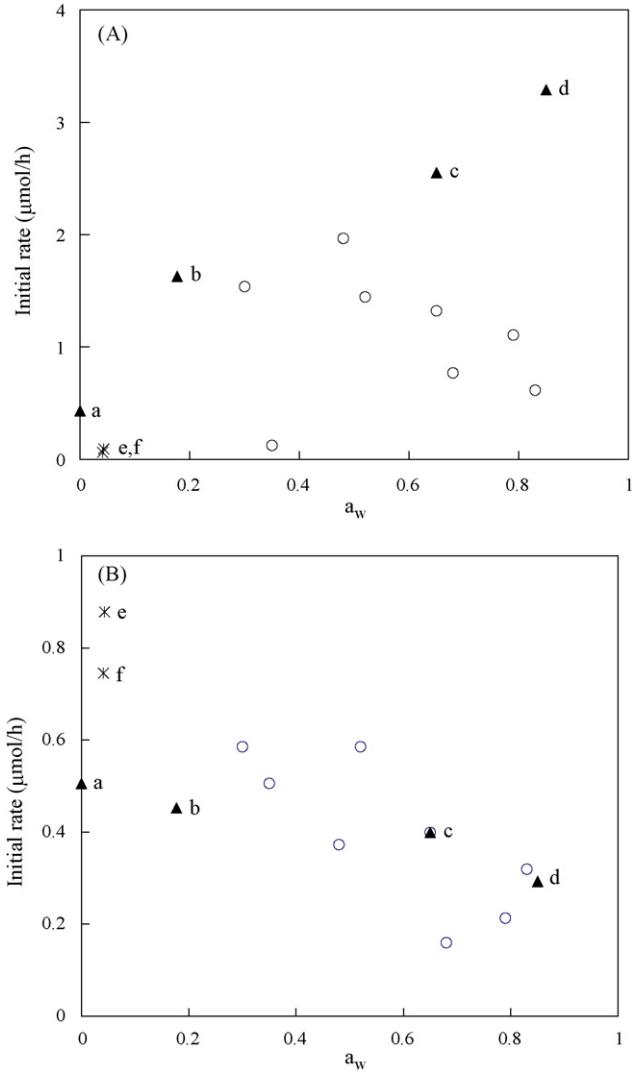


Fig. 5. Correlation between PEL's hydrolytic activity in [BMIm][PF_6] (A) and hexane (B) and the water activity (a_w) offered by the salt hydrate added to the reaction system at 30 °C. Different salt hydrates (0.5 g) were added to the reaction system containing 2.0 ml of pNPP solution (10.0 mM in hexane and 2.3 mM in [BMIm][PF_6]) and 160 mg enzyme powders, and the reactions were carried out at 30 °C with agitation of 240 rpm. Both solvents were dried over molecular sieves prior to use. The salt hydrates used and their water activities at 30 °C (obtained from [18] and listed in brackets) are: $MgCl_2{\cdot}6H_2O$ (0.041), $CaCl_2{\cdot}2H_2O$ (0.043), $NaAc{\cdot}3H_2O$ (0.3), $CuSO_4{\cdot}5H_2O$ (0.35), $K_4Fe(CN)_6{\cdot}3H_2O$ (0.48), $Na_4P_2O_7{\cdot}10H_2O$ (0.52), $Na_2B_4O_7{\cdot}10H_2O$ (0.65), $ZnSO_4{\cdot}7H_2O$ (0.68), $Na_2CO_3{\cdot}10H_2O$ (0.79), $Na_2SO_4{\cdot}10H_2O$ (0.83), $Na_2HPO_4{\cdot}2H_2O$ (0.177), $Na_2HPO_4{\cdot}7H_2O$ (0.65), and $Na_2HPO_4{\cdot}12H_2O$ (0.85). The data points labeled with the letter 'a' refers to the reaction rate obtained without addition of any salt hydrate, whereas those labeled with 'b', 'c', 'd', 'e', and 'f' represent the rates obtained with addition of the salt hydrates $Na_2HPO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}7H_2O$, $Na_2HPO_4{\cdot}12H_2O$, $CaCl_2{\cdot}2H_2O$, and $MgCl_2{\cdot}6H_2O$, respectively.

correlation with the water activities presented by the salt hydrates (Fig. 5A), and the data for the three hydrate forms of Na_2HPO_4 (see points b, c, and d on Fig. 5A) has even shown the opposite trend, as compared to Fig. 5B. The possible reason for this unexpected result might be related to the specific salt effect.

As the activity of an enzyme in nonaqueous media depends on its hydration, the enzyme is active when there is a microaqueous phase associated with it [15]. The salt hydrates added to the reaction system can partially penetrate and dissolve in this microaqueous phase and dissociate into individual cations and anions to function on the enzyme. It has long been realized that different ions affect the enzyme performance in aqueous solution following a common order known as the Hofmeister series [35]. Although the mechanism regarding this Hofmeister effect is still not clearly known, it is believed to be correlated with the kosmotropic/chaotropic properties of the ions, which can be characterized by the Jones–Dole viscosity B coefficients (B_+ for cations and B_- for anions, available in [36]) (for recent reviews, see [37–39]). In a recent study of salt effects on the hydrolytic activity of *C. rugosa* lipase in aqueous solution, Salis et al. [40] found that NaSCN is a strong inhibitor to the enzyme, Na_2SO_4 acts as an activator, whereas NaCl shows a quasi-neutral behavior. This has been attributed to the role of anions in modifying both the surface pH and the tertiary structure of the enzyme. In particular, a chaotropic anion (such as SCN^-) has a higher tendency of binding to the enzyme surface. As a result, the two residues (Glu^{341} and His^{449}) involved in the catalytic triad mechanism are protonated and hence cannot assist the other residue Ser^{209} in the nucleophilic attack of the substrate, leading to inhibition to the lipase. Previously, the same research group has already observed a similar effect of anions on the hydrolytic activity of *Aspergillus niger* lipase in aqueous solution [41]: the enzyme activity varied in the order of $Br^- > Cl^- \sim NO_3^- > ClO_4^-$, basically following the Hofmeister series.

If there is indeed a thin layer of water surrounding the enzyme molecule for its catalysis, it is reasonable to speculate that the salt hydrates added to the reaction system offers dual functions in affecting the enzyme activity: they can not only exist as intact molecules in the nonaqueous reaction medium to control the hydration level of the enzyme (the water activity) via their hydration/dehydration equilibria; but also penetrate into the microaqueous phase surrounding the enzyme molecule and dissolve and dissociate into their corresponding cations and anions, exhibiting their specific ion effect (the Hofmeister effect) on the enzyme. Correspondingly, a reasonable V vs. a_w or V vs. ($B_- - B_+$) relationship would be expected for the former or latter salt hydrate effect, respectively. For our case of PEL in the ionic liquid, although the enzyme did not show an obvious V–a_w correlation (Fig. 5A), it did show a clear trend of increasing activity upon an increase in the ($B_- - B_+$) value of salts added (Fig. 6A). This is in line with the above literature reports regarding the higher lipase activity in aqueous solution in the presence of more kosmotropic anions (with higher B_- values, such as SO_4^{2-} and HPO_4^-) and can be explained by the specific ion effect on the surface pH, structure, and catalytic mechanism of the enzyme [40]. Our recent study about the Hofmeister effect on the activity of alkaline phosphatase has provided further evidence for the idea that kosmotropic cations and anions have opposite effect on activity [25], supporting the viewpoint that the ($B_- - B_+$) value of a salt is a better parameter for quantifying the salt effect [42]. The experimental results obtained in this study have therefore suggested that for PEL suspended in [BMIm][PF_6], it is the Hofmeister effect rather than the water buffering effect, resulted from addition of salt hydrates, that has a stronger impact on the enzyme activity. In fact, the dual functions of the salt hydrates may work together to affect the enzyme catalysis, and this may offer a plausible explanation for the higher lipase activity in the ionic liquid containing a higher hydrate form of Na_2HPO_4, as can be observed in Fig. 5A. Addition of the higher hydrate form of this salt provides a higher a_w, thus introducing more water onto the enzyme surface where more salt is dissolved and ionized to get direct contact with the enzyme molecule. As the anion HPO_4^{2-} is a strong kosmotrope, it is not surprising for lipase to be more active in the ionic liquid with addition of a higher hydrate form of Na_2HPO_4. Interestingly, when PEL was suspended in hexane, the enzyme activity was well correlated with a_w (Fig. 5B) but not with the ($B_- - B_+$) value (Fig. 6B) of the salt hydrate added, suggesting that in this case, the water buffering effect offered by the salt hydrates is predominant, as compared to their specific ion effect.

Actually, the different results obtained in the two different solvent systems can be attributed to the different water requirements by the solvents: [BMIm][PF_6] is a highly polar ionic liquid that requires a large amount of water, some of which may be attracted by the enzyme to form a thin layer surrounding the enzyme molecule; whereas hexane is such a hydrophobic and nonpolar solvent that there may be only a small amount of water avail-

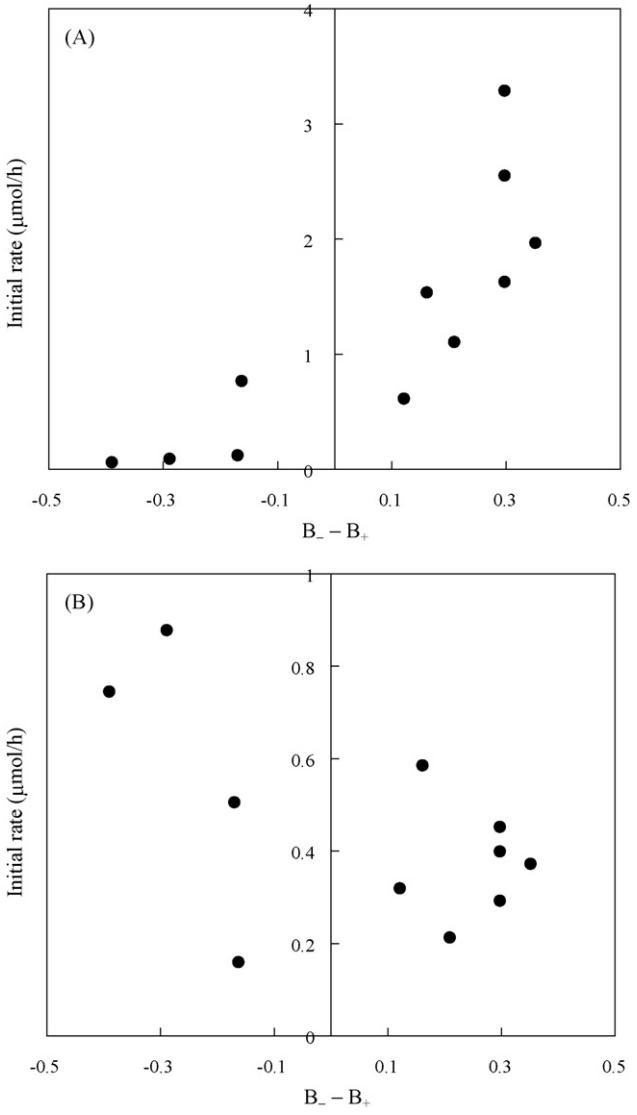


Fig. 6. Correlation between initial rate of PEL-catalyzed hydrolysis of pNPP in [BMIm][PF_6] (A) and hexane (B) and the ($B_- - B_+$) value of the salt hydrate added to the reaction system. Reaction rates obtained from Fig. 5 were used in this presentation, and the viscosity B coefficients for both cations (B_+) and anions (B_-) of the salt hydrates added were obtained from [36] to work out the ($B_- - B_+$) value for each salt: $MgCl_2$ (−0.39), $CaCl_2$ (−0.289), NaAc (0.161), $CuSO_4$ (−0.17), $K_4Fe(CN)_6$ (0.351), $ZnSO_4$ (−0.163), Na_2CO_3 (0.209), Na_2SO_4 (0.121), and Na_2HPO_4 (0.297).

able for the enzyme, far insufficient to form a monolayer around the enzyme molecule. Indeed, the optimal water activity level for immobilized *C. antarctica* lipase B to catalyze the esterification of geraniol in hexane is 0.1, much lower than the value of 0.6 for the enzyme suspended in [BMIm][PF_6] [20]. This is different from what was observed in another study: the initial rate of esterification catalyzed by an immobilized lipase (lipozyme) showed similar dependence on water activity (with the same optimal $a_w = 0.5$) in different organic solvents ranging in polarity from pentan-3-one to hexane [43]. It is worth mentioning that for the above two references, water activity was acquired by using the pre-equilibration method without the involvement of salt hydrates. The fact that lipase requires a higher a_w when suspended in [BMIm][PF_6] may be related to the extremely high polarity and the unique ionic nature of the ionic liquid.

3.4. Effect of reaction temperature

Variation of enzyme activity in both nonaqueous solvents upon an increase in the reaction temperature is shown in Fig. 7. A higher substrate concentration of 10 mM was used in hexane again because of the extremely low activity of PEL in this reaction medium. Comparing the two sets of initial conversion rates (i.e., percentages of the substrate converted per hour) supported the above result that PEL presented a higher activity in [BMIm][PF_6] than in hexane. PEL's hydrolytic activity in the ionic liquid was optimal at 70 °C, higher than the optimal temperature of 34 °C obtained in the hydrolysis of acylglycerols [6]. Higher reaction temperatures could not be studied in hexane due to its low boiling point (69 °C), after which the solvent evaporates seriously. The data in Fig. 7 were used to calculate the activation energy of the enzyme, which is 25.8 kJ/mol in hexane and 38.9 kJ/mol in [BMIm][PF_6]. It is interesting that although the enzyme was more active in the ionic liquid, its activation energy in this system was higher. A plausible explanation might be related to the water content in the solvent available for the hydrolytic reaction.

3.5. PEL-catalyzed methanolysis of corn oil for biodiesel production

The transesterification activity of PEL was investigated by conducting the PEL-catalyzed methanolysis of corn oil for biodiesel

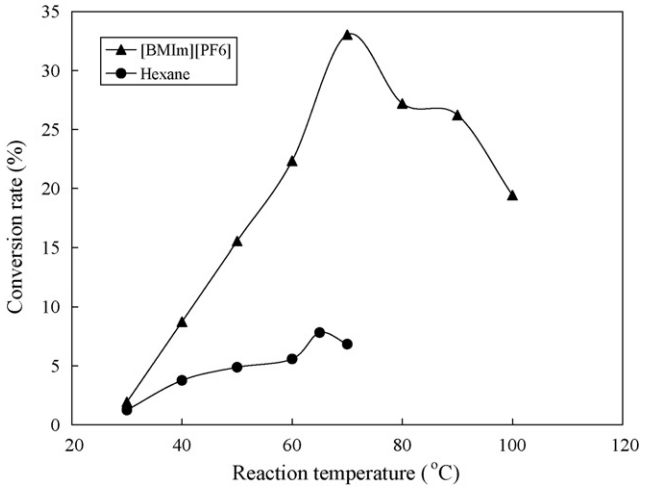


Fig. 7. Dependence of PEL's hydrolytic activity in hexane and in [BMIm][PF_6] on reaction temperature. Enzyme powders (160 mg) were added to 2 ml of hexane or [BMIm][PF_6] containing 10 or 2.3 mM of pNPP, respectively, and the reaction mixtures were incubated at different temperatures with agitation of 240 rpm. The conversion rate refers to the percentage of the substrate being converted per hour. Both solvents were used directly without drying pretreatment.

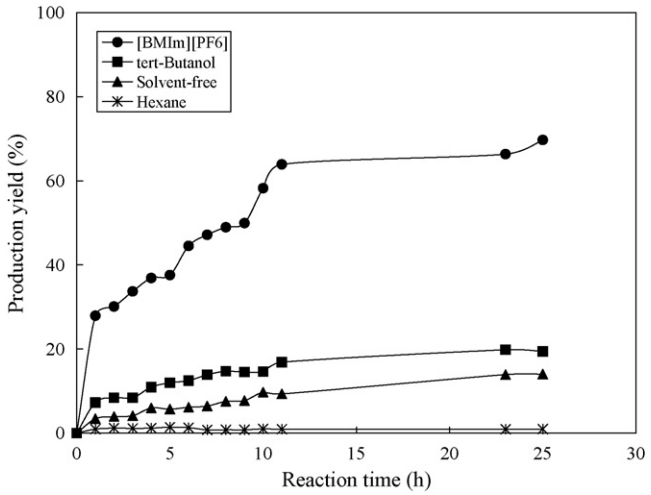


Fig. 8. PEL-catalyzed methanolysis of corn oil in [BMIm][PF_6], *tert*-butanol, hexane, and solvent-free system. Please refer to Section 2.6 for experimental details.

production. Fig. 8 shows the production of FAMEs in [BMIm][PF_6], *tert*-butanol, hexane, and in the solvent-free system. Obviously, the highest yield was achieved in [BMIm][PF_6], whereas hexane was the solvent to promote the lowest conversion. The yield obtained in the ionic liquid was 3.6-, 5.0-, and 72.5-fold as high as the one obtained in *tert*-butanol, solvent-free system, and hexane, respectively. The extremely low production yield obtained in hexane may be related to the low ability of hexane to solubilize glycerol (a by-product) and methanol (a co-substrate). A major problem for biocatalytic biodiesel production is the accumulation of these two materials on the surface of the enzyme [8]. Glycerol is so viscous that it blocks the active site of the enzyme in access with the substrate; while methanol has a strong power of inactivating enzymes. This latter situation indeed occurred to PEL, as was demonstrated in our experiments mentioned above. Taking these into consideration, the ionic liquid [BMIm][PF_6] shows its potential advantage as a solvent for PEL-catalyzed biodiesel production.

PEL has been utilized by Zong's group in catalyzing biodiesel production from corn oil [8] and waste oil [9]. A series of conventional organic solvents (petroleum ether, hexane, cyclohexane, *tert*-butanol, and *tert*-amyl alcohol) have been tested, and *tert*-amyl alcohol was found to be the optimal reaction medium for PEL-mediated biodiesel production presumably due to their good ability of solubilizing the co-substrate, methanol, and the by-product, glycerol. So far there has been only one paper dealing with lipase-catalyzed biodiesel production in ionic liquids [23]. Immobilized *C. antarctica* lipase was used to catalyze methanolysis of soybean oil for biodiesel production in 23 different ionic liquids. [EMIm][TfO] was found to be the solvent in which the highest yield was achieved, followed by *tert*-butanol, a commonly used solvent for lipase-catalyzed biodiesel production. The conversion in [BMIm][PF_6] was even poorer than that obtained in the solvent-free system.

It has to be mentioned that although our reaction conditions have not been optimized, the yield of FAMEs obtained in [BMIm][PF_6] was comparable to the results presented in the above three references. Our results of higher production yield in [BMIm][PF_6], as compared to that obtained in the other solvent systems, have clearly demonstrated that PEL is a promising lipase used for biodiesel production and that [BMIm][PF_6] is a promising reaction medium for such biotransformations. Our study has also indicated that as compared to its activity in hexane, PEL is much more active in [BMIm][PF_6], not only in catalyzing hydrolysis but also in catalyzing transesterification reactions.

4. Conclusions

This work has clearly shown that PEL offers a significantly enhanced activity, both in hydrolysis and transesterification reactions, when assayed in the ionic liquid [BMIm][PF_6] relative to the organic solvent hexane. The higher hydrolytic activity in the ionic liquid was well demonstrated in the experiments investigating the effects of enzyme amount (Fig. 3), water content (Fig. 4), water activity (Fig. 5), and reaction temperature (Fig. 7). We have also demonstrated the potential use of ionic liquids as the reaction media for PEL-catalyzed biodiesel production. It is worth noting that although [BMIm][PF_6] is so far still one of the most commonly used ILs, salts containing PF_6^- as the anion may be potentially hazardous because their hydrolysis might induce the formation of HF [44]. This study is aimed to demonstrate that the catalytic activity of PEL can be significantly improved in ionic liquids. For our later research regarding IL effects on PEL's activity and stability, 'greener' ionic liquids will be selected.

With regard to the water effect, PEL does require an essential amount of water for its activity in [BMIm][PF_6] as well as in hexane, and the significantly greater polarity of the ionic liquid can account for the much higher optimal water content it requires. The essential water can be introduced into the reaction system by direct addition of water or a salt hydrate. When added to the non-aqueous reaction system, the salt hydrate may play two roles in affecting the enzyme activity: water buffering effect and specific ion effect. To the best of our knowledge, this latter effect caused by salt hydrates has not been stressed in any studies before. Depending on the water requirement by the solvent, the former or latter effect may be dominating. Therefore, care must be taken when trying to use salt hydrates for controlling the water activity in the biocatalytic ionic liquid system.

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