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Lipase-Catalyzed Bioconversion in Ionic Liquids

Dang Thanh Dung
Lipase-Catalyzed Bioconversion in Ionic Liquids

Dang Thanh Dung
이 논문을 Dang Thanh Dung의 학위 논문으로 인정함

2007년 2월

주심

부심

위원
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Finally, I am especially grateful to my family and I really love all of them as much as they love me.
ABSTRACT

Room temperature ionic liquids (ILs) have been popular as potential green solvents due to their unique physicochemical properties, such as negligible vapor pressure, non-flammability, excellent thermal stability and a strong ability to dissolve a wide range of organic and inorganic compounds. In this study, the applications of ILs in biocatalysis were investigated. Firstly, ILs were used as pretreating reagents of lipase to increase activity and stability of *Mucor javanicus* lipase. The activities of lipase pretreated with ILs such as [Bmim][PF₆], [Emim][Tf₂N], [Bmim][BF₄] and [Emim][BF₄] were 1.8, 1.6, 1.5 and 1.6 times, respectively, higher than that of untreated lipase for the hydrolysis reaction in an aqueous medium. Furthermore, activities of lipase in ILs were well maintained even after seven days of incubation in ILs at 60°C, while untreated lipase in a phosphate buffer was fully inactivated only after 12 hours of incubation at the same temperature. Secondly, lipase-catalyzed kinetic resolution of 1-phenylethanol and 1-chloro-3-phenoxy-2-propanol with vinyl acetate were studied in various ILs and organic solvents. The enantioselectivities of lipase in ILs were much higher than those
in organic solvents and the activities of lipase were similar to those in general organic solvents for biocatalysis. For the kinetic resolution of 1-phenylethanol, operational and thermal stability of Novozym 435 lipase were drastically increased in ILs. Among ILs tested, the highest enantioselectivity, activity, and stability were observed in [Edmim][Tf$_2$N]. The activity of lipase in [Edmim][Tf$_2$N] was well maintained even after incubation of 24 hours at 80 °C. Furthermore, enzyme/IL mixture can be reused at least 5 times without loss of enzyme activity. Thirdly, lipase-catalyzed esterifications of glucose with fatty acid and fatty acid vinyl ester were carried out, because the ILs are the most suitable solvents to dissolve polar substrates such as sugars, peptides, and water miscible vitamins. The synthetic process using supersaturated glucose solution in [Bmim][BF$_4$] and [Bmim][TfO] could overcome the problem of low productivity caused by low glucose solubility in organic solvents. In addition, the use of water-mediated supersaturated glucose solution in ILs induced a higher initial rate and final conversion than those of conventional methods.
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ABBREVIATIONS

[Emim][BF₄]: 1-ethyl-3-methylimidazolium tetrafluoroborate

[Bmim][BF₄]: 1-butyl-3-methylimidazolium tetrafluoroborate

[Omim][BF₄]: 1-methyl-3-octylimidazolium tetrafluoroborate

[Emim][TfO]: 1-ethyl-3-methylimidazolium trifluoromethanesulfonate;

[Bmim][TfO]: 1-butyl-3-methylimidazolium trifluoromethanesulfonate;

[Hmim][TfO]: 1-hexyl-3-methylimidazolium trifluoromethanesulfonate;

[PPmim][TfO]: 1-phenylpropyl-3-methylimidazolium trifluoromethanesulfonate;

[Emim][MS]: 1-ethyl-3-methylimidazolium methylsulfate

[Emim][Tf₂N]: 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

[Bmim][Tf₂N]: 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

[Hmim][Tf₂N]: 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

[Omim][Tf₂N]: 1-methyl-3-octylimidazolium bis(trifluoromethylsulfonyl)imide

[Bzmim][Tf₂N]: 1-bezyl-3-methylimidazolium
bis(trifluoromethylsulfonylimide)

[PPmim][Tf$_2$N]: 1-phenylpropyl-3-methylimidazolium

bis(trifluoromethylsulfonylimide)

[Edmim][Tf$_2$N]: 1-ethyl-2,3-dimethylimidazolium

bis(trifluoromethylsulfonylimide)

[Bmim][PF$_6$]: 1-butyl-3-methylimidazolium hexafluorophosphate

[Hmim][PF$_6$]: 1-hexyl-3-methylimidazolium hexafluorophosphate

[Omim][PF$_6$]: 1-methyl-3-octylimidazolium hexafluorophosphate

[Hmim][SbF$_6$]: 1-hexyl-3-methylimidazolium hexafluoroantimonate

[Empyr][SbF$_6$]: 1-ethyl-1-methylpyrrolidinium hexafluoroantimonate

IL: ionic liquid

DMF: dimethylformamide

PB: phosphate buffer

MTBE: Methyl tert-butyl ether

HPLC: high performance liquid chromatography

NMR: nuclear magnetic resonance

LC-MS: liquid chromatography mass spectrometry
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I. INTRODUCTION

1. Ionic liquids

Ionic liquids (ILs) are generally defined as salts that contain cations and anions (Figure 1). Most of the ILs are free-flowing liquids at room temperature. These can be called Room Temperature Ionic Liquids (RTILs). Of course, these latter liquids have real advantages over higher melting salts in terms of the practicalities and handling.

The first IL (EtNH$_3$NO$_3$) was reported in 1914, but the list of ILs grows daily. The flexibility of cations and anions makes the number of ILs reach over $10^{18}$ kinds nowadays.

ILs are generally immiscible with many organic solvents especially when the latter are nonpolar, such as hexane; whereas some

<table>
<thead>
<tr>
<th>Cations</th>
<th>Anions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyridinium</td>
<td>Cl$^-$, Br$^-$, I$^-$</td>
</tr>
<tr>
<td>pyrrolidinium</td>
<td>[CF$_3$COO]$^-$, [CF$_3$SO$_2$]$^-$</td>
</tr>
<tr>
<td>Imidazolium</td>
<td>[BF$_4$]$^-$</td>
</tr>
<tr>
<td></td>
<td>[PF$_6$]$^-$</td>
</tr>
<tr>
<td></td>
<td>[(CF$_3$SO$_2$)$_2$N]$^-$</td>
</tr>
</tbody>
</table>

**Figure 1.** Common cations and anions used in combination to prepare ILs
may be miscible with polar solvents like dichloromethane and tetrahydrofuran [1]. The immiscibility of ILs with either water or organic solvents has made them feasible to be used to form two-phase systems.

Compared to typical organic solvents, ILs are much more viscous (35-500 cP viscosity for commonly used ILs versus 0.6 cP for toluene and 0.9 cP for water at 25 °C) [1]. The viscosity of an IL represents its tendency to form hydrogen bonding and the strength of its Van der Waals interactions can be lowered by increasing the temperature or by adding some organic co-solvents. Normally, an IL with longer alkyl chains on the cation and a larger anion size presents a higher viscosity.

One obvious advantage of using ILs over the use of normal organic solvents is that the physical and chemical properties of the ILs, including their hydrophilicity, hydrophobicity, viscosity, and solvent miscibility, can be finely tuned by altering both the nature of the cation and anion. This is important, because by manipulating the solvent properties, one is allowed to design an IL for specific reaction conditions, such as to increase the substrate solubility, to modify the enzyme selectivity, or to tailor the reaction rate. Generally, ILs have been widely researched as potential “green solvents” for a number of
reasons [2]:

- They have essentially no vapor pressure and thus serve as potential replacements for volatile organic compounds in the chemical industry.
- They possess good thermal stability and do not decompose over a large temperature range, thereby making it feasible to carry out reactions requiring high temperature conveniently in ILs.
- They are able to dissolve a wide range of organic, inorganic and organometallic compounds.
- They serve as a good medium to solubilize gases as H₂, CO, O₂ and CO₂ and many reactions are now being performed using ILs and supercritical CO₂.
- The solubility of ILs depends upon the nature of the cations and counteranions.
- They generally do not co-ordinate to metal complexes, enzymes and different organic substrates.
- Their ionic character enhances the reaction rates to a great extent in many reactions including microwave-assisted organic synthesis.
- Most of the ILs can be stored without decomposition for a long
time.

They show a high degree of potential for enantioselective reactions as a significant impact on the activities and selectivities due to their polar and non-coordinating properties. In addition, chiral ILs have been used to control stereoselectivity.

The viscosity of 1-alkyl-3-methyl imidazolium salts can be decreased by using highly a branched and compact alkyl chain, as well as by changing the nature of anion. The viscosity decreases in the order: $\text{Cl}^-$, $\text{PF}_6^-$, $\text{BF}_4^-$, $\text{Tf}_2\text{N}^-$.

2. Biocatalysis in ILs

Biocatalysis in nonaqueous environments instead of in aqueous media has been widely published and its importance and applicability have been well recognized [3, 4]. In many cases, it has been proven beneficial to use organic solvents instead of water (water is the solvent traditionally used for enzymatic conversion). During the last couple of decades, organic solvents have been used as good media in this area. There are a lot of advantages of using enzymes in organic solvents compared with in water, such as increased solubility of nonpolar substrates, high activity and thermal stability. However, organic
solvents have several unfavorable characteristics such as evaporability, flammability and toxic properties. As a result, ILs were paid attention as an alternative with some remarkable results such as enzyme activity, enzyme stability and enzyme selectivity. Generally, there are three types of operations for ILs in biocatalytic process. They can be used as a co-solvent in an aqueous system, in a biphasic system, or as a pure solvent. One early study can be dated back to 1984, which demonstrated the activity and stability of alkaline phosphatase in aqueous mixtures of [EtNH$_3$][NO$_3$]. The authors reported an increased activity of alkaline phosphatase at low [EtNH$_3$][NO$_3$] concentrations. Moreover, Kragl et al. (2002) have studied galatosylation with β-galactosidase from Bacillus cirrulans for the synthesis of N-acetyllactosamine for lactose and N-acetylglucosamine in 25% [Emim][MeSO$_2$] IL-water mixture. This mixture suppressed the secondary hydrolysis of the product, resulting in a doubling of the yield to almost 60% [5].

The investigation of new biphasic reactions using ILs is gaining special interest. The possibility to adjust solubility properties using different cation/anion combinations allows for the systematic optimization of a biphasic reaction. Even three-phase systems are
possible, consisting of water, IL and organic solvent. This is beneficial for systems where organic solvents in combination with an aqueous phase either do not dissolve enough substrate or lead to increased enzyme deactivation. Recently, Ley et al. (2000) reported the use of a biphasic [Bmim][PF$_6$]/H$_2$O medium for the conversion of 1,3-dicyanobenzene to 3-cyanobenzamide and 3-cyanobenzoic acid catalyzed by whole cells of *Rhodococcus* R312 [6]. The ILs act as reservoirs for substrate and product, thereby decreasing substrate and product inhibition observed in water, and hence, increasing the catalytic productivity. This work established ILs as a potential alternative to organic solvents for multiphase biotransformations.

It was only recently, however, that the first results describing the use of pure ILs as an enzymatic reaction medium were published [7,8]. In these studies, ILs such as [Bmim][PF$_6$] or [Bmim][BF$_4$] were simply used to replace organic solvents. The enzymes, thermolysin and *Candida antarctica* lipase B, showed the same activity and selectivity when compared with the original medium. Therefore, ILs can indeed be used for biocatalytic reactions.

Although polar organic solvents inactivate enzymes, surprisingly, ILs do not inactivate enzymes. Enzymes usually do not dissolve in ILs
those are remained in suspended form. This feature extends enzyme-catalyzed reactions to a solvent polarity range that was previously inaccessible. The ability to use solvents with greater polarity increases the solubility of polar substrates, such as glucose, maltose or ascorbic acid [9], leading to faster reactions and changes in selectivity. For example, in the *Candida Antarctica* lipase B (CAL-B)-catalyzed acylation of ascorbic acid with oleic acid in an IL, the conversion was higher (83%) than compared with typical results in organic solvents (50%) [10].

### 2.1 Enzyme activity in ILs

Not all ILs are suitable for biocatalysis. Enzymes are usually active in ILs containing BF$_4^-$, PF$_6^-$, TfO and Tf$_2$N anions, but not in ILs containing Cl, NO$_3^-$, CF$_3$SO$_3^-$ or acetate anions [11]. A possible reason for this difference is the lower hydrogen-bond basicity of the enzyme-compatible anions. The BF$_4^-$ anion spreads its negative charge over four fluorine atoms, the PF$_6^-$ anion over six fluorine atoms and the Tf$_2$N anion over five atoms. The lower hydrogen-bond basicity minimizes interference with the internal hydrogen bonds of an enzyme. Consistent with this notion, enzymes are inactive in [Bmim][Cl]
(liquid at 65°C), which has high hydrogen-bond basicity [12]. Many researchers have reported that while present in ILs, a variety of enzymes [Table 1] are capable of performing catalytic activities, which are generally comparable with or higher than those observed in conventional organic solvents [13,14]. When preparing the active enzyme encapsulated cellulose films reconstituted from ILs, Turner et al. [15] found that pre-coating the enzyme (laccase) with a second, hydrophobic IL prior to dispersion in the cellulose/IL solution can provide an increase in enzyme activity relative to that of untreated films, and this seemed to suggest that the IL is capable of providing a stabilizing microenvironment for the enzyme. A systematic study on the lipase-catalyzed resolution of amino acid ester in ILs has suggested that enzyme activity can be improved by adjusting the solvent parameters of the ILs such as their nature, polarity, and concentration [16]. Obviously, it is necessary to work out the controlling factors that affect the enzyme activity in ILs so as to be able to optimize and to take advantage of biocatalysis in such a new reaction medium.
<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Reaction</th>
<th>IL</th>
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<tbody>
<tr>
<td>Lipase</td>
<td>Transesterification</td>
<td>[Bmim][PF$_6$]</td>
</tr>
<tr>
<td></td>
<td>Alcoholysis, ammonialysis, perhydrolysis</td>
<td>[Bmim][PF$_6$], [Bmim][BF$_4$]</td>
</tr>
<tr>
<td></td>
<td>Kinetic resolution of chiral alcohols</td>
<td>[Bmim][Tf$_2$N]</td>
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<tr>
<td></td>
<td>Resolution of amino acid ester</td>
<td>[Epy][BF$_4$], [Emim][BF$_4$]</td>
</tr>
<tr>
<td></td>
<td>Esterification of carbohydrates</td>
<td>[MOEmim][BF$_4$]</td>
</tr>
<tr>
<td></td>
<td>Synthesis of polyesters</td>
<td>[Bmim][PF$_6$]</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Enantioselective reduction of 2-octanone</td>
<td>[Bmim][Tf$_2$N]</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>Synthesis of Z-aspartame</td>
<td>[Emim][CF$_3$CO$_2$]</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>Transesterification</td>
<td>[Bmim][PF$_6$]</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>Synthesis of (N)-acetyllactosamine</td>
<td>[Mmim][MeSO$_4$]-H$_2$O</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Oxidation of guaiacol</td>
<td>[Bmim][PF$_6$]</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>Regeneration of NADH</td>
<td>[MmIm][MeSO$_4$]-H$_2$O</td>
</tr>
<tr>
<td>Baker’s yeast</td>
<td>Enantioselective reduction of ketones</td>
<td>[Bmim][PF$_6$]-H$_2$O</td>
</tr>
</tbody>
</table>
2.2 Enzyme stability in ILs

Given the well-known fact that enzyme stability is usually much higher in organic media, especially at a low water activity, than in aqueous solution, it is expected that ILs should have the same effect on stabilizing enzymes. Indeed, the activity of thermolysin was well-retained after incubation in [Bmim][PF₆] at 37°C for 144 hr, whereas the same treatment in ethyl acetate resulted in the loss of almost half of the enzyme activity [7]. The stability of the esterase from *B. stearothermophilus* at 40 °C was also found to be considerably increased in the ILs, [Bmim][BF₄] and [Bmim][PF₆] as compared to that in hexane and MTBE: a half-life of >240 hr was obtained in [Bmim][PF₆], which was >30-fold and >3-fold higher as compared to that in hexane and MTBE [17]. Moreover, the stability of α-chymotrypsin was studied by incubation in four different ILs at 2% (v/v) water content and 50 °C [18]. The deactivation profiles fitted with the first-order kinetic model and the half-life of the enzyme (1.08–2.63 h) was significantly enhanced, as compared to that of 0.15 hr in 1-propanol. The increase in the enzyme’s half-life seemed to be in agreement with the increase in hydrophobicity of the ILs. It is reasonable that a hydrophobic reaction medium would allow the preservation of the essential water molecules surrounding the protein structure, thereby reducing the protein–ion direct contact and enhancing the enzyme stability toward denaturation. The same phenomenon was also observed when *C. antarctica* lipase B was used to catalyze the ester synthesis reactions in IL/supercritical CO₂ biphasic systems [19]. By fitting the kinetic data obtained at 50 °C to a two-step kinetic
deactivation model, the authors reported that all the five ILs based on quaternary ammonium cations that were tested acted as stabilizing agents to the enzyme with respect to hexane, producing an increase in the free energy of deactivation and an improvement in the half-life of the enzyme (2000-fold), which agrees with the observed increased hydrophobicity of the cation alkyl side chain. Frater et al. [20] have also reported that at elevated temperatures, C. rugosa lipase in ILs kept its activity and enantioselectivity much better than in traditional organic solvents. The stabilization effect of the ILs on enzymes can be further improved by incubating the enzyme in the presence of the substrate, in this way α-chymotrypsin obtained a 10-fold increase in its half-life [21]; more severely, the reuse of free lipase (C. antarctica lipase B) in [BMIm][PF$_6$] in continuous operation cycles showed a half-life of 2300 times greater than that observed when the enzyme was incubated in the absence of substrate [22]. The association of the substrate to the enzyme may cause a conformational change that activates the enzyme, and this active conformation is well-retained during the presence of the substrate. Studies on enzymes after incubation in ILs have also revealed that enzymes are not only strongly tolerant against ILs, but they can even be activated. The thermostability of C. antarctica lipase B, either free (Novozym SP525) or absorbed on a macroporous acrylic support (Novozym 435), was investigated by incubating the enzyme preparation in anhydrous [BMIm][PF$_6$] at 80°C for a specific period and then after dilution with water, measuring the residual activity in triacetin hydrolysis [22]. Both enzyme forms showed a significant increase in their activities: 120% increase for the free enzyme after
incubation for 20–100 hr, and 350% increase for Novozym 435 after 40 hr incubation. The mechanism involving this enzyme activation is still unknown and deserves a further investigation. It is necessary to mention the two recent spectroscopic studies of protein thermostability in ILs. By conducting fluorescence studies of a single tryptophan protein, monellin, Baker et al. [23] reported the first detailed protein spectroscopy in ILs, which indicated that the significant thermodynamic stabilization effect offered by the IL may result from alteration in the protein hydration level and structural compaction. The authors also suggested that some other factors such as free volume contributions, ionic interactions and confinement effects may also contribute to the protein stabilization by ILs. More recently, Iborra’s research group reported a more detailed spectroscopic study on protein thermostability [24]. They studied the stability of α-chymotrypsin in the IL [Emim][Tf$_2$N] at elevated temperatures and compared that to the stability in other liquid media such as water, 3M sorbitol, and 1-propanol. Their results have revealed that enzyme stabilization by ILs seemed to be related to the associated structural changes of the protein. Differential scanning calorimetry (DSC) revealed that both the melting temperature and heat capacity of the enzyme was enhanced by the IL as compared to the other media. The fluorescence spectra clearly showed the ability of the IL to compact the native structural conformation of the enzyme, preventing the usual thermal unfolding which occurs in other media. The circular dichroism (CD) spectra have also demonstrated the changes in the secondary structure of the protein in the presence of the IL, reflecting its stabilization power.
2.3 Enzyme selectivity in ILs

There is an increasing demand for enantiomerically pure compounds in the pharmaceutical, fine chemical, and material science industries both for reasons of patient safety and to encourage the development of more efficient chemical processes. Because only one of the enantiomers of the drug showed pharmacological activity while the other enantiomer may exhibit negative side effects [25]. Berglund (2001) investigated how to control lipase enantioselectivity for organic synthesis that emphasized some important aspects for the control of lipase enantioselectivity [26]. There were three strategies (engineering of the reaction medium, the substrate molecule, and the enzyme) for exploring lipase enantioselectivity at a molecular level. These three different approaches represent powerful tools for understanding the molecular basis for lipase enantioselective catalysis and can guide the rational improvement and tailoring of catalyst performance. During the last two decades, organic solvents were known as good media with numerous papers published on various aspects of enzyme-catalyzed reactions of chiral compounds. The nature of the solvent is also known to be of importance for a high enantioselectivity in lipase reactions. However, demand for ‘green solvents” makes organic solvents less important in lipase enantioselectivity for organic synthesis. Recently, ILs have gained increasing attention for performing all types of reactions with sometimes remarkable results. Several research groups have reported that lipase showed higher enantioselectivity when used for kinetic resolution of chiral alcohols in ILs [13,27,28,29] And the requirement for enantiomerically pure products has been fully
improved [30] by using lipase-catalyzed transterifications in ILs with markedly enhanced enantioselectivity. Kim et al showed the enantioselectivity of lipase in ILs were up to 25 times higher than that in common organic solvents [27]. In addition, Dynamic kinetic resolution of racemic substrates that provide a useful method for the preparation of enantimomerically has been also studied. These results have demonstrated that the racemization of secondary alcohols exhibited more efficiency in ILs than in organic solvents [28]. The enzyme-mediated kinetic resolution in ILs has shown its advantages when conducted at elevated temperatures due to the high enzyme thermostability in ILs. The kinetic resolution of (R,S)-phenylethanol catalyzed by a lipase from Pseudomonas sp. in [Bmim][Tf2N] remained highly enatoselective, with only a minimal decrease in the $E$-value from 200 to 150 when temperature was raised from 25 to 90 °C, while in MTBE, the $E$-value dropped dramatically to 4 at 55 °C [14]. Moreover, effect of water activity on enantioselectivity of lipase from Pseudomonas sp. has been reported by Eckstein’s group. They demonstrated that a higher selectivity existed in the IL than that in methyl tert-butylether at low water activities and even high temperature [29].

2.4 Glucose ester production in ILs

Glucose fatty acid esters have a wide range of commercial applications (pharmaceuticals, cosmetics, food industry, etc.) due to their surfactant properties [30]. Although most of these compounds are chemically synthesized, enzymatic synthesis of glucose esters has
gained great consideration in making environmental-friendly processes [31]. However, a major problem in synthesizing glucose esters by using enzymes in non-aqueous media is the low solubility of glucose in most organic solvents. To dissolve glucose of high concentration, hydrophilic organic solvents are desirable as the reaction media, but most enzymes are rapidly inactivated under hydrophilic organic solvents. Therefore, the saturated glucose solution in the presence of crystal or metastable supersaturated solution can be used for the enzymatic reaction in less harmful organic solvents such as acetonitrile, acetone, t-butanol and 2-methyl 2-butanol [30,32,33]. However, synthetic reactions may be still limited by low solubility and low dissolution rate of glucose. Alternatively, the solubility can be increased by using protected glucoses or alkyl glycosides, but the use of these substrates requires extra steps [34].

When used as solvents for chemical processes, ILs exhibit excellent physical characteristics including the ability to dissolve polar and nonpolar organic, inorganic, and polymeric compounds. Moreover, the number of combinations of anions and cations encompassed by ILs is vast, and their associated synthetic flexibility has lead to ILs being referred to as ‘designer solvents’ [35]. A few groups have reported that ILs have great potential as alternative reaction media for biocatalysis and biotransformation. It was observed that their use enhanced the activity, selectivity, and stability of enzymes. Spear et al. reported the good solubility of various mono and disaccharides in ILs containing the [Cl] anion [36]. However, ILs containing the [Cl] anion cannot be used to carry out enzyme reaction by the inactivation of most enzymes [37].
Only ILs containing dicyanamide ([dca]) anion have been reported as good solvents for sugar dissolution and enzyme reaction [31]. Recently, pure [Bmim][BF₄] and [Bmim][PF₆] were used as reaction media in the lipase-catalyzed transesterification of glucose with fatty acid vinyl ester, although the solubility of glucose in these ILs is very low [34].
II. OBJECTIVES

In the present study, firstly, the activity and stability of *Mucor javanicus* lipase pretreated with various ILs were investigated for *p*-nitrophenyl butyrate hydrolysis reaction in an aqueous medium. Secondly, the kinetic resolution of 1-phenylethanol and 1-chloro-3-phenoxy-2-propanol with vinyl acetate catalyzed by lipase Novozym 435 was carried out in ILs of different hydrophobicities and common organic solvents. Among the ILs, we selected the best candidates for enantioselectivity, thermal and operational stability that compared with conventional organic solvents. One of potential advantages of ILs, recycling of enzyme/[Edmim][Tf$_2$N] mixture after the conversion as well as enantioselectivity was studied. Thirdly, we investigated the solubility of sugars in various ILs and prepared the supersaturated solution of sugars in ILs. The stability of the supersaturated solution was also studied for the application to the biocatalytic reaction. In addition, lipase-catalyzed transesterification and direct esterification of glucose with vinyl laurate and lauric acid were successfully achieved in pure IL. And water-mediated supersaturated glucose solution in IL was used to increase the dissolved concentration of glucose in ILs. Summarily, there were some objectives in this study:

- Enhance lipase (from *Mucor javanicus*) activity and stability by ILs-treated lipase
- Investigate ILs as green solvents for Novozym 435 lipase of selectivity, operational, thermal stability and recycling in biocatalysis
Investigate the supersaturated solution in ILs that dissolve carbohydrate in high concentrations

Use ILs as media for glucose ester synthesis to overcome the problem of low productivity in organic solvents
II. EXPERIMENTAL

1. Enhanced activity and stability of lipase by pretreated with ILs

1.1 Materials

Lipase from *Mucor javanicus* was purchased from Sigma. [Bmim][BF₄] was purchased from Merck (Germany) and [Emim][BF₄], [Bmim][PF₆], [Emim][TfO], [Hmim][TfO] and [Emim][Tf₂N] were kindly supplied by C-Tri (Korea). p-Nitrophenyl butyrate, used as the substrate in the hydrolysis reaction, and dimethylformamide (DMF) were purchased from Sigma-Aldrich. Sodium phosphate was purchased from Oriental Chemical Industry (Korea). Water bath (Vision Company, Korea), circulator (Cole-Parmer Instrument Company, USA) and UV/VIS spectronic (Milton Roy Company, USA) instrument were used for this experiment.

1.2 Treatment of lipase (*Mucor javanicus*) with ILs

10 mg of lipase was suspended in 1 ml of various ILs for 20 minutes. The mixture including lipase and IL was used in the hydrolysis reaction.

1.3 Activity of lipase

Enzyme activity was determined by measuring the increase in absorbance at 400 nm produced by the release of p-nitrophenol during
the hydrolysis of 0.5 mM p-nitrophenyl butyrate in 20 mM phosphate buffer (PB) at pH 6.5 and 37°C. The reaction was started by adding 10 µl of lipase solution or lipase/IL mixture which contained 0.02 mg of lipase to 990 µl of substrate solution and carried out at 37 °C in the water bath with shaking at 200 rpm or in the circulator with stirring at 300 rpm. To investigate the effect of different concentrations of ILs in the reaction medium on enzyme activity, the reaction was started by adding 2, 5, 10, 20 and 30 µl of lipase/IL to the substrate solution to make 0.2, 0.5, 1, 2, and 3 % (v/v) of lipase/ILs in 1 ml of the reaction medium. Lipase concentration in reaction medium was kept at 0.02 mg/ml throughout the experiment.

1.4 Stability of lipase

Lipase was suspended in PB or various ILs, and then incubated at 60°C. At regular time intervals, the enzyme was withdrawn and the activity was measured as described above.

2. Lipase-catalyzed kinetic resolution of chiral compounds in ILs

2.1 Materials

Commercial lipase, Novozym 435, was purchased from Danmark. All of the ILs, 1-chloro-3-phenoxy-2-propanol and phenylethyl acetate were kindly supplied by C-Tri (Korea). 1-Phenylethanol, vinyl acetate and other chemicals which were used in this work were purchased from
Sigma-Aldrich, USA.

2.2 HPLC analysis

The extracts were analyzed by HPLC using a chiral column (250mm × 4.6mm, OJ-H, Daicel). The mobile phase was used as n-hexane:iso-propanol:ethanol (965:30:5) for 1-phenylethanol and n-hexane:iso-propanol (90:10) for 1-chloro-3-phenoxy-2-propanol with a flow rate of 1 ml/min. A wavelength of 205 nm was used to be able to detect all compounds in the extracts. With these conditions, the enantiomers of the 1-phenylethanol or 1-chloro-3-phenoxy-2-propanol and of product 1-phenylethyl acetate or 1-chloro-3-phenoxy-2-propanyl acetate could be significantly separated without any influence of the IL. The conversion (C) in percentage was calculated by following equation: \[ C = \frac{EE_s}{(EE_s + EE_p)}, \]
where \( EE_s = \frac{(R_s-S_s)}{(R_s+S_s)} \) and \( EE_p = \frac{(R_p-S_p)}{(R_p+S_p)} \) are enantiomeric excess of substrate and product, respectively. The enantioselectivity (E) was calculated by using the equation \[ E = \ln\left(\frac{(1-C)(1-EE_s)}{(1-C)(1+EE_s)}\right)/\ln\left(\frac{(1-C)(1+EE_s)}{(1-C)(1-EE_s)}\right). \]

2.3 Kinetic resolution of 1-phenylethanol and 1-Chloro-3-phenoxy-2-propanol

In this part of the experiment, 100 µmol 1-phenylethanol and 100 µmol vinyl acetate (in case of 1-chloro-3-phenoxy-2-propanol, 200 µmol 1-chloro-3-phenoxy-2-propanol and 200 µmol vinyl acetate) were dissolved in 1ml ILs or organic solvents. The reaction was started by adding 5 mg Novozym 435 in the mixture and run by stirring at 1000 rpm at 50 °C for 20 hr. After completing the reaction, a 10 µl of
sample was taken and extracted with 0.1 ml of \( n \)-hexane: iso-propanol (9:1). The extracts were analyzed by HPLC following the conditions as described above.

**2.4 Operational stability of lipase**

Operational stability of enzyme was carried out by measuring the conversion and enantioselectivity of enzyme in ILs or organic solvents. Here, we maintained the reaction for 12 hours until the next recycling operation began. During each cycle, enzyme was collected from the IL or organic solvent media and washed with hexane to eliminate the coating of IL on the enzyme. Then another reaction was begun by adding reused enzyme to the reaction solution including fresh ILs or organic solvents (1 ml) and the fresh substrates (100 µmol 1-phenylethanol and 100 µmol vinyl acetate).

**2.5 Thermal stability of lipase**

We added 10 mg of lipase and 1 ml of ILs (or organic solvents) into different screw-capped vials (4 ml capacity). The mixtures were then incubated at 80 °C in the absence of substrates. At different intervals, 100 µmol 1-phenylethanol and 100 µmol vinyl acetate were added to different vials to initiate the reaction. The initial rate, conversion and enantiomeric excess were determined as described above.

**2.6 Recycling of enzyme/IL mixture**

[Edmim][Tf₂N] was selected as the best candidate for recycling of the enzyme/IL mixture. 100 µmol 1-phenylethanol and 100 µmol vinyl
acetate were introduced to the enzyme/IL mixture (10 mg of enzyme and 1 ml of [Edmim][Tf$_2$N]) in the vial to initiate the reaction time of 5 hours. For extraction, 20 ml of hexane and isopropanol (9.5:0.5) were added into the finished reaction vial to make a biphasic mixture. This mixture was significantly shaken to extract all unreacted substrates and products into the hexane:isopropanol phase. The top of phase (organic solvents) was carefully removed and the enzyme/IL mixture was washed more two times with fresh hexane:isopropanol. The IL phase was analyzed by HPLC to ensure that all of products and unreacted substrates had been eliminated. Then next batch reaction was initiated by the addition of the fresh substrates (100 µmol 1-phenylethanol and 100 µmol vinyl acetate) into the enzyme/IL mixture. After each cycle, the initial rate and enantiomeric excess were determined as described above.

3. Supersaturated sugar solution in ILs

3.1 Materials

All ILs were synthesized and purified by C-TRI (Suwon, Korea) and had a residual chloride content of less than 30 ppm. ILs were dried in vacuum oven at 60°C for several days before use. Sucrose and D-(+)-glucose were purchased from Sigma (St. Louis, USA). All other chemicals used in this work were of analytical grade and used without further purification.
3.2 Determination of sugar content

The glucose concentration was determined by dinitrosalicylic acid (DNS) method [38] with glucose standard. To measure the concentration of sucrose, sucrose was firstly hydrolyzed with concentrated HCl solution and then analyzed by DNS method with sucrose standard. It was observed that ILs does not interfere on the DNS methods in the measured concentration range (0.1 g/L – 1.0 g/L).

3.3 Solubility of sugar

Glucose (250 mg) or sucrose (50 mg) was added to the glass vials containing ILs (1 ml). The suspension was stirred for 12 hr at 25 °C and 60 °C. After centrifugation for 5 min with 14000 rpm, supernatant was obtained.

3.4 Supersaturated sugar solution by direct method

Glucose (250 mg) or sucrose (50 mg) was added to the glass vial containing ILs (1 ml) and the suspension was stirred for 12 hr at 60 °C. The saturated solution in the presence of excess sugar was slowly cooled to 25 °C and incubated for 2 hr at 25 °C. After centrifugation, supernatant was obtained.

3.5 Supersaturated sugar solution by water-mediated method

Firstly, glucose (50 mg) or sucrose (50 mg) was dissolved in water (0.3 ml). Then ILs (1 ml) were added to these solution at room temperature. The water content could be increased to dissolve glucose
of higher than 50 mg, but the concentration of glucose in water (167 g/l) was maintained to obtain clear solutions. The contained water in the mixtures was removed by vacuum evaporation for 12 hr at 60°C. The residual water content was measured with weight difference and confirmed by Karl-Fischer Titration. The saturated sugar solutions were slowly cooled to 25°C and incubated for 2 hr at 25°C. After centrifugation, the supernatant was obtained.

4. Lipase-catalyzed glucose ester production by using supersaturated glucose solution in ILs

4.1 Materials

Novozym 435 (Candida antarctica type B lipase immobilized on acrylic resin) was provided by Novo Nordisk ( Bagsvaerd, Denmark). [Bmim][TfO] and [Bmim][BF_4] were synthesized and purified by C-TRI (Suwon, Korea) and had a residual chloride content of less than 30 ppm. ILs were dried in vacuum oven at 60°C for several days before use. D-(+)-Glucose, vinyl laurate, and lauric acid were purchased from Sigma (St. Louis, USA). All other chemicals used in this work were of analytical grade and used without further purification.

4.2 Esterification of glucose with vinyl laurate

Substrate solutions were prepared by the previously described method. For each reaction, 0.5 ml of a prepared substrate solutions were added to 4ml vials containing Novozym 435 (50 mg). The mixtures were incubated in a shaking incubator at 40°C. At the end of
the reaction, deionized water (1 ml) was added to the reaction vials in order to remove unreacted glucose. The supernatant was obtained after filtration and centrifugation of reaction media. The content of unreacted glucose in water was measured by DNS method. The precipitated product was dissolved in tetrahydrofuran and the concentration was determined by HPLC analysis.

4.3 Esterification of glucose with lauric acid

Substrate solutions were prepared by the previously described method. For each reaction, 0.5 ml of a prepared substrate solutions were added to 4ml vials containing Novozym 435 (50 mg). The mixtures were incubated in a shaking incubator at 50°C. At the end of the reaction, deionized water (1 ml) was added to the reaction vials in order to remove unreacted glucose. The supernatant was obtained after filtration and centrifugation of reaction media. The content of unreacted glucose in water was measured by DNS method. The precipitated product was dissolved in tetrahydrofuran and the concentration was determined by HPLC analysis.

4.4 HPLC analysis

The concentrations of 6-O-lauroyl-D-glucose were measured by HPLC. Separation was accomplished using a Shimadzu HPLC system (Model LC-10A, Japan) equipped with a reverse-phase C\textsubscript{18} column (SYMMETRY, Waters, USA) and a RI detector (Waters 410, USA). The mobile phase consists of methanol : water = 90 : 10 with a flow rate of 1.0 ml/min.
III. RESULTS AND DISCUSSION

1. Enhanced activity and stability of lipase by pretreated with ILs

1.1 Activity of lipase (*Mucor Javanicus*) pretreated with ILs

The hydrolytic activities of ILs-pretreated lipase in aqueous media with various concentrations of [Emim][BF₄] are shown in Figure 2. To compare enzyme activities between [Emim][BF₄]-pretreated lipase and untreated lipase with [Emim][BF₄] in reaction, free lipase in the reaction medium with different concentrations of [Emim][BF₄] was prepared by adding lipase solution and [Emim][BF₄] into the reaction medium as additives. The activities of ILs-pretreated lipase were much higher than those of free lipase at various ILs concentrations (0.2-3 %) in the reaction medium. The highest activity was obtained when [Emim][BF₄]-pretreated lipase was dissolved in the reaction medium with 0.5 % (v/v) of [Emim][BF₄]. Since there was no separation of enzyme from ILs after treatment in this study, the effect of ILs on the hydrolysis reaction was examined. ILs generally are more viscous than water and common organic solvents ([Emim][BF₄] 66.5 cP at 20°C, [Bmim][BF₄] 154 cP at 20°C; H₂O 0.9 cP at 25°C; toluene 0.59 cP) [39]. The high viscosity limits the rate of diffusion of solute molecules, and thus, may limit the rate constants of bimolecular chemical reactions.
To find out whether the viscosity was related to lower enzyme activities in higher ILs concentration, the reaction was carried out in the reaction block with stirrer to provide perfect mixing. As shown in Table 2, there were little differences in enzyme activities in reaction media with over 1 % of [Emim][BF$_4$] in between the water bath and the reaction block as reaction system (2.6 % and 4.2 % for 1 % and 3 % of [Emim][BF$_4$]), respectively. However, a much higher enzyme activity (26 % increase) was observed in the reaction block compared to that in the water bath at 3 % of [Bmim][BF$_4$] in the reaction medium. These results imply that the viscosity of ILs does not seriously affect enzyme activity in the range of IL concentrations in reaction media tested except 3 % of [Bmim][BF$_4$]. Table 2 also shows that the highest enzyme activity was obtained at 0.5 % of ILs in the reaction medium as observed in Figure 2. It implies that there exists an optimum concentration of ILs (0.5 % in this case) in reaction medium for enzyme activity.

1.2 Stability of lipase (*Mucor Javanicus*) in ILs

The thermal stability of lipase in ILs was investigated by incubating *Mucor javanicus* lipase in [Emim][BF$_4$] at 60°C. Figure 3 shows that the residual activities of lipase were well maintained around 78 %, 83 %, 72 %, and 68 % at 0.5 %, 1 %, 3 %, and 5 % (v/v) of [Emim][BF$_4$] in reaction medium, respectively, even after 7 days of incubation in [Emim][BF$_4$] at 60°C. The amount of untreated lipase was 6 % only after 12 hours of incubation at 60°C which indicates the denaturation of untreated lipase by high temperature (Figure 3). A
possible explanation for enhanced activity and stability of [Emim][BF₄]-pretreated lipase is the change on the secondary structure of lipase in ILs. De Diego et al. have demonstrated that the stabilization of lipase by ILs seems to be related to the observed evolution of α-helix and β-sheet secondary structure of the enzyme, leading to a more compact enzyme conformation able to exhibit more activity and stability [40].

Since the optimal concentrations of [Emim][BF₄] in reaction medium for the activity and stability of lipase were found to be 0.5 % and 1 % (v/v), respectively, lipase was pretreated with various ILs to investigate the effect of change in ILs structure on enzyme activity. Figure 4 shows that the activity of lipase pretreated with ILs were higher than that of untreated lipase in the hydrolysis reaction in an aqueous medium. The activities of lipase pretreated with [Bmim][BF₄], [Bmim][PF₆], [Emim][BF₄] and [Emim][Tf₂N] were 1.5, 1.8, 1.6 and 1.6 times higher than that of untreated lipase in phosphate buffer, respectively. The activities of lipase pretreated with hydrophobic ILs ([Bmim][PF₆] and [Emim][Tf₂N]) were a little higher than those in hydrophilic ILs ([Bmim][BF₄] and [Emim][BF₄]). It means that hydrophobic ILs may be favorable for enzyme activity in hydrolysis reaction rather than hydrophilic ILs.

The thermal stability of lipase in various ILs was also studied by incubating lipase in ILs at 60°C. Figure 5 shows the residual activity of lipase pretreated with hydrophobic ILs such as [Bmim][PF₆] and [Emim][Tf₂N] were well maintained at 85 % and 82 % even after 7 days of incubation at 60°C, respectively. Hydrophilic ILs
([Bmim][BF₄] and [Hmim][TfO])-pretreated lipase also retained their initial activity more than 80% even after 7 days of incubation at 60°C. However, [Emim][TfO]-pretreated lipase whose activity was significantly decreased after 3 days of incubation as shown in Figure 6. One possible explanation of high thermal stability of enzyme in ILs is that the structure of enzyme may be compact in ILs which prevents the thermal denaturation of enzyme under the temperature condition tested. This enhancement of thermal stability of lipase in ILs was a coincidence with the results of Erbeldinger et al. [7]. ILs might stabilize enzyme to a greater extent than the commonly used organic solvents. Lozano et al. have also shown that ILs could stabilize the enzyme and increase enzyme half-life greatly [18].
Table 2. Activity of lipase pretreated with [Emim][BF$_4$] or [Bmim][BF$_4$] at different concentrations of ILs in the hydrolysis reaction

<table>
<thead>
<tr>
<th>ILs</th>
<th>Concentration [%(v/v)]</th>
<th>Water bath</th>
<th>Circulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Emim][BF$_4$]</td>
<td>0.2</td>
<td>0.73</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.82</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.73</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.65</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>0.57</td>
<td>0.60</td>
</tr>
<tr>
<td>[Bmim][BF$_4$]</td>
<td>0.2</td>
<td>0.79</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.80</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.77</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>0.45</td>
<td>0.62</td>
</tr>
</tbody>
</table>
Figure 2. The effect of [Emim][BF₄] concentration in the reaction medium on activity of [Emim][BF₄]-pretreated enzyme ([Emim][BF₄]-pretreated lipase (black bar), free lipase with [Emim][BF₄] in reaction mixture as an additive (grey bar)).
Figure 3. Stability of lipase was carried out by incubating lipase in [Emim][BF₄] at 60°C. The activity was measured in aqueous solution containing 0.5% (▲), 1% (▲), 3% (●) and 5% (▼) of [Emim][BF₄] in reaction mixture and untreated lipase in phosphate buffer (▼) at 60°C.
Figure 4. Activities of lipase pretreated with various ILs in hydrolysis reaction (A: unpretreated; B: [Emim][BF$_4$]; C:[Bmim][BF$_4$]; D: [Bmim][PF$_6$]; and E: [Emim][Tf$_2$N]).
Figure 5. Stability of lipase was carried out by incubating lipase in pure hydrophobic ILs at 60°C. The activity was measured in an aqueous solution containing 1% [Bmim][PF$_6$] (□), 1% [Emim][Tf$_2$N] (▲), Phosphate buffer (△).
Figure 6. Stability of lipase was carried out by incubating lipase in pure hydrophilic ILs at 60°C. The activity was measured in an aqueous solution containing 1% [Emim][BF$_4$] (■), 1% [Bmim][BF$_4$] (▲), 1% [Emim][TfO] (▼), 1% [Hmim][TfO] (▲), Phosphate buffer (■).
2. Lipase-catalyzed kinetic resolution of chiral compounds in ILs

2.1 Kinetic resolution of 1-phenylethanol and 1-chloro-3-phenoxy-2-propanol

The conversion and enantioselectivity in the lipase-catalyzed acetylation of 1-phenylethanol using various ILs containing different cations and anions as reaction media were compared with those in normal organic solvents such as Hexane and DMSO. Table 3 showed that the reaction conversions in ILs were lower than that in hexane. However, the enantioselectivity in ILs were significantly higher ($E > 200$).

The influence of IL properties on kinetic resolution of 1-phenylethanol derivatives was investigated. Considering the same cation (1-butyl-3-methylimidizolium) and different anions, higher conversion was obtained in [$\text{Tf}_2\text{N}^-$] ILs and conversion decreased with the following order [$\text{Tf}_2\text{N}^-$] < [$\text{PF}_6^-$] < [$\text{TfO}^-$] < [$\text{BF}_4^-$]. These trends can be correlated with the dipolarity/polarizability values of ILs ([$\text{Tf}_2\text{N}^-$] < [$\text{PF}_6^-$] < [$\text{TfO}^-$] < [$\text{BF}_4^-$]). Considering same anion, ILs containing long alkyl chain cation showed lower conversion. The influence of cation alkyl chain on the conversion can be explained with viscosity and hydrogen bond basicity. The increase of cation alkyl chain length leads to increase of viscosity and hydrogen bond basicity and results in mass transfer limitations and the interference with the internal hydrogen bonds of enzyme, respectively [14,41,42]. Furthermore, different substrate solubilities may have an influence on
the conversion. If the substrates are well solubilized in the ILs, the increased activation energy will cause lower conversion in this solvent [13]. Table 3 showed that [Edmim][Tf$_2$N] was the best IL for both conversion (50%) and enantioselectivity ($E > 200$), compared with other ILs.

Lipase-catalyzed kinetic resolution of 1-chloro-3-phenoxy-2-propanol was also investigated. The best enantioselectivity of lipase was observed in [Edmim][Tf$_2$N] ($E = 33$) (Table 4). Although the conversions in ILs were lower than those in organic solvents, enantioselectivities in ILs were significantly higher than those in organic solvents. Therefore, ILs can be used as reaction media for kinetic resolution of 1-phenylethanol derivatives instead of organic solvents.

### 2.2 Operational stability of lipase

Operational stability of Novozym 435 lipase was studied in hydrophobic ILs ([Edmim][Tf$_2$N], [Emim][Tf$_2$N], [Bmim][BF$_6$]), hydrophilic ILs ([Emim][BF$_4$], [PPmim][TfO]) and organic solvents (toluene and acetone). Figure 7 shows that lipase in hydrophobic ILs retained the transesterification activity without any loss of activity and residual activities in these ILs were significantly higher than those in organic solvents. Residual activity of lipase in acetone was lower than 50% after 1 time reuse, while the activity of lipase in hydrophobic ILs was well maintained even after 5 times of recycling. However, the transesterification activity was drastically decreased in hydrophilic ILs. The high stability of lipase in hydrophobic ILs can be explained with
high log $P$ values which allow the preservation of essential water and maintenance of active structure in enzymes. In the hydrophilic ILs, the essential water of lipase can be stripped by its hydrophilic nature. On the other hand, hydrophobic ILs which showed lower hydrogen bond basicity might minimize interference with the internal hydrogen bonds of enzyme compared to hydrophilic ILs [12]. Interestingly, ILs can alter the protein hydration level, structure compaction, and ionic interactions that might contribute to the stabilization of protein. These characteristics of ILs may be a main reason for the high stability of lipase in ILs [23].

2.3 Thermal stability of lipase

Thermal stability of lipase was investigated in hydrophobic ILs and common organic solvents such as benzene, iso-octane and pentanone at 80 °C (Figure 8). The activities of lipase were well maintained in hydrophobic ILs, while the half-life time of lipase in pentanone was 5 hours at 80 °C. The residual activities of lipase were gradually decreased and dropped to 84 % and 74 % in benzene and iso-octane, respectively, after 1 day of incubation. The higher thermal stability of lipase in ILs have been also reported by other researchers. Iborra’s research group investigated the spectroscopic study on protein thermostability. They observed that enzyme stabilization by ILs seemed to be related to the associated changes of protein structure, the melting temperature and heat capacity of lipase was enhanced by the ILs compared to that in the other media [24].
### 2.4 Recycling of enzyme/IL mixture

Recycling of enzyme/IL mixture was a strategy for green solvents due to economics and environment-friendly considerations. As we mentioned above, [Edmim][Tf$_2$N]a is good reaction media which can induce high reaction conversion, enantioselectivity, operational and thermal stability. Therefore, this IL may be the most suitable reaction media for Novozym 435 lipase. Recently, Yuan et al have carried out reuse of *Candida rugosa* lipase in [Bmim][PF$_6$] or hexane. They showed that the enzyme activity had decreased after several recycles [41]. Itoh et al also showed that activity of free enzyme in ILs was significantly decreased after several recycles [48]. However, the activity of Novozym 435 in [Edmim][Tf$_2$N] was slightly decreased after 6 times reuse (Figure 9). Residual activity was maintained at 92% even after 6 times of recycling. Therefore, this IL is the most suitable reaction media for biocatalysis.
Table 3. Conversion and enantioselectivity of the acetylation of 1-phenylethanol with vinyl acetate from lipase, Novozym 435, in ILs and conventional organic solvents

<table>
<thead>
<tr>
<th>ILs/Org solvents</th>
<th>$EEs$</th>
<th>$EEp$</th>
<th>$C$</th>
<th>$E$</th>
</tr>
</thead>
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<tr>
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<td>61</td>
<td>&gt;99</td>
<td>38</td>
<td>&gt;200</td>
</tr>
<tr>
<td>[Bmim][BF$_4$]</td>
<td>2</td>
<td>91</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>[Omim][BF$_4$]</td>
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<td>&gt;200</td>
</tr>
<tr>
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<tr>
<td>[Bmim][TfO]</td>
<td>5</td>
<td>&gt;99</td>
<td>5</td>
<td>&gt;200</td>
</tr>
<tr>
<td>[Hmim][TfO]</td>
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<td>&gt;99</td>
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<td>&gt;200</td>
</tr>
<tr>
<td>[PPmim][TfO]</td>
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<td>97</td>
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<td>157</td>
</tr>
<tr>
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<td>&gt;200</td>
</tr>
<tr>
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<td>40</td>
<td>&gt;99</td>
<td>28</td>
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<td>[PPmim][Tf$_2$N]</td>
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<td>75</td>
<td>54</td>
<td>21</td>
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<tr>
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<td>&gt;200</td>
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<td>18</td>
<td>&gt;99</td>
<td>15</td>
<td>&gt;200</td>
</tr>
<tr>
<td>[Omim][PF$_6$]</td>
<td>57</td>
<td>&gt;99</td>
<td>36</td>
<td>&gt;200</td>
</tr>
<tr>
<td>[Hmim][SbF$_6$]</td>
<td>41</td>
<td>&gt;99</td>
<td>29</td>
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<td>DMSO</td>
<td>0</td>
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</table>
Table 4. Conversion and enantioselectivity of the acetylation of 1-Chloro-3-phenoxy-2-propanol with vinyl acetate from lipase, Novozym 435, in ILs and conventional organic solvents

<table>
<thead>
<tr>
<th>ILs/Org</th>
<th>$EE_s$</th>
<th>$EE_p$</th>
<th>C</th>
<th>E</th>
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<tr>
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<td>67</td>
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</tr>
<tr>
<td>[Emim][Tf$_2$N]</td>
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<td>60</td>
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<td>[Bmim][PF$_6$]</td>
<td>90</td>
<td>61</td>
<td>59</td>
<td>12</td>
</tr>
<tr>
<td>[Emim][BF$_4$]</td>
<td>22</td>
<td>80</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>[Bmim][BF$_4$]</td>
<td>57</td>
<td>59</td>
<td>49</td>
<td>7</td>
</tr>
<tr>
<td>[PPmim][TfO]</td>
<td>25</td>
<td>79</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Hexane</td>
<td>&gt;99</td>
<td>17</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>Toluene</td>
<td>&gt;99</td>
<td>26</td>
<td>79</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 7. Operational stability of lipase at 50 °C in ILs [Edmim][Tf$_2$N] (▪), [Emim][Tf$_2$N] (○), [Bmim][PF$_6$] (▲); [PPmim][TfO] (▼), [Bmim][BF$_4$] (△) and organic solvents toluene (□), acetone (○), the enzyme activity in hydrophobic ILs was significantly maintained even after five times of recycling compared to that in hydrophilic and organic solvents.
Figure 8. Thermal stability of lipase at 80 °C in ILs [Edmim][Tf$_2$N] (■), [Emim][Tf$_2$N] (●), [Bmim][PF$_6$] (▲) and organic solvent benzene (□), iós-octane (○) and 3-pentanone (△), the enzyme activity were well maintained in hydrophobic ILs, while the half-life of lipase in 3-pentanone was 5 hours at 80 °C.
Figure 9. Effect of recycling of lipase/[Edmim][Tf$_2$N] mixture on enzyme activity.
3. Supersaturated sugar solution in ILs

3.1 Solubility of sugars in ILs

The solubilities of D-(+)-glucose in the various hydrophilic and water miscible ILs at 25°C and 60°C are shown in Table 5. When the ILs containing [Emim] cation were considered, the solubility of glucose was highly influenced by the anion structure of ILs. Some researchers have explained that the high solubility of carbohydrates in several ILs can be attributed to the hydrogen bond acceptor properties depending on anion structure [12]. The ILs containing [dca] anion have been reported the best ILs to dissolve glucose [31]. The glucose solubilities in [dca] ILs ranged from 66 g/l to 145 g/l at 25°C. The glucose solubility in [Emim][MS] at 25°C was similar to those in [dca] ILs. The solubilities of glucose in ILs containing [TfO] and [BF₄] were greatly influenced by temperature. The solubility of glucose in these ILs increased by a factor of 2-5 when the temperature was increased from 25°C to 60°C.

The solubilities of sucrose in the various ILs at 25°C and 60°C are shown in Table 6. The solubilities of sucrose in ILs were much less than those of glucose. The severe temperature dependency of solubility in ILs containing [TfO] anion was also shown. After 12 hr incubation of excess sucrose (50 g/l) in [Emim][MS] at 60°C, the solution changed to viscous brown liquid although no sucrose crystal was observed. It seems like that sucrose can be degraded or reacted in [Emim][MS] at an excessively high temperature.
3.2 Supersaturation of sugars in ILs

If a saturated solution is prepared at an elevated temperature and undissolved solute is removed, the solution can sometimes be cooled without crystallization of solute and the solution contains more dissolved solute than the solubility limit, forming a supersaturated solution [43]. Sugars are known to produce supersaturated solutions in aqueous or organic systems, and the metastable supersaturated solutions of sugars have been used for biotransformation. Millqvist-Fureby et al [44] produced supersaturated solution containing glucose of about 50% higher concentration than solubility by preparing a saturated glucose solution at 95°C and cooling it at 37°C. In recent, Flores et al. [30] made supersaturated glucose solution with a concentration of 300% higher than solubility in 2-methyl 2-butanol by heating to 102°C and cooling down to 60°C.

The supersaturated solutions are usually prepared by heating at high temperature to dissolve excess solutes and then slowly cooling down to a low temperature. This general process was defined as the direct method. After heating at 60°C with excess sugars and cooling down to 25°C, metastable supersaturated solutions in ILs were obtained (Table 5,6). The supersaturated glucose solution in ILs prepared by the direct method were stable in the presence of solid glucose or under the shaking of solution, while the supersaturated sucrose solution in an aqueous system is usually unstable and easily crystallized. The supersaturated glucose solutions in ILs containing [TfO] anion have a concentration of about 400% higher than its solubility. To our knowledge, the metastable supersaturated solutions of glucose in ILs
have not been reported and the degree of supersaturation in ILs were higher than those in aqueous (150% in ethylene glycol-citrate buffer) [44] or organic systems (300% in 2-methyl 2-butanol) [30].

The crystallization process of the supersaturated sugar solutions in water is known to include at least two steps. Firstly, sugars diffuse from the bulk solution to the thin layer at the interface crystal/solution. Then, sugar molecules in the crystal incorporate after the release of their hydration water. The dissociation of hydration water may be a major hurdle in this process [45]. However, among the energy barriers in the crystallization process of the supersaturated solution in ILs, viscosity of ILs seems to be a major obstacle and the dissociation between ILs and sugar molecules a minor one. The high viscosity of ILs can decrease the diffusion rate from the bulk solution to the interface crystal/solution and collision probability of sugar molecules. Therefore, the crystallization rate of supersaturated solution in ILs may be very low. On the other hand, the supersaturated sucrose solution through direct method is unstable and easily crystallized. The higher stability of supersaturated glucose solution than supersaturated sucrose solution must be studied further.

3.3 Water-mediated supersaturation of sugars in ILs

The dissolution of glucose in ILs is simply understood by the detachment of glucose molecules from the solid surface at the solid-liquid interface and the transport of glucose molecules from the solid-liquid interface to the bulk ILs solution [46]. However, it took a long time to dissolve glucose in ILs, so an alternative dissolution process
which uses water as a mediator was developed to overcome this disadvantage. As ILs are non-volatile, the contained water in ILs can be easily removed by evaporation after the dissolution process. To make the supersaturated glucose solution in ILs, glucose was firstly dissolved in water and then this solution was mixed with ILs. The contained water in this mixture was removed by vacuum evaporation. The residual water content in ILs was measured by weight difference and was confirmed by Karl-Fischer Titration to be lower than 0.1% (w/w). The supersaturated glucose solution was obtained after centrifugation and it was fairly stable in the presence of excess glucose. This process was defined as the water-mediated method.

The glucose concentration of supersaturated solution in ILs prepared by the water-mediated method was extremely higher than the solubility at 25°C. Especially, the supersaturated glucose solutions in [Emim][TfO] and [Bmim][TfO] had 19 and 10 times higher concentrations than solubility at 25°C, respectively (Table 5). As for the stability of supersaturated glucose solution in [Bmim][TfO] at 25°C (Figure 10), the solution was stable for 1 day and the glucose concentration was 87% of the initial content even after 3 days. The degree of supersaturation was particularly high in ILs containing [TfO]. The [TfO] ILs have special properties; [Bmim][TfO] is fully water miscible but the Hildebrand solubility parameter of this IL is similar to that of [Bmim][Tf2N] known as the very hydrophobic and water immiscible IL [47]. The Hildebrand solubility parameter has been widely used for predicting the solubilities of various chemicals in organic solvents. The maximum solubility is observed when the
Hildebrand solubility parameters of solute and solvent are identical. Therefore, it means that [Bmim][TfO] can dissolve not only hydrophilic substrates but also hydrophobic organic compounds. The [Bmim][TfO] was reported as a good reaction media for lipase-catalyzed reaction [50]. Recently, lipase-catalyzed transesterification of glucose with fatty acid ester was successfully carried out by using supersaturated glucose solution in [Bmim][TfO] [49]. The ILs containing [TfO] anion may be useful for the enzyme reaction using hydrophilic substrate like sugars.

The supersaturated sucrose solutions were also prepared with water-mediated method. These solutions were highly stable, while the supersaturated sucrose solutions made by direct method were unstable and quickly crystallized. The supersaturated sucrose solutions through water-mediated method in all ILs had about 5 times higher concentration than their solubility at 25°C.

As the remaining water of less than 0.1% can increase the solubility of glucose, it was investigated whether high supersaturation was induced by the remaining water. However, the glucose solubility in [Bmim][TfO] increased from 4.8 g/l to 8.2 g/l by adding 1% water, which was much lower than the glucose content of 46.3 g/l in supersaturated solution.
Table 5. Solubility and supersaturated concentration of glucose in ILs

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility (g/l)</th>
<th>Supersaturated concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>60°C</td>
</tr>
<tr>
<td>2-Methyl-2-butanol</td>
<td>2.6 (^c)</td>
<td></td>
</tr>
<tr>
<td>tert-Butanol</td>
<td>0.3 (^d)</td>
<td></td>
</tr>
<tr>
<td>[Emim][MS]</td>
<td>89.6</td>
<td>133.2</td>
</tr>
<tr>
<td>[Emim][TfO]</td>
<td>6.1</td>
<td>27.8</td>
</tr>
<tr>
<td>[Bmim][TfO]</td>
<td>4.8</td>
<td>18.1</td>
</tr>
<tr>
<td>[Emim][BF(_4)]</td>
<td>1.1</td>
<td>4.8</td>
</tr>
<tr>
<td>[Bmim][BF(_4)]</td>
<td>0.9</td>
<td>3.5</td>
</tr>
<tr>
<td>[Omim][BF(_4)]</td>
<td>0.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(^a\) Slowly cooled from 60°C to 25°C, and then supernatant was carefully obtained after centrifugation.

\(^b\) Evaporated to remove water at 60°C, slowly cooled to 25°C, and then supernatant was carefully obtained.

\(^c\) From ref. 30.

\(^d\) From ref. 31.
### Table 6. Solubility and supersaturated concentration of sucrose in ILs

<table>
<thead>
<tr>
<th>Solvent</th>
<th>25°C</th>
<th>60°C</th>
<th>direct a</th>
<th>water-mediated b</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Emim][MS]</td>
<td>12.4</td>
<td>ND c</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>[Emim][TfO]</td>
<td>3.1</td>
<td>7.1</td>
<td>3.2</td>
<td>15.3</td>
</tr>
<tr>
<td>[Bmim][TfO]</td>
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<td>5.3</td>
<td>2.1</td>
<td>10.2</td>
</tr>
<tr>
<td>[Emim][BF₄]</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>5.1</td>
</tr>
<tr>
<td>[Bmim][BF₄]</td>
<td>0.5</td>
<td>0.6</td>
<td>0.6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

a Slowly cooled from 60°C to 25°C, and then supernatant was carefully obtained after centrifugation.

b Evaporated to remove water at 60°C, slowly cooled to 25°C, and then supernatant was carefully obtained.

c Sucrose was degraded at 60°C and the color of solution changed to brown.
Figure 10. Stability of supersaturated glucose solution in [Bmim][TfO] prepared by water-mediated method. After incubation at 25 °C, the supernatant was periodically obtained and diluted with deionized water to analyze the glucose content.
4. Lipase-catalyzed glucose ester production by using supersaturated glucose solution in ILs

4.1 Transesterification of glucose with fatty acid vinyl ester in IL

The lipase-catalyzed transesterification of glucose with vinyl laurate was performed with partially dissolved glucose solution and supersaturated glucose solutions (Figure 11). Dissolved concentrations of glucose in saturated solution and supersaturated solution prepared by direct and water mediated method were initially 110, 190, and 440 mM, respectively. The transesterification of glucose with vinyl laurate was successfully carried out by using Novozym 435. The initial reaction rate (180 mM/hr) for the reaction with supersaturated glucose solution prepared by water-mediated method was extremely higher than those of reaction with supersaturated solution in the presence of solid crystal prepared by direct method (10 mM/hr) and partially dissolved glucose solution (2 mM/hr). The conversion of 96% at 1 day of reaction was also significantly higher than those of other reactions. Only the monoester, 6-\(\text{O}\)-lauroyl-\(\text{D}\)-glucose, was formed and confirmed by \(^1\)H NMR and LC-MS. The higher dissolved concentration of glucose in the supersaturated solution lead to an increase of reaction rate and conversion. These results indicate that homogeneously dissolved glucose in supersaturated solution can be easily transported to the active site of lipase, while low dissolution rate of partially dissolved glucose in [Bmim][TfO] may limit the productivity of enzyme. In addition, the increase of dissolved glucose concentration yielded a better conversion because the equilibrium was forced toward synthesis.
4.2 Direct esterification of glucose with fatty acid in ILs

The direct esterifications of glucose with fatty acids are usually preferred to transesterifications of glucose with fatty acid vinyl esters, because water as by-product is non-toxic and can be easily removed during the reaction. However, the choice of solvent for this reaction is very difficult because one reactant is polar (glucose), the other is nonpolar (fatty acid) and the product is amphiphilic (glucose ester). Most ILs possess both a hydrophilic ionic head and hydrophobic organic chain, which are one category of surfactants. Therefore, ILs may be good solvents for direct esterification of glucose. Ganske and Bornscheuer [34] carried out the direct esterification of glucose with fatty acid in mixture of ILs ([Bmim][BF₄] or [Bmim][PF₆]) and t-butanol, but no sugar ester synthesis took place in pure ILs. It may be caused by low solubilities of substrates in these ILs. The glucose solubilities at 25°C in these ILs were less than 1.0 g/L [31,32]. To dissolve high concentrations of glucose and lauric acid, we selected [Bmim][TfO] as reaction media for lipase-catalyzed direct esterification. The [Bmim][TfO] has special properties; [Bmim][TfO] is fully miscible with water (\( \delta_H = 47.8 \)) but the Hildebrand solubility parameter of this IL (\( \delta_H = 25.4 \)) is similar to that of allyl alcohol (\( \delta_H = 25.7 \)) or [Omim][Tf₂N] (\( \delta_H = 25.0 \)) known as very hydrophobic and water immiscible IL [47]. The Hildebrand solubility parameter has been widely used for predicting the solubilities of various chemicals in organic solvents. The maximum solubility is observed when the Hildebrand solubility parameters of solute and solvent are identical. Therefore, it means that [Bmim][TfO] can dissolve not only
hydrophilic substrates but also hydrophobic organic compounds. In addition, [Bmim][TfO] was reported as a good reaction media for lipase-catalyzed reaction [49].

Lipase-catalyzed direct esterification of glucose with lauric acid was achieved for the first time in pure IL with high concentrations (220 mM) of substrates (Figure 12). However, the reaction rate and final conversion were too low, because the solubility of glucose at 50°C is still low (14.2 g/L), while lauric acid is fully miscible. To increase dissolved concentration of glucose in [Bmim][TfO], we used supersaturated glucose solution prepared by the water mediated method. The lipase-catalyzed direct esterification of glucose with lauric acid was efficiently carried out with the supersaturated glucose solution in [Bmim][TfO] (Figure 12). The initial reaction rate (12.2 mM/hr) for the reaction with supersaturated glucose solution in [Bmim][TfO] was 20 times higher than that of reaction with saturated glucose solution in the presence of glucose crystal (0.6 mM/hr). The produced ester concentration of 0.10 mmol (91% conversion) at 100 hr of reaction with supersaturated solution was also significantly higher than that (0.03 mmol, 27% conversion) of reaction with saturated solution. To compare with general IL for biocatalysis, same reaction was carried out in [Bmim][BF₄]. The initial reaction rate and final conversion in [Bmim][BF₄] were lower than those in [Bmim][TfO]. However, the use of supersaturated glucose solution in [Bmim][BF₄] also increased the reaction rate of Novozym 435, compared with the method using saturated solution in the presence of glucose crystal. Therefore, water-mediated supersaturation is useful method to increase the dissolved
concentration of glucose in ILs.

4.5 Structure determination

$^1$H NMR spectra of 6-$O$-lauroyl-D-glucose were recorded on a 400MHz FT-NMR spectrometer (Varian Inova 400, CA) in CD$_3$OD. The molecular weight of product was analyzed by LC-MS (Varian 1200L, CA). Calculated for C$_{18}$H$_{34}$O$_7$Na (M + Na$^+$) 385.4, found 385.0.
Figure 11. Lipase-catalyzed transesterification of glucose with vinyl laurate in IL. Reaction conditions: 0.11 mmol glucose, 0.22 mmol vinyl laurate, 0.5 ml [Bmim][TfO], 50 mg Novozym 435, 40°C (●: reaction with supersaturated glucose solution prepared by water-mediated method, ▲: reaction with supersaturated glucose solution prepared by direct method, ▼: reaction with crystalline glucose).
Figure 12. Lipase-catalyzed direct esterification of glucose with lauric acid in IL. Reaction conditions: 0.11 mmol glucose, 0.11 mmol lauric acid, 0.5 ml IL, 50 mg Novozym 435, molecular sieve (4Å), 50°C (▲: reaction with supersaturated glucose solution in [Bmim][TfO] prepared by water-mediated method, ▼: reaction with supersaturated glucose solution in [Bmim][BF₄] prepared by water-mediated method, empty symbols represent the reaction with saturated glucose solution in the presence of crystalline glucose).
V. CONCLUSION

Firstly, the activity and stability of ILs-pretreated lipase were investigated with hydrophobic and hydrophilic ILs in the hydrolysis reaction. Activities of ILs-pretreated lipase were obviously higher than that of untreated lipase. This may be explained by the change on the secondary structure of lipase in ILs, which gives rise to the enhancement in the interaction between substrate and active site. Moreover, the activities of lipase in ILs were well maintained after seven days of incubation at 60°C compared with the stability of free lipase. These results suggest that pretreatment of lipase with ILs might form ILs-coated lipase which causes the structural change of lipase, and thus, enhances the activity and stability of lipase in aqueous solution. Secondly, lipase-catalyzed kinetic resolution of alcohol derivative compounds for intermediate products was achieved in the IL system. This work showed that lipase significantly maintained higher enantioselectivity and better stability during long-term operational recycling and high temperature of incubation in ILs than those in conventional organic solvents. With enhancing enantioselectivity, operational and thermal stability of lipase in ILs demonstrated a great potential as an alternative media for biocatalysis and bioconversion. In addition, the potentiality of recycling of an enzyme/IL mixture was more important for “green solvents” due to economic and environmental benefits. Since [Edmim][Tf₂N] was investigated as the best of the ILs in enantioselectivity, stability and potential recycling of enzyme compared to the others, it was suggested that this IL would
become an excellent candidate for enzyme-catalyzed kinetic resolution of alcol derivative compounds in bioconversion. Furthermore, this process can be applied to large scale in industry. Thirdly, we were able to make the supersaturated sugar solutions in ILs through the direct and water-mediated method. The water-mediated supersaturation method can be used to dissolve excess hydrophilic substrates such as sugars, peptides, and water miscible vitamins without their degradation at high temperature. The prepared supersaturated solutions were fairly stable in the presence of excess sugar molecules and their sugar content did not change much for 1 day. In addition, we successfully achieved lipase-catalyzed esterifications of glucose in pure ILs. [Bmim][TfO] is a very useful reaction media for the reaction containing both hydrophilic and hydrophobic substrates. The initial rate and final conversion can be significantly improved by using supersaturated glucose solution with higher concentration than its solubility. Therefore, lipase-catalyzed esterification in the supersaturated solution through water-mediated method will be able to overcome the problem of limited glucose solubility in various reactions. Generally, ILs can be an environmentally clean alternative to organic solvents for biocatalysis in non-conventional media.
REFERENCES


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