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Review

Strategies for the development of enzymatic reactions in ionic liquids.

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Ionic liquids are liquid salts at room temperature that have properties as solvents that allows a wide range of applications. Their use in enzymatic reactions is increasing since 1991 because ionic liquids (ILs) serve as a highly efficient reaction medium for biocatalytic reactions that cannot be carried out with traditional solvents such as toluene, methanol or acetone, characterized by high volatility. The present project aims to analyze recent advances on enzymatic reactions in ionic liquids, and their possible applications in obtaining value-added products that may be of interest to the national industry. To achieve this, a systematic review of the literature will be carried out using the Springer link and Science Direct databases available at Universidad ICESI to establish the state of the art. Subsequently, the advantages and disadvantages of traditional ionic liquids and solvents in enzymatic reactions and how their structure and design relate to the properties of ILs are presented. Finally, applications for biofuel production using ILs were shown. Hydrophobic ionic liquids were found to have potential as reaction media with enzymes in different applications due to high conversion rates, high selectivity and enhanced enzymatic stability.

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1. Introduction

Currently, the industry makes use of organic solvents as reaction and extraction media that has strongly contributed to pollution, and other detrimental effects such as mass transfer limitations [1], safety and health risks has promote the minimization of their use, and replacement with new solvents that generate less emissions.

Ionic liquids (ILs) are liquid salts formed by organic cations and anions with melting points below 100°C [2]. These solvents are adaptable because they can be designed to have specific physicochemical properties through structural changes in the cation and anion [3]. Unlike conventional volatile organic solvents, ILs have many favorable properties, such as low vapor pressure, high ionic conductivity, wide liquid range, high thermal stability, and the ability to dissolve a variety of solutes

[4]. Therefore, during the last few years there has been a growing interest in the application of ionic liquids in the development of new applications.

Ionic liquids have a modular nature so that structural alterations can be introduced through changes or substituents in both the anion and the cation. Most of the cations that make up these liquids are of the imidazolium, ammonium, phosphonium, pyridinium or pyrrolidinium type [4]. By modifying the cation, properties such as melting point, viscosity, and miscibility, can be modified to obtain values in a specific range [5]. Similarly, the simplest anions to purify are hexafluorophosphate [PF_6], tetrafluoroborate [BF_4] and bis(trifluromethane) sulfonimide [TF_2N-]. Then, by having many anions and cations available to design these liquids, compounds with very different physicochemical properties can be designed for specific applications such as enzymatic polymerization, protein, and enzyme extraction for biocatalysis and fuel production.

On the other hand, studies on enzymatic reactions in IL over the past ten years have revealed that not only are these alternatives environmentally friendly, but also in these solvents the enzymes exhibit excellent selectivity including substrate selectivity, regioselectivity and enantioselectivity. In addition, many enzymes maintain very high thermal and operational stability when ILs were used as solvents [6]. Therefore, enzymatic reactions in ILs have increased exponentially in recent years in applications such as enzyme-catalyzed polymerization, use as solvents in enzymatic catalysis for biofuel production, biodiesel production by lipase-catalyzed alcoholysis of vegetable oils, etc. [3].

On the other hand, ionic liquids have a wide field of applications, therefore it is necessary to develop a review on the different enzymatic reactions in ionic liquids, where their possible uses and the prospective of each application are identified and classified [7]. For this purpose, a search and selection of articles related to the topic was carried out in different databases such as Springer link and Science direct available at Universidad Icesi. Subsequently, a database was created to support all the information collected. Finally, the coincidences or disagreements between the different authors were shown in a logical and coherent line of argument throughout the present article. In view of the above, the present project was aimed at carrying out an exhaustive review of the properties and possible applications of ILs, emphasizing their potential use in enzymatic reactions to obtain value-added products that can be obtained in the national context.

2. Applications of ionic liquids in enzymatic reactions: State of the art.

ILs can play a similar role as an organic solvent by influencing enzyme function as follows: the IL replaces the water surrounding the enzyme; then, upon entering the microaqueous phase, the IL interacts with the enzyme by modifying the conformation, dynamics or active site; and finally, the IL interacts with the products and substrates by reacting or changing their partitioning between the aqueous and non-aqueous phases [6]. As a result of their adaptability, some enzymes exhibit higher activities in the presence of ionic liquids than without these solvents. Careful selection of ionic liquids is necessary to evaluate enzyme activity and stability, as well as the kinetic parameters of enzyme reactions in ILs. Here are some of the recent developments that have proven to be very effective in biocatalysis using these solvents as reaction media.

On the other hand, Kitamura *et al*, (2010), developed an efficient procedure for the transesterification of methyl caffeate to produce caffeic acid phenethyl ester analogues. For this purpose, the activity of 4 commercially available lipases was evaluated. The lipase from *Candida antarctica* (Novozyme 435) showed the highest activity at [Bmim] [Tf_2N], this is because the difference in substrate recognition by the lipases is probably in accordance with the difference in substrate orientation at the active center [8].

As for the activity of Novozyme 435 on various ILs, the transesterification of 2-cyclohexylethanol and 3-cyclohexylpropyl caffeate was examined obtaining conversion yields of 97.6% and 93.8% respectively on [Bmim] [Tf_2N], the results indicated that the enzymatic activity on ILs is anion dependent. Furthermore, it was suggested that [BF_4] and [CF_3SO_3] anions are more nucleophilic than [Tf_2N] and [PF_6], anions, thus they coordinate more strongly with positively charged sites in the structure of an enzyme causing conformational changes in its structure and deactivating the enzyme [8].

Wei *et. al.* (2016), investigated the effects of ionic liquids as co-solvents on the enzymatic synthesis of 6-sucrose acetate (S6A) for sweetener manufacture by fructosyltransferase from *Aspergillus oryzae*. Fifteen ionic liquids containing six different cations and six

different anions were studied. The yield of S6A achieved using [Dmim] [PF$_6$] was 87.2% and thus much higher than that obtained with the other ILs because it led to significant changes in the structural elements of the enzyme, including a decrease in its α-helical content and an increase in its β-sheet content. Therefore, these results indicate that the observed decrease in the α-helical content of the enzyme would have some impact on the active site of the enzyme, which could lead to an increase in its transfructosylation activity.

On the other hand, the yield of S6A decreased dramatically with increasing IL concentration from 20% to 50%. A concentration of [Dmim] [PF$_6$] at 20% was the optimal condition for fructosyltransferase-catalyzed S6A synthesis. Changes in the enzyme conformation are a possible reason for the differences between S6A yields at different [Dmim] [PF6] concentrations. A higher concentration of IL not only results in a higher ionic strength in the enzyme reaction medium, which could inactivate the enzyme, but also leads to a higher viscosity of the reaction mixture, which limits the diffusion of reaction substrates to the enzyme active site or inhibits the enzyme due to excess substrate, resulting in a drop in enzymatic efficiency [9].

Very recently, Bento *et al*, (2020), have shown that the use of IL increases the degradation rate of indigo carmine (IC) dye reaching a decolorization efficiency of 82% in 0.5 h using [N1$_{0111}$] Br and laccase, whereas without IL only 6% of the IC is degraded. Furthermore, after 24 h, 93% decolorization of the dye was achieved with [N$_{10111}$] Br. [10].

Table 1 summarizes other work carried out in the last 30 years on applications of enzymatic reactions using ionic liquids, highlighting the enzymatic yield obtained in comparison with organic solvents and without these solvents in reactions including ester synthesis, medicinal applications, biodiesel production and transesterification.

Table 1: Summary applications of ILs in enzymatic reactions.

Ref.	Application	Enzyme	Ionic Liquid	Results
[11]	Synthesis of nucleoside drug esters.	Lipase B	1. [Bmim] [BF$_4$]	An eight-fold increase in yield and three times faster reaction rates.
[12]	Biodiesel production from vegetable oil.	Lipase B	1. [C16mim] [NTf$_2$] 2. [C18mim] [NTf$_2$]	Yields close to 100% during 9 days of operation in ([C16mim] [Tf$_2$N]).
[13]	Synthesis of Z-aspartame.	Metalloproteinase (Thermolysin)	1. [Bmim] [PF$_6$]	The conversion of reached 95% at 48 h and Z-aspartame (Z-APM) was formed.
[14]	Enzymatic degradation of corn stalk.	Cellulase	1. [Emim] OAc	The enzymatic degradation rate was 96.6% and was 2.1 times faster using [Emim]OAc.
[15]	Simultaneous saccharification and fermentation of sugarcane bagasse	Xylitol dehydrogenase, Xylose isomerase	1. [Emim] [OAc] 2. [Emim] [Cl] 3. [Emim] [DEP] 4. [Bmim] [OAc] 5. [Bmpy] [Cl]	An ethanol yield of 84.0% was obtained using [Emim] [OAc] compared to 26.7% produced by the strain in the absence of ionic liquids.
[16]	Acetylation of methyl-α-D-glucopyranoside with vinyl acetate.	Lipases from*Burkholderia contaminans* LTEB11	1. [Emim][MeSO$_3$]	A 76% conversion was obtained in 72 h from 6-OAc-α-MetGlc. This is the highest conversion produced by enzymatic catalysis.
[17]	Saccharification of bamboo.	Extremozymes	1. [Emim]Ac	The sugar yields were higher than without pretreatment.
[18]	Synthesis of poly-L-l-lactide and poly-L-lactide-co-glycolide.	Lipase B	1. [Hmim] [PF$_6$]	A yield of poly-L- lactide in IL of 63% was obtained at 90 °C.
[19]	Synthesis of poly(ε-caprolactone).	Lipase B	1. [C2mim] [PF$_6$] 2. [C4(C6mim)$_2$][PF$_6$] 3. [C6mim] [PF$_6$] 4. [C12mim] [PF$_6$].	The highest yield was obtained with [C4 (C6mim) $_2$] [PF$_6$] with a value of 62%.

[20]	Synthesis of indolyl 4H-chromenes.	Lipase from *Mucor miehei*	1. [Bmim] [OTf] 2. [Bmim] [Tf$_2$N] 3. [Emim] [BF$_4$] 4. [Bmim] [PF$_6$] 5. [Emim] [BF$_4$] 6. [Hmim] [BF$_4$]	A yield of 93% was obtained when [Emim] [BF$_4$]. In addition, this IL showed good reusability.
[21]	Rapid and improved synthesis of galactose oleate ester.	Lipase from *Rhizomucor miehei*	1. [Emim] [BF$_4$] 2. [Bmim] [BF$_4$] 3. [Bmim] [PF$_6$] 4. [Bmim] [NTf$_2$] 5. [Bmim] [TfO].	A conversion of 87% was obtained after only 2 h under optimal synthesis conditions with [Bmim] [BF$_4$].
[22]	Synthesis of glycerol synapate.	Feruloyl esterase	1. [Bmim] [PF$_6$] 2. [Omim] [PF$_6$] 3. [C$_2$OHmim] [PF$_6$] 4. [C$_5$O$_2$mim] [PF$_6$]	A conversion yield of 76.7% was obtained with [C$_2$OHmim] [PF$_6$]. The feruloyl esterase retained its activity after 5 consecutive 24-h reaction cycles.
[23]	Enzymatic transesterification and selective formation of lipophilic feruloylated or hydrophilic ferulate glyceryl (FG) acylglycerols.	Lipase from *Candida antarctica*	1. [Emim] [TF$_2$N] 2. [Bmim] [TF$_2$N] 3. [Mmim] [TF$_2$N] 4. [Emim] [PF$_6$] 5. [Bmim] [PF$_6$] 6. [OMIM] [PF$_6$] 7. [C12mim] [PF$_6$] 8. [C14mim] [PF$_6$] 9. [Bmim] [BF$_4$] 10. [HMIM] [BF$_4$].	[Emim] [TF$_2$N] showed a protective effect on enzyme stability at high temperature (110 °C). High EF conversion (99.7 ± 0.3%) was also obtained.
[24]	Enzymatic hydrolysis of chitin.	*Streptomyces albolongus* chitinase ATCC 27414	1. [Emim] [OAc]	IL-pretreated chitin provided efficient production of N-acetylglucosamine (175.62 mg/g chitin) and N, N '-diacetylchitobiose (341.70 mg/g chitin) with 61.49% conversion at 48 h.
[25]	Enzymatic esterification of esculin.	*Candida antarctica* B Lipase (Novozym 435)	1. [Bmim] [BF$_4$] 2. [Bmim] [PF$_6$] 3. [OMIM] [PF$_6$] 4. [MeOcPy] [BF$_4$] 5. [TOMA] [Tf$_2$N]	The conversion of esculin using acetone was 71% and with [TOMA] [Tf$_2$N] was 80%, being the highest among the 6 ionic liquids studied. The kinetic parameters of the enzymatic reaction mediated by the binary system ([TOMA] [TF2N]-hexane) were analyzed in which the highest values of Vmax (6.55 mM/h) and the lowest of Km (10.93 mM) were obtained.
[26]	Enzymatic esterification of furoic acid to methyl-2-furoate.	CAL-B Lipase TLL Lipase RML Lipase CRL Lipase	1. [Bmim] [BF$_4$] 2. [Bmim] [PF$_6$]	MF yields were obtained for enzymatic esterification by CAL-B and [Bmim] [PF$_6$] of 82.5%, whereas for the other three immobilized lipases it was 10%. The difference in yield is attributed to variation in enzyme structure, since part of the α-helix (α-10), formed by the amino acid residue A281 of CAL-B, is in a highly hydrophobic microenvironment.

[27]	Enzymatic synthesis of phenethyl ester of caffeic acid.	*Candida antarctica* B Lipase (Novozym 435)	1. [Emim][Tf₂N] 2. [Bmim][Tf₂N] 3. [Hmim][Tf₂N] 4. [Emim] [BF₄] 5. [Bmim] [PF₆]	[Emim] [Tf$_2$N] was the best solvent with 92% conversion after 48 h due to the similar characteristics in chemical structure between CA and the cationic substituent [Bmim].
[28]	Enzymatic hydrolysis of *Asclepias syriaca*.	*Aspergillus niger* cellulase	1. [Bmim] Cl 2. [Emim] Cl 3. [BMP] Cl	After 120 h, the degree of hydrolysis of A. syriaca fibers treated with [BMP]Cl was higher than the saccharification conversion achieved using [BMP]Cl. [Emim] Cl or [Bmim] Cl.
[29]	Enzymatic synthesis of amoxicillin by penicillin G acylase.	*Candida antarctica* B Lipase (Novozym 435).	1. [Bmim][Tf₂N] 2. [Bmim] [BF₄] 3. [Bmim] [PF₆]	Increases as high as 400% in selectivity were achieved when 75% v/v of [Bmim][PF$_6$] was used. Selectivity was found to have an inverse relationship with water activity. In this case, selectivity was lower in [Bmim][TF$_2$N] compared to [Bmim][BF$_4$].
[30]	Enzymatic peptide synthesis of N-acetyl-1-tryptophan ethyl ester.	α-chymotrypsin	1. [Emim] [FSI] 2. [Emim] [Tf₂N] 3. [Emim] [BF₄] 4. [Emim] [PF₆]	The initial rate of peptide synthesis was improved 16-fold by switching from an organic solvent, acetonitrile, to an ionic liquid, [Emim] [FSI], a 25 °C. at 25 °C. Likewise, the activity of α-chymotrypsin in peptide synthesis in [Emim] [FSI] was 17-fold higher than in acetonitrile at 60 °C.
[31]	Synthesis of low pour point plant lubricants.	*Candida antarctica* B Lipase (Novozym 435)	1. [Hmim][PF₆] 2. [Hmim] [Cl]	83.74% conversion with 76.56% selectivity was obtained with H$_2$SO$_4$ in 3 hours and with ionic liquid [Hmim] [PF$_6$] 95.92% conversion with 89.09% selectivity was obtained. Furthermore, the result indicated that the activity (conversion rate 83.32%) and selectivity (84.71%) of [Hmim] [PF$_6$] remained in high stabilization after four times of recycling.
[32]	Degradation of chitosan.	Cellulase	1. [Gly] [BF4].	It was observed that at pH 5.0, the highest catalytic activity is obtained, the molecular weight of chitooligosaccharides (chitosan degradation product) and the yield reached the maximum of 76.36%.

Several authors have successfully reported on the yields obtained using hydrophobic ionic liquids in enzymatic reactions [25,27]. It was observed that the more hydrophobic the IL is, the less water is required to maintain enzymatic activity and optimal enantioselectivity. As a consequence, higher or equal conversion compared to conventional organic solvents is obtained. When the IL is hydrophilic more water is needed because they tend to remove the essential water from the enzyme thus it might cause its deactivation [6]. Furthermore, Moniruzzaman et al, (2010), reported that hydrophobic ILs containing anions such as [PF$_6$] and [Tf$_2$N] are less toxic to cell membranes than organic solvents [33].

In several cases, the enzymes exhibit good thermal stability in the presence of ILs. For example, in the case of Pedersen, *et al*, (2020), the results showed a positive correlation between reaction rate and reaction temperature in [TOMA][Tf$_2$N]-Hexane because the esculin conversions showed a significant improvement as the reaction temperature was increased from 40 to 50 °C.

The high temperature-dependent reactivity in [TOMA][Tf$_2$N]- binary solvent systems indicates that [Tf$_2$N]- could have a protective effect on enzyme structures at elevated temperatures [25]. Also, enzymes such as lipases are very stable in anhydrous IL due to their conformational rigidity in the dehydrated state [33].

Similarly, several studies indicates that, although ionic liquids exhibit higher viscosity than conventional organic solvents, the high viscosity of ILs slows down the conformational changes of enzymes, resulting in proteins maintaining their native structures and activities [6]. Generally, high viscosities of ILs can be translated into good enzyme stabilization. The excellent stability of enzymes at elevated temperatures in highly viscous ILs has opened up the possibilities for biotransformations with several different incubation protocols [34].

For example, Pereira *et al*. (2012), reported on the catalytic activity of penicillin G acylase for the synthesis of amoxicillin using different ILs, they observed that the range of viscosities vary in the following order [Bmim][PF$_6$] > [Bmim][BF$_4$]> [Bmim][TF$_2$N] where the higher symmetry of the inorganic anions (PF$_6$ or BF$_4$) compared to the organic anion (Tf$_2$N) may play an important role, that is, the geometry and molar mass of the anions have a strong influence on the viscosity of this class of ILs, since Bmim combined with PF$_6$ or Tf$_2$N produces ILs with significantly different viscosities. These differences in viscosity are one of the reasons behind the recent development of Tf$_2$N-based ILs, which are relatively less viscous compared to ILs containing other anions. However, the enzyme exhibited higher selectivity with IL [Bmim] [PF$_6$] because the high viscosity allowed protein domain changes to slow down and the enzyme conformation to remain active [29]

Now, the differences in the improved yields with ionic liquids and organic solvents can be attributed to the variation in the structure of the currently most commonly used lipase enzyme, due to the fact that the α-helix part (α-10), formed by the amino acid residue A28, is in a highly hydrophobic microenvironment that is favorable for enzyme-substrate interaction in non-aqueous media [26]. Indeed. Tian et al , (2009) in their study found that the electrostatic interactions occurring between the protein and the ionic liquid result in a more rigid protein, which needs to overcome a relatively high kinetic barrier to unfold [35]

Nevertheless, in the research of Wei, *et al*, (2016), they found a higher yield of sucrose 6 acetate with [Dmim][PF$_6$] in such case the anion and cation of the IL led to significant changes in the structural elements of the enzyme, including a decrease in its α-helical content and an increase in its β-sheet content. Therefore, these results indicate that the observed decrease in the α-helical content of the enzyme would have some impact on the active site of the enzyme, which could lead to an increase in its transfructosylation activity [9]. Those examples shows that a highly understanding of the interactions between IL and proteins must be achieved to select the best IL necessary for a particular application.

3. Advantages and disadvantages of ILs and organic solvents in enzymatic reactions.

Ionic liquids have been widely investigated as possible "green" substitutes for organic solvents; having low vapor pressures they can be reused more efficiently than organic solvents. However, a greener technology is generally not sufficient to justify a new process [36]. Some important advantages and disadvantages of organic solvents and ionic liquids as reaction media for enzymes are presented in Figure 1.

The main advantage of ionic liquids is they can be adjusted by selecting the appropriate cation and anion, to obtain a wide range of properties, which is not possible with organic solvents. In fact, due to the large amounts of cations and anions, the number of ILs is theoretically estimated to be around 10^8, which provides many possibilities to design specific ILs by adjusting the chemical structure; thus ILs could be designed to be hydrophilic or hydrophobic, acidic or alkaline, protic or aprotic, chiral or achiral, metal-containing or metal-free, magnetic or non-magnetic [26].

The other advantage of IL is that they increase the enzyme stability. For example, Zhang, et al, (2020), investigated the effect of the temperature of the oleic acid esterification reaction from 120 °C to 170 °C. With increasing temperature, the esterification rate increased dramatically from 67.34% to 98.80% with [Hmim][PF$_6$]. At higher temperatures, the substrate molecules gained more kinetic energy, and the mass transfer rate between the reactants would be faster, which would benefit the esterification. The optimum temperature for esterification was set at 150 °C, and the esterification and selectivity were 95.28% and 86.12%, respectively [31].
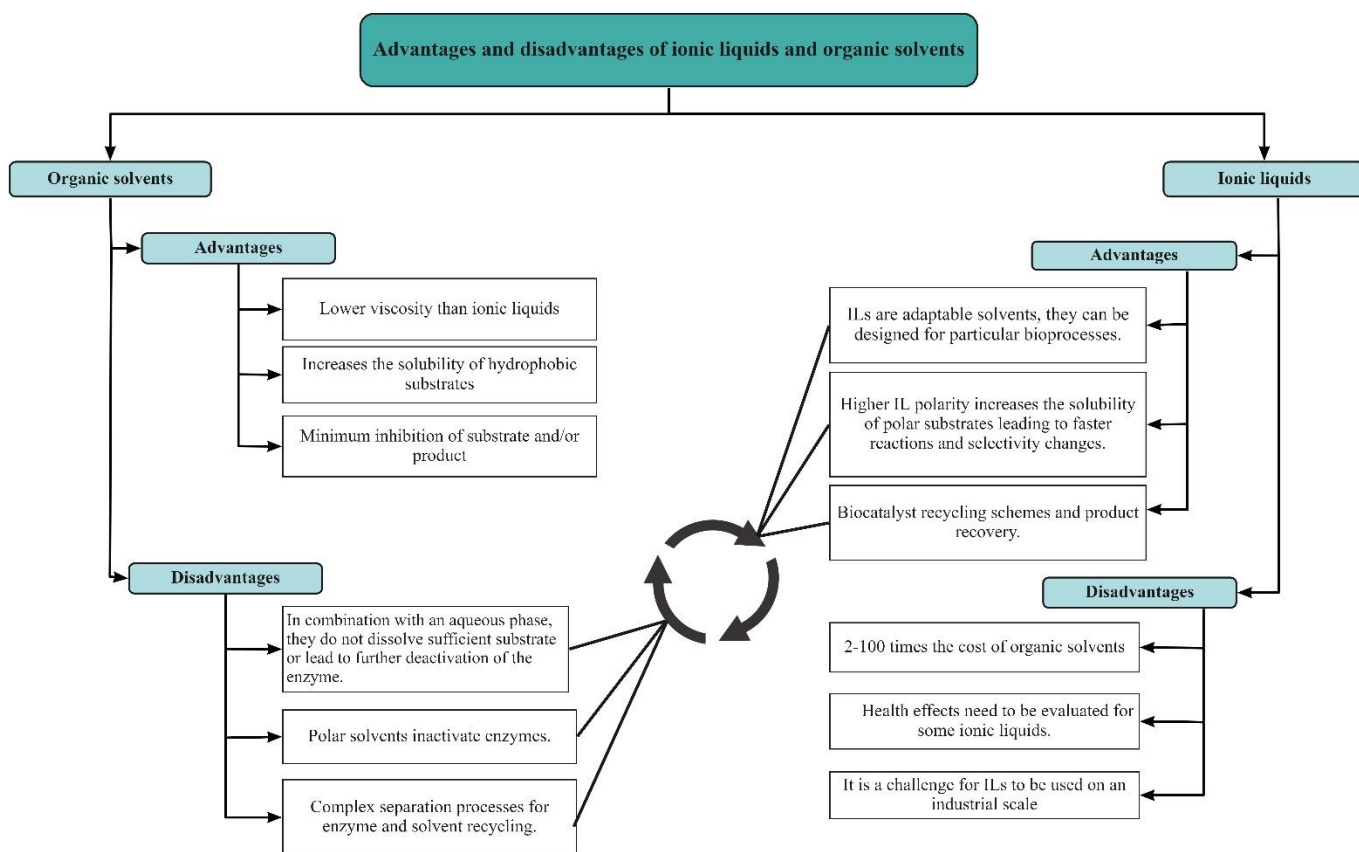
FIgure 1: Advantages of ionic liquids and organic solvents.

In the same way, Zhang, *et al,* (2013), studied porcine pancreatic lipase, which was submmited to a higher temperature of 150 °C for 24 h to test its stability at high temperatures, as a result the enzyme retained about 80% activity in [Bmim] [PF$_6$]. In both cases using a hydrophobic ionic liquid such as [Bmim] [PF$_6$] increases the stability of the enzyme because it allows the preservation of the essential water molecules surrounding the protein structure, thus reducing the direct protein-ion contact and improving the stability of the enzyme leading to no denaturation [37].

Persson, et al, (2003), investigated the stability of *Bacillus stearothermophilus* esterase in hexane, MTBE, [Bmim] [BF$_4$] y [Bmim] [PF$_6$]. The results indicated a half-life of 240 and 220 hours in [Bmim] [PF$_6$] and [Bmim] [BF$_4$], respectively. This was 3.0- and 2.8-fold higher compared to the stability in MTBE, and 30- and 27-fold higher than that found for n-hexane, respectively [38].

Similarly, Galai et al, (2015), observed that using IL [Chol] [H$_2$PO$_4$] allowed a significant increase in laccase activity at 50 °C, 60 °C and 70 °C (10.5%, 46.5% and 19.5% residual activity, respectively), confirming the thermal stabilizing effect of [Chol] [H$_2$PO$_4$]. Improving laccase stability at very basic pH by [Chol] [H$_2$PO$_4$] would be very interesting for the application of this enzyme in the treatment of textile or olive mill wastewater as the pH of conventional media is usually high [39].

Regarding the above mentioned, it has been demonstrated that the stability of enzymes increases notably under conditions of water scarcity, which allows biotransformations to be carried out at higher temperatures than those used in conventional aqueous solutions [37]. It was also shown that the temperature at which a protein undergoes thermal denaturation is highly dependent on the amount of water associated with the protein. All the data shown indicate that hydrophobic ionic liquids change the enzyme conformation making it easier to access the active site, this can be attributed to the strong interactions between enzymes and anions such as [BF$_4$] y [PF$_6$] [40].

On the other hand, it has also been observed that enzyme stability in ionic liquids depends on their viscosity. For

example, Moniruzzaman, *et al,* (2010), indicates that enzyme conformational-change is slow in high viscosity IL solvents, thus it is effective in maintaining enzyme activity and stability over a prolonged period [33]. In addition, enzymes have been shown to be highly stable in anhydrous ILs due to their conformational rigidity in the dehydrated state. Enzyme stability increases with increasing alkyl chain length of cations, as cations interact with enzymes through van der Waals force, which plays an important role in protein-IL interactions [41]. From the above, a better understanding of the IL decomposition process would help to design and synthesize ionic liquids that meet the "green" requirement for biocatalysis.

It is important to note that polar organic solvents inactivate enzymes. However, ionic liquids do not; this feature extends enzyme-catalyzed reactions to a range of solvent polarity that was previously inaccessible. The ability to use solvents with higher polarity increases the solubility of polar substrates, such as glucose, maltose or ascorbic acid [36]. Let us look at some cases.

Park*, et al* (2001), showed that glucose acetylation catalyzed by lipase B from *Candida antarctica* (CAL-B) was more regioselective in [Emim] [BF4] because glucose is up to one hundred times more soluble in ionic liquids. Acetylation of insoluble glucose in organic solvents yielded the more soluble 6-O-acetylglucose, which underwent further acetylation to give 3,6-O-diacetylglucose. Even so, acetylation of glucose in ionic liquids yielded only 6-O-acetylglucose. Furthermore, it was observed that the polarity values of the ILs is between 0.63 to 0.71, the most polar being [Emim] [BF$_4$] and the least polar, [BmPyr] [BF$_4$]. With normal organic solvents, the trend is toward higher reaction rates in less polar solvents. However, for lipase-catalyzed acetylation of *Pseudomonas cepacia* in ionic liquids the trend was in the opposite direction toward higher reaction rates in the more polar ionic liquids [42]. Indeed, Ulbert, et al, (2004), demonstrated that *Pseudomonas cepacia* lipase activity is inversely proportional to conventional solvent polarity, while the enzyme activity is directly propotional to IL polarity [43].

Another advantage of ILs is their recycling scheme, because ionic liquids are more expensive than organic solvents (2-100 times more than organic solvents), a key to their industrial use is their efficient recovery, product isolation and reuse. Also, these issues are the key to the environmental performance of ionic liquids [44].

The recovery of both enzyme and IL has been evaluated in different investigations. For example, Vidya, *et. al.* (2009), investigated the recyclability of *Pseudomonas cepacia* (PS) lipase mixture and hydrophobic IL, it was observed that both PS -IL lipase mixtures can be reused five times without any decrease in yields [45].

Ulbert, *et al,* (2004), evaluated the recyclability of *Candida rugosa* lipase in ionic liquids. Surprisingly, lipase retained 92 and 95% of its original activity after recycling five times in [Bmim] [PF$_6$] and [Onim] [PF$_6$]. Whereas in n -hexane and toluene, the initial activity decreased to 55 and 50%, respectively [43]. Similarly, Yuan, et al, (2006), demonstrated that *Candida rugosa* lipase shows better stability and enantioselectivity in [BMIM] [PF$_6$] than in hexane, so it is worth checking whether the enzyme and ionic liquid can be recycled. It was observed that the conversion of menthol decreased dramatically during recycling, although the conversion in ionic liquid was somewhat higher than in hexane. This may be due to enzyme mass loss and/or decreased activity in the recycling operation [46].

On the other hand, Xu, *et al,* (2017), explored the reuse performance of [Amim] Cl and [Bmim][OAc] for pretreatment and enzymatic hydrolysis of eucalyptus. It was observed that IL can be reused without substantial losses in pretreatment and hydrolysis efficiencies of eucalyptus at least three times. A conversion yield of 54.3% was achieved for the fourth recycling of [Amim] Cl, which was almost 5.0 times higher than that of untreated eucalyptus. However, the hydrolysis rate of eucalyptus pretreated with [Bmim] OAc (72,8%) was significantly higher than that of the untreated sample (10.9%) even if the IL was reused for four cycles, suggesting that [Bmim] OAc exhibited high recyclability in biomass pretreatment compared to [Amim] Cl [47].

Given these advantages, there is a substantial value proposition for recycling ionic liquids, both in terms of lowering the cost of IL processes and the environmental impact. However, the means to economically recycle ILs on an industrial scale still requires further research. Several IL extraction methods have been tested including distillation, liquid-liquid extraction, adsorption, induced phase separation, and membrane-based methods [48]. Efforts to effectively separate hydrophilic ILs from the reaction process are still scarce, as the biphasic layers offered by the ILs, enzyme and product have proven to be challenging [34].
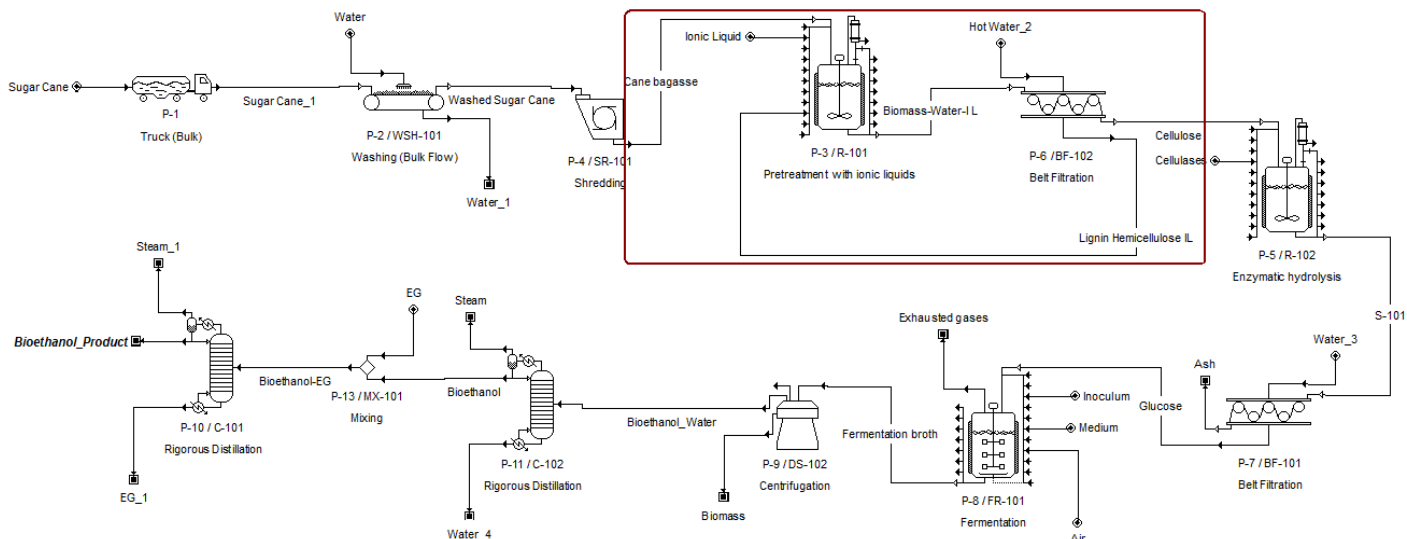
Figure 2: Bioethanol production using ionic liquids in pretreatment.

4. Biofuel production with ionic liquids.

In Colombia, sugarcane is the main raw material, especially for sugar as a food additive and for bioethanol production. Likewise, the amount of bagasse destined to ethanol processing is 40%, since in the current processing scheme in the country, 60% of the bagasse generated in milling is used in the cogeneration system. The ethanol production process can be described in four stages. The first stage is pretreatment, the second corresponds to hydrolysis, the third to fermentation and the fourth to ethanol separation [49].

Lignocellulosic biomass is composed of cellulose ($C_6H_{10}O_5$), hemicellulose ($C_5H_{10}O_5$) and lignin ($C_{10}H_{11}O_{3,5}$). With respect to sugarcane bagasse, it contains 11.4% lignin, 31.1% hemicellulose and 43.1% cellulose [2]. Multiple factors such as the complexity of cell wall components, structural heterogeneity, cellulose crystallinity, and degree of lignification are responsible for the recalcitrance of biomass that makes it resistant to chemical degradation. To overcome the recalcitrance of the feedstock, the pretreatment process becomes an important step in the utilization of lignocellulosic biomass for industrial applications [50].

Different methods have been adopted to treat lignocellulosic biomass prior to its use for bioethanol fermentation [51]. Figure 2 shows bioethanol production using ionic liquids in the pretreatment of sugarcane bagasse. The section marked with a red line refers to the pretreatment of lignocellulosic biomass using ionic liquids that have the capacity to significantly decrystallize cellulose, simultaneously breaking the lignin and hemicellulose network and decreasing the enzymatic load required for hydrolysis. For subsequent IL regeneration, the cellulose recovered by the addition of anti-solvents such as hot water is transferred to the enzymatic hydrolysis operation while the IL, hemicellulose and lignin are recirculated back to the pretreatment [50].

To illustrate, Yang, *et al,* (2020), selected three types of ionic liquids with the aim of establishing a reaction system with Tween-80 surfactant for in situ enzymatic hydrolysis of lignocellulose from sugarcane bagasse. The results indicated that the highest activity occurred with [Emim] OAc and the rates of bagasse cellulose hydrolysis in the first 12 h dramatically increases by 2.5-fold. Interestingly, [Emim] OAc showed a synergistic effect and performed better with Tween-80 than [Emim] DEP. Among the possible reasons, the cell wall degradation effect is eliminated, and the Tween-80 stabilize *Paenibacillus* cellulase to retain the active conformation, implying a unique interaction mechanism of [Emim] OAc and Tween-80 [52].

On the other hand, Bian, *et al,* (2014), found that pretreatment with [Emim]Ac ionic liquid led to 92.8% glucose conversion, compared to 80.0% in the original cellulose [53]. Also, Da silva, et al, (2011), analyzed six ILs for their ability to improve the rate of enzymatic saccharification. Enzymatic hydrolysis of IL-treated products resulted in higher saccharification yields of glucose and xylose than when no treatment was

employed. [Emim] [Ac] proved to be the most effective IL, resulting in glucose and xylose yields of 98.2% and 60.7%, respectively, after a saccharification time of 48 h [54].

With respect to the above, the enhancement of enzymatic hydrolysis with treatment with ILs such as [Emim]Ac and [Emim]OAc causes both the reduction of crystallinity and the degree of polymerization, leading to the improvement of cellulose conversion. Moreover, the structure of the original cellulose preserves its complete crystalline structure in the form of long and well-separated microfibrils, which has a strong interchain hydrogen bonding network leading to high resistance to enzymatic hydrolysis [52]. After pretreatment with IL, the cellulose is observed to coalesce and agglomerate, the surfaces are rougher and with more porosity. Also, the accessibility of the substrate to cellulolytic enzymes is one of the main factors influencing the hydrolysis process, cellulases can be trapped in the pores if the internal area is much larger than the external area. Thus, one of the objectives of pretreatment is to increase the porosity and surface area available for enzymatic attack [53].

In the case of biodiesel, Colombia has been stimulating the planting of African palm as the main raw material for biodiesel production, becoming the fifth country in the world in terms of African palm production; likewise, a whole legislation has been enacted to stimulate biodiesel production in such a way that by 2011 it is mandatory that all diesel used in the country must be mixed with at least 10% of biodiesel [55]. Currently in Colombia there are 11 biodiesel production plants distributed in several areas of the country, being Bio D the plant that produces more tons, 200,000 tons per year. Likewise, companies such as Ecodiesel Colombia, Aceites Manuelita,

Inversiones la Paz, ALPO and Biocosta Green Energy stand out [56].

On the other hand, the reaction of biodiesel or fatty acid alkyl esters (FAME) is obtained by catalytic transesterification of triglycerides with an alcohol (generally methanol or ethanol). Chemical transesterification has been used for the industrial production of biodiesel, although the process has several drawbacks: it is energy intensive; recovery of the glycerol by-product is difficult; the acid or alkali catalyst must be removed from the product; alkaline wastewater requires treatment; and both free fatty acid and water interfere with the reaction [57].

In this regard, some alternatives that lead to a more sustainable catalyst such as the use of lipolytic enzymes has been encouraged. These enzymes biocatalyze the cleavage of ester bonds in a wide range of substrates (e.g., alcoholysis, acidolysis, aminolysis) as well as synthetic reactions such as transesterifications. These have been reported to exhibit unique characteristics such as high degree of chemoselectivity, enantioselectivity and regioselectivity, which make them attractive for different applications, such as biodiesel production. In this context, ionic liquids emerge as adjuvants to lipase action to increase selectivity, reaction rate, transesterification yield, and improve enzyme recycling [58].

Figure 3 shows the transesterification reaction, which consists of the reaction between a triglyceride and an excess of methanol in the presence of enzymes and ionic liquid, at a temperature that can range from 40°C to 110°C, resulting in three methyl esters (when methanol is used) and glycerin as products. It is estimated that using one ton of oil produces approximately one ton of biodiesel and 100 kg of glycerin, which is used in the
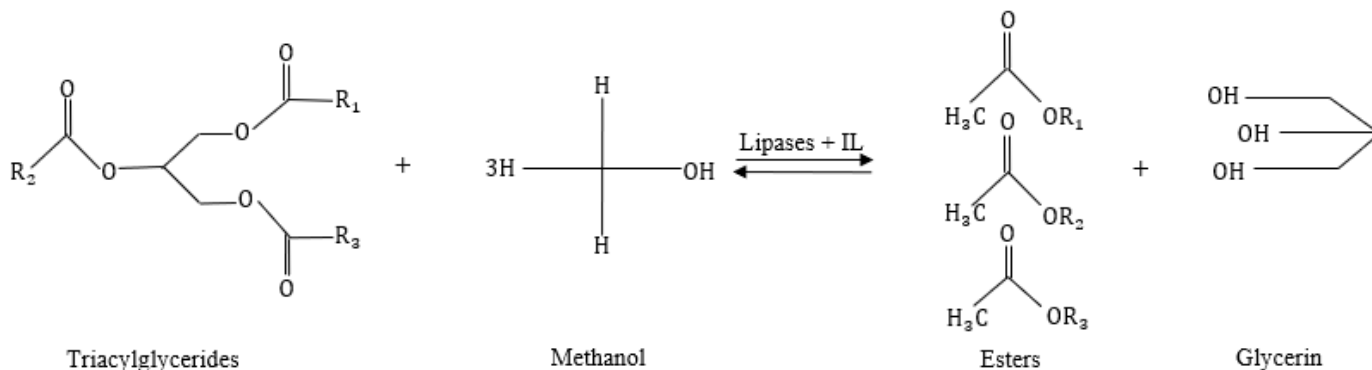

Figure 3: Transesterification reaction

chemical industry for the manufacture of soaps, detergents and, in some countries, as an additive to biodiesel [59]

Now, there are several reports on the production of biodiesel using IL and enzymes, for example, De Diego, et al, (2011), found that by using immobilized Candida antarctica lipase B enzyme (Novozym® 435) is able to transform triacylglycerides into biodiesel with [C_{16}mim] [Tf_2N] obtaining a yield of 98% after 6h at 60°C. Also, the IL was reused in 6 cycles, after the seventh cycle the reaction yield decreased probably due to the low stabilization power of the high hydrophobicity of [C_{16}mim] [Tf_2N], produced by the 18 alkyl chains in the cation [57].

In addition, when the transesterification reaction was analyzed in a solvent-free system without ionic liquid, a yield of 45.46% was obtained at 24 h. These results clearly demonstrated the protective capacity of these ILs against enzymatic deactivation, as they preserve the lipase activity against the denaturing effect of aliphatic alcohol (i.e. methanol) and the by-product glycerol [57].

In fact, Ha, *et al*, (2007), demonstrated that the highest FAME production was obtained in [Emim] [TfO] and [Omim] [Tf_2N]. with conversions 8 and 3 times higher than the solvent-free system respectively, after 24 hours FAME production in hydrophobic anion containing ILs such as [PF_6] and [Tf_2N] increased with increasing alkyl chain length in the cation, but this relationship was not observed in other ILs. These phenomena may be partly explained by a higher solubility of palm oil in more hydrophobic ILs, since hydrophobicity increases as the alkyl chain length in the cation increases [60].

On the other hand, Yang, *et al*, (2010) investigated the transesterification of *Penicillium expansum* lipase because it has a high percentage of hydrophobic residues (51.4%) in the N-terminal region, by making use of ionic liquid [Bmim] [PF_6] a yield of 69.7% was obtained compared to 19.4%, 14.0% and 1.0% obtained in tert-butanol, solvent-free system and hexane, respectively [61]. Similarly, Nara, et al, (2002) demonstrated that with lipase from Pseudomonas cepacia immobilized on diatomite particles a relatively high degree of transesterification was obtained in [Bmim] [PF6] compared to [Bmim] [BF4] and dichloromethane [62].

From these research works, it can be highlighted that PF6 anion is the most studied one for lipase-catalyzed biodiesel synthesis since it involves conversions close to 100% [58]. This anion also proved to be suitable with different substrates and in various substrate: alcohol ratios. In studies such as Su, et al, (2016) and Ha, et al, (2007), the highest yields were obtained with a methanol: oil molar ratio of 4:1 because more methanol could lead to inhibition of the transesterification reaction and, meanwhile, too much oil would increase the production cost, so the molar ratio of 4:1 was selected as the optimal one [60,63].

Overall, the biodiesel production application revealed an increase in conversion when hydrophobic ILs were used. Different hypotheses were suggested to explain this behavior, but the most plausible one refers to the ability of hydrophilic organic solvents to remove essential water molecules that hydrate the enzyme [58]. An enzyme is usually inactive under completely dry conditions, but the addition of a small amount of water to the non-aqueous reaction medium normally accelerates enzymatic reactions, and the main function of water is to ensure that the enzyme has its optimal flexibility [61]. It is evident that the implementation of an efficient process depends not only on the phobicity of the ionic liquid, but also on the type of substrate, operating conditions, and the biocatalyst [58].

Conclusions

In conclusion, in reactions catalyzed by lipases and laccases in ionic liquids, the same or higher yields could be obtained than in conventional solvents used in the industries. Also, the enzymes showed higher activity and thermal stability with hydrophobic ILs. However, further research on enzyme inactivation and activation with ILs is required for industrial scale application.

Another interesting aspect is that ionic liquids can be reused up to 5 times without significant losses in the reaction yield, which is an opportunity to reduce the costs of these liquids and that they can be used in the industry. Also, ILs with high viscosity are useful in some biocatalytic reactions because they favor the slow change of the enzyme conformation.

In Colombia, ionic liquids could be implemented in the production of biofuels both for the pretreatment of sugarcane bagasse and the transesterification of palm oil, which are alternatives that reduce the environmental impact and obtain yields similar to those of organic solvents. Although the transesterification reaction can be

carried out in a relatively short time, it is necessary to look for different methods such as microwaves to accelerate the reaction.

Abbreviations

[Amim] Cl	1-allyl-3-methylimidazolium chloride
[Bmim] [BF$_4$]	1-butyl-3-methylimidazolium tetrafluoroborate
[Bmim] [PF$_6$]	1-Butyl-3-methylimidazolium hexafluorophosphate
[Bmim] [Tf$_2$N]	1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) Imide
[Bmim] [CF$_3$SO$_3$]	1-butyl-3-methylimidazolium trifluoromethanesulfonate.
[Bmim] [OTf]	1-butyl-3-methylimidazolium trifluoromethanesulfonate
[Bmim] Cl	1-butyl-3-methylimidazolium chloride
[BmPyr][BF$_4$]	N-butyl-N-methylpyrrolidinium tetrafluoroborate
[BMPyr] Cl	N-butyl-N-methylpyrrolidinium chloride
[C$_2$OHmim][PF$_6$]	1-hydroxyethyl-3 Methylimidazolium hexafluorophosphate.
[Chol] [H$_2$PO$_4$]	Choline dihydrogen phosphate
[Dmim] [PF$_6$]	1-decyl-3-methylimidazolium hexafluorophosphate
[Emim] Cl	1-ethyl-3-methylimidazolium chloride
[Emim] OAc	1-ethyl-3-methylimidazolium acetate
[Emim] DEP	1-ethyl-3-methylimidazolium Diethylphosphate
[Emim] [MeSO3]	1-ethyl-3-methylimidazolium methanesulfonate
[Emim] [FSI]	1-ethyl-3-methylimidazolium bis(fluorosulfonyl) imide
[Gly] [BF$_4$]	Glycine tetrafluoroborate
[Hmim] [PF$_6$]	1-hexyl-3-methylimidazolium hexafluorophosphate
[Hmim] [Cl]	1-hexyl-3-methylimidazolium chloride
[Omim] [BF$_4$	1-octyl-3 methylimidazolium tetrafluoroborate
[Omim] [PF$_6$]	1-octyl-3-methylimidazolium hexafluorophosphate
[Mmim] [MeSO4]	1,3-dimethylimidazolium methylsulfate
[N$_{10111}$] Br	Decyltrimethylammonium bromide
[Omim] [PF$_6$]	1-octyl-3-methylimidazolium hexafluorophosphate
[TOMA] [Tf$_2$N]	Trioctylmethylammonium bis(trifluoromethylsulfonyl) imide.

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