Biocatalysis in ionic liquids

State-of-the-union

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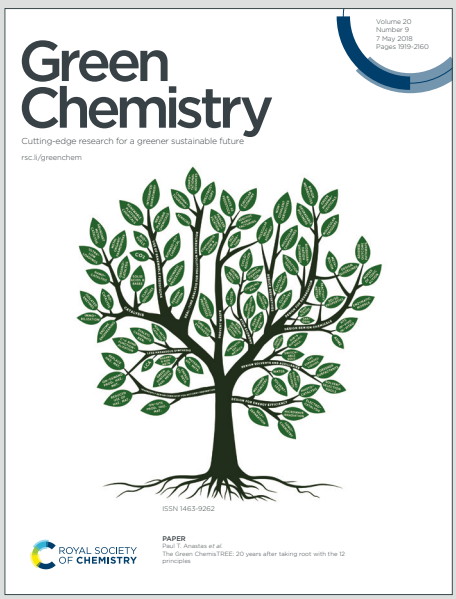

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ARTICLE

Biocatalysis in Ionic liquids: State-of-the-Union.

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This perspective reviews the current status and prospects of biocatalysis in ionic liquids. Although they are not strictly speaking ionic liquids, deep eutectic solvents are included because of the close similarities of their properties and potential applications with those of ionic liquids. One consequence of the ongoing transition from an economy based on fossil resources to a circular economy based on renewable biomass is the burgeoning interest in the use of biocatalysis for the selective conversion of carbohydrates and triglycerides to liquid fuels and chemicals in biorefineries. The use of inexpensive, environmentally attractive ionic liquids as solvents, in the pre-treatment and subsequent biocatalytic conversions, for example, is expected to play an important enabling role in this process.

Introduction to Non-Aqueous Enzymology

Enzymes are Nature's sustainable catalysts. They are derived from renewable resources and are biodegradable and biocompatible. Enzymatic processes are more step economic, cost-effective, energy-efficient, generate less waste and are, consequently, greener and more sustainable than conventional chemical processes. These substantial environmental and economic benefits of enzymatic processes provided the major driving force for the wide-spread application of biocatalysis in industrial organic syntheses, particularly in the pharmaceutical industry, in the preceding two decades.[1,2,3,4,5]

Advantages and limitations of enzymes in organic media

It is generally perceived as an important ecological benefit that enzymes function optimally in aqueous media. However, this can be a serious limitation in organic synthesis where the majority of organic substrates are sparingly soluble in water. Sporadic reports of employing enzymes in organic media go back as far as the beginning of the last century,[6] but it was the seminal paper by Klibanov and Zaks[7] in 1984, describing enzymatic catalysis in organic solvents at elevated temperatures, that heralded the advent of non-aqueous enzymology. Their observation that certain hydolases, particularly lipases, are actually more stable in hydrophobic organic solvents, such as toluene at 100°C, was a complete revelation, particularly for organic chemists like the author. It led, in subsequent decades, to the widespread application of enzymes in organic solvents and, eventually, to the adoption of biocatalysis as a mainstream synthetic tool with broad industrial scope.[8,9]

Further advantages of biocatalysis in organic media include facile product recovery from volatile organic solvents and elimination of microbial contamination. In addition, transformations that are problematical in water owing to equilibrium limitations, e.g. esterifications and amidations, are eminently feasible in organic solvents.

However, there are disadvantages associated with the use of enzymes in organic solvents. Enzymes can function as suspensions in organic solvents but generally with catalytic activities that are two or more orders of magnitude lower than those observed with the soluble enzymes in water. It was known, however, that activities of enzymes in organic media can be dramatically increased by lyophilisation in the presence of relatively large amounts of salts, such as potassium chloride.[10]

This led us, at the turn of the century, to surmise that a suspension of an enzyme in an ionic liquid (IL), with its salt- and water-like character, could possibly afford significant rate enhancements compared with analogous suspensions in organic solvents.

Enzymes in ionic liquids

Ionic liquids (ILs) are molecules that are composed entirely of ions and are liquids at or close to ambient temperature. If they are mixed with water they become ionic solutions. ILs have been widely advocated as green alternatives to volatile organic solvents based on their negligible vapour pressures coupled with good thermal stability and tunable properties such as viscosity, polarity, hydrophobicity and miscibility with organic solvents. Some ILs are, by an appropriate choice of cation and anion, capable of dissolving a variety of biopolymers, including proteins and recalcitrant polysaccharides[11], which is of particular interest in the context of biocatalysis.[12] We note, however, that whether or not ILs can be considered as green depends on their method of synthesis and individual physical and chemical properties, such as viscosity, volatility, ecotoxicity and

biodegradability. In this respect, they are not assessed any differently than organic solvents.

We are now two decades further on. What has happened in the intervening twenty years with regard to solvent use in biocatalytic processes? First of all, the best solvent is no solvent: reactions can be performed with neat liquid substrates, e.g. in biodiesel production from triglycerides[13] and a variety of biocatalytic oxidations and reductions.[14] Various mono- and bi-phasic solvent systems have been proposed as alternatives for volatile organic solvents with undesirable ecological properties.[15,16,17] These include, for example, solvents derived from renewable raw materials, such as bio-ethanol,[18] 2-methyltetrahydrofuran,[19] γ-valerolactone (GVL)[20] and cyrene (dihydrolevoglucosenon),[21,22] glycerol,[23] and glycerol derivatives such as glycerol carbonate.[24] Interestingly, lipases dissolve in the latter solvent with retention of activity.[24]

In this Perspective we present an assessment of the current status and future prospects of biocatalysis in ILs. Because of their close similarity to ILs, both in structure and properties, we include a discussion of deep eutectic solvents (DESs). The latter consist of mixtures of hydrogen bond acceptors (HBAs), usually comprising organic salts, and hydrogen bond donors (HBDs).

Structures and Properties of Ionic Liquids

ILs are sometimes defined as salts with melting points below 100 °C. If they are liquid at ambient temperature they are often referred to as room temperature ionic liquids (RTILs) but we will use the general abbreviation, ILs. They are not new. Ethylammonium nitrate, which is liquid at ambient temperature, was first described in 1914.[25,26] Interest in ILs as solvents for catalytic processes led to the use of alkylpyridinium and dialkylimidazolium chloroaluminates ($AlCl_4^-$ or $Al_2Cl_7^-$) as solvents and catalysts for Friedel-Crafts acylations.[27] These ILs are generally referred to as 1st generation ILs.

However, the high reactivity of chloroaluminates towards water is a serious impediment for their application in organic synthesis with enzymes. This stimulated the development of 2nd generation quaternary ammonium and phosphonium salts consisting of non-coordinating, water- and air-stable (at least at room temperature), anions, such as, tetrafluoroborate (BF_4^-) and hexafluorophosphate (PF_6^-). Unfortunately, these anions do undergo slow hydrolysis at elevated temperatures, resulting in the generation of hazardous HF. Consequently, dialkylimidazolium salts containing a wide variety of anions, such as $CF_3SO_3^-$, $[CF_3SO_2]_2N^-$, $CF_3CO_2^-$, $CH_3CO_2^-$, HCO_2^-, $PhSO_3^-$, HSO_4^-, $CH_3SO_3^-$, $ArSO_3^-$, $H_2PO_4^-$, $R_2PO_3^-$, $(CN)_2N^-$ and many more were prepared, e.g. by anion exchange in the quaternary halide.[28,29,30]

Motivated by the pressing need for waste reduction and efficient recycling of homogeneous catalysts, such 2nd generation ILs (see Figure 1 for structures) were used as green and sustainable solvents for organic synthesis[31] including a wide variety of catalytic processes.[32,33,34,35,36,37]

Figure 1. Structures of 2nd generation ILs

Biocatalysis in 2nd generation ILs : origins.

A logical extension of this was to use the same ILs to perform biocatalytic transformations. In this case another property of ILs becomes important, namely water miscibility. The latter is largely determined by the structure of the anion but rationalising the effect of different anions on miscibility with water is not a simple matter.[38] Many ILs are completely miscible with water while others are hydrophobic and form a separate phase with water. For example, 1-butyl-3-methylimidazolium (bmim) ILs containing the perfluorinated anions, PF_6^- and $[CF_3SO_2]_2N^-$, are hydrophobic and with nitrate, chloride and acetate anions they are completely water miscible while bmim ILs with BF_4^- and $CF_3SO_3^-$ are somewhere in between and are water miscible. The lipophilicity of dialkylimidazolium salts, or other ILs, can be increased by increasing the chain length of the alkyl substituents. It is also worth noting, in this context, that even hydrophobic ILs are hygroscopic and will absorb up to several volume percent of water, which can have a significant effect on their properties.

As noted in the Introduction, the use of ILs for conducting biotransformations was motivated by the possibility of replacing volatile organic solvents with non-volatile, thermally stable ILs. The first example was reported by Lye and Seddon and coworkers[39] in 2000. It involved the hydrolysis of 1,3-dicyanobenzene to 3-cyanobenzoic acid (Figure 2a) catalysed by whole cells of *Rhodococcus* R312 containing a nitrile hydratase and an amidase to catalyse the hydrolysis of the initially formed 3-cyanobenzamide. The use of a two-phase mixture of [bmim][PF_6] and water avoided the flammability and toxicity issues associated with the standard use of toluene as the co-solvent. Moreover, the microbial cells were better dispersed, more stable and afforded higher activities and product yields. Nonetheless, strictly speaking, it is not an example of biocatalysis in an IL as the reaction takes place in the water phase and the IL is merely acting as a reservoir for the substrate and product, thereby suppressing substrate and product inhibition.

Figure 2. Early examples of biocatalysis in hydrophobic ILs.

In the same year Russell and coworkers[40] reported the use of [bmim] [PF_6] containing 5 vol % water as a solvent for thermolysin catalysed synthesis of protected aspartame. Activities and yields were comparable with those observed in water/organic solvent mixtures and the enzyme could be recycled with no apparent loss in activity. However, it is highly likely that the enzymatic reaction took place in the separate water phase created from the 5 volume % water. Also in the same year, we reported[41] the use of anhydrous [bmim][PF_6] and [bmim][BF_4] as reaction media for *C. antarctica* lipase B (CalB) catalysed amidations and transesterifications (see Figure 2). . Both the enzyme and the IL were dried over phosphorus pentoxide prior to use. Similarly, the groups of Kragl[42] and Itoh[43] independently reported the first examples of lipase catalysed enantioselective transesterifications in the same ILs in 2001. Following these initial reports at the turn of the century, biocatalysis in ILs has been widely studied and forms the subject of numerous reviews.[44,45,46,47,48,49,50,51,52]

Biocatalysis in 3rd generation biocompatible ILs

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Although reactions in 2nd generation ILs were important for demonstrating proof of concept, ILs such as [bmim] [BF_4] and [bmim] [PF_6] have serious shortcomings, namely a propensity for hydrolysis to highly corrosive HF coupled with aquatic ecotoxicity[53] and poor biodegradability.[54] In addition, their syntheses are neither cost-effective nor green.[55] This led to the emergence of 3rd generation ILs, containing more biocompatible cations and anions, derived from inexpensive, readily available and, in many cases, renewable raw materials, including carbohydrates and amino acids (see Figure 3 for examples).

Cholinium salts as ILs

Choline-based ILs are obtained by reacting inexpensive choline hydroxide with a (natural) carboxylic acid to produce a range of choline alkanoates that combine biocompatibility with low toxicity and good biodegradability.[56,57,58,59,60] A wide variety of choline ILs were suitable for use in aqueous biphasic systems (ABS).[61] Choline-based ILs in particular have been widely studied in connection with their pronounced stabilising effect on enzymes and therapeutic proteins.[62] In particular, choline dihydrogen phosphate, [Ch][H_2PO_4], was shown[63,64] to have excellent biocompatibility, which is perhaps not so surprising when one considers that relatively high concentrations of phosphate ions are present in all living cells, e.g. in DNA and ATP. ILs based on the dimethylphosphate (dmp) anion appear to be cost-effective and compatible solvents for enzymes and polysaccharides but have not been extensively studied for conducting biocatalytic transformations.[65]

ILs derived from amino acids (AAILs)

A wide variety of ILs has been described[66,67] in which the anions are derived from naturally occurring amino acids (AAs) and, hence, are generally non-toxic, biocompatible, biodegradable and bio-renewable. They are collectively referred to as AAILs and include ILs based on both a choline cation and an AA anion (ChAAILs).[68,69,70] AAILs based on alkyimidazolium cations were shown to form biphasic mixtures with water by salting out with, for example, potassium phosphate,[71] with the hydrophobicity of the AAIL increasing in the order Ser<Gly<Ala<Leu. This creates the possibility of performing biocatalysis in aqueous biphasic systems.

In another study, cholinium glycinate, alaninate and lysinate were shown to enhance the activities of *Thermomyces lanuginosus* lipase (TlL) in mixtures with water.[72] Biocompatible AAILs are of particular interest in connection with biomass pre-treatment (see later). It is worth noting, on the other hand, that ILs containing AA anions are not always more biocompatible than, for example, cholinium halides.[73]

Hydrophobic ILs from fatty acids

ILs produced from readily available mixtures of long chain fatty acids (LCFAs), and tetraalkyl ammonium and phosphonium and dialkylimidazolium cations, similarly represent a family of hydrophobic bio-based and biodegradable FAILs with a reduced environmental footprint.[74,75]

Designer ILs

ILs are clearly amenable to fine-tuning of their physical and chemical properties by an appropriate choice of cation and anion. The current trend is, therefore, towards designer ILs that are readily available, cost effective, compatible with enzymes and have a negligible environmental footprint. For example, Zhao and coworkers[76] designed enzyme compatible ILs based on carboxylate salts of quaternary ammonium cations containing oligoether side-chains (see Figure 3 for structures) that are able to dissolve large amounts of carbohydrates, including glucose and cellulose. It is also possible to design polar hydrophobic ILs that are essentially the IL equivalent of chlorinated hydrocarbons such as dichloromethane. by combining tetraalkyl phosphonium cations with alkylphosphonate anions.[77]

Figure 3. Structures of 3rd generation ILs

Protic ionic liquids (PILs)

The quest for inexpensive ILs with low toxicity combined with improved biodegradability and enzyme compatibility led to the idea of using protic ILs (PILs)[78] as media for conducting biocatalyic reactions.[79] Their synthesis is exceedingly simple - by mixing an amine with e.g. sulfuric or a carboxylic acid - and PILs were known to exhibit better biodegradability and lower toxicity than the corresponding quaternary ammonium salts.[80] Moreover, they have H-bond donating properties that enable the stabilisation of enzymes and, when they are carboxylate salts, they are self-buffering (see Figure 4 for examples of PILs).

Similarly, 2-hydroxyethylammonium lactate is a PIL consisting of a cation derived from inexpensive ethanolamine and an anion of a natural, biodegradable carboxylic acid.[81]

Figure 4. Examples of PILs

We found that a series of trialkylammonium carboxylates were suitable solvents for the kinetic resolution of 1-phenylethanol by C. antarctica lipase B (CaLB) catalysed acylation with vinyl acetate at 40 °C.[77] Good rates and high enantioselectivities (92->99%) were observed with suspensions of the free CaLB or immobilised as cross-linked enzyme aggregates. *A priori* one might expect competition from catalysis by small amounts of the free amine, formed by dissociation, but the high enantioselectivities can only be rationalised by invoking an enzyme catalysed reaction.

Inexpensive PILs are readily available from bulk, commodity amines, such as triethylamine and triethanolamine, and inexpensive acids, such as sulphuric, phosphoric, formic and acetic acids. For example, the cost-price for large scale production of triethylammonium hydrogen sulfate, $(Et)_3NH^+$ HSO_4^-, was estimated at $ 1.24 per kg.[82]

Deep Eutectic Solvents (DESs)

Deep Eutectic solvents (DESs) constitute another class of so-called neoteric solvents for biocatalytic reactions. They are formed by mixing certain hydrogen bond acceptors, such as a quaternary

ammonium salt, and hydrogen bond donors (HBDs) such as alcohols, carboxylic acids and amides, and were first reported by Abbott and co-workers in 2003.[83] For example, a mixture of the solids urea (m.p. 132°C) and choline chloride (m.p. 302°C) is a liquid at ambient temperature (m.p. 12°C) as a result of interactions through intermolecular hydrogen bonds leading to lowering of their melting points. Although they are, strictly speaking, not ILs since they also contain uncharged components, they have properties, such as low volatility and high thermal stability, strongly resembling those of ILs. Indeed, they are sometimes called 4th generation ILs.

They are easily synthesized by mixing the two components and gently heating the mixture. Hence, they are readily available and relatively inexpensive. For example, choline chloride/glycerol (ChCl/Gly; 1:2) is prepared by mixing one equivalent of choline chloride, an inexpensive feed additive produced in >1 mio tonnes per annum, and 2 equivalents of glycerol, a byproduct of biodiesel manufacture. Natural deep eutectic solvents (NADESs) have been prepared from combinations of a variety of relatively simple, primary metabolites, including sugars, amino acids and organic acids, such as citric, itaconic, malic, lactic and succinic acids (see Figure 5 for examples). The fact that they are present in relatively large amounts in all living cells[84] strongly suggests that NADESs play the role of reaction media in the intracellular synthesis of secondary metabolites, such as flavonoids and steroids, which are sparingly soluble in water.[85] Indeed, they have been called "Solvents for the 21st century".[86] As a result of the strong hydrogen bonding, they tend to be more viscous than ILs and are, therefore, often used as mixtures with other solvents, such as water.

Figure 5. Examples of deep eutectic solvents

Kazlauskas and coworkers[87] were the first to report, in 2010, the use of DESs as solvents for biocatalytic reactions and referred to them as advanced ionic liquids. In the following decade they were widely applied in biocatalysis,[88,89,90] both whole cell biocatalysis and with isolated enzymes, including chemo-enzymatic cascade processes[91] Since, the number of possible combinations of hydrogen bond acceptors and donors, both hydrophobic and hydrophilic, is enormous, a DES can, in principle, be designed for a particular enzymatic conversion.[92]

Following the seminal work of Kazlaukas and co-workers, DESs have been extensively used for conducting lipase catalysed reactions, [93] including hydrolyses, (trans)esterifications, amidations, and chemo-enzymatic epoxidations.[94] DESs have also been widely used in biocatalytic redox processes.**[14]** For example, a ChCl/Gly/aqueous buffer mixture was successfully used as the solvent for the enantioselective reduction of prochiral ketones catalysed by Baker's yeast[95] or by various ketoreductases overexpressed in *E.coli* cells.[96] Biocompatible DESs are also promising solvents for biocatalytic conversions of carbohydrates and triglycerides, e.g. in lignocellulose deconstruction and biodiesel production (see later).

In common with PILs, rather than classical ILs, DESs are prepared in a single, 100% atom efficient step. They are mostly derived from renewable, biocompatible raw materials and are, hence, carbon neutral, non-toxic and biodegradable. From an application viewpoint it is important to have access to both hydrophilic and hydrophobic. DESs, as is the case with ILs. However, DESs are generally hydrophilic, in many cases hygroscopic, and are unsuitable for use in aqueous biphasic catalysis or as solvents for extracting products from aqueous mixtures. The first examples of hydrophobic ionic liquids were reported by Kroon and coworkers[97] in 2015. They consisted of mixtures of decanoic acid and various quaternary ammonium salts. They are of interest, for example, for use in liquid-liquid extraction of various components of aqueous mixtures in lignocellulose bio-refineries, e.g. hydroxymethylfurfural (HMF) and furfural,[98] or even in the synthesis of HMF from fructose.[99]

Activities and Stabilities of Enzymes in ILs.

A wide variety of enzymes has been used with success in ILs. Early examples generally involved suspensions of free enzymes in ILs based on weakly coordinating anions such as BF_4^-, PF_6^- and $(CF_3SO_2)_2N^-$, which do not dissolve the enzyme. Enzymes suspended in such ILs showed excellent storage and operational stabilities. For example, the thermostable *Geobacillus thermocatenulatus* lipase catalysed the esterification of phthalic acids at 120°C (Figure 6a) in [bmim][BF_4].[100] More recently, the same enzyme was shown to catalyse smooth transesterification of O-acylmandelic acid with ethanol at 120°C (see Figure 6b) in [bmim][BF_4], with exclusive formation of *R*-mandelic acid (E>200). Remarkably, when the more hydrophilic 1-ethyl-3-methylimidazolium tetrafluoroborate, [emim][BF_4], was used as the solvent an even faster reaction was observed and *S*-mandelic acid was formed exclusively (E >200).[101]

Figure 6. Lipase catalysed (trans)esterifications in ILs at 120°C

Hydrophilic ILs containing coordinating anions such as halides, sulfate and nitrate can dissolve biopolymers, such as polysaccharides and proteins, by disrupting intermolecular hydrogen bonds. Dissolution of an enzyme in such ILs can lead to deactivation as a result of disruption of intramolecular hydrogen bonds that are essential for its catalytic activity. For example, the rates of transesterifications catalysed by suspensions of CaLB in ILs with the [bmim] cation and BF_4^-, PF_6^- and $CFSO_3^-$ anions were comparable to those observed in tert-butanol. In contrast, CaLB dissolved in the corresponding nitrate and lactate salts and virtually no conversion (< 5%) was observed.[102] Similarly, the cellulose from *Trichoderma reesei* dissolved in [bmim][Cl} with loss of activity.[103] In contrast, CaLB dissolves in [Et_3MeN][$MeSO_4$] with retention of its activity and the FT-IR spectrum is consistent with the dissolved enzyme retaining its active conformation in this IL. [104]

That inorganic salts exhibit different propensities for precipitating proteins from aqueous solution has been known for more than a century and follows a sequence known as the Hofmeister series.[105] Similarly, the behaviour of ILs towards proteins, based on the structures of constituent anions and / or cations, follows the same series.[106,107,108] Ions are divided into two groups: kosmotropes (structure making) and chaotropes (structure breaking). The former strongly interact with water, have a high charge density and are exemplified by SO_4^{2-}, AcO^-, Mg^{2+}, and Ca^{2+}. In contrast, chaotropes, such as ClO_4^-, PF_6^-, I^-, NH_4^+, and Cs^+, have a low charge density and interact weakly with water (Figure 7).

Owing to their strong interaction with water, kosmotropic anions, such as SO_4^{2-}, HPO_4^{2-} and AcO^-, effectively remove water molecules

from the protein surface causing the protein to minimise the surface area exposed to the solvent to restore its compact native state. In contrast, chaotropic anions, such as PF_6^-, Cl^- and I^-, have a low affinity for water and preferably bind to the protein-water interface, resulting in destabilization of the protein

Figure 7. The Hofmeister series and classification of kosmotropic and chaotropic ions.

The effect of cations in the ILs is generally less dominant but kosmotropic cations, such as Mg^{2+} and Ca^{2+}, tend to denature proteins by "salting in" the peptide moiety, thereby interacting more strongly with the unfolded form of the protein than with the native form.

Enzymes in biocompatible ILs

Generally speaking, there are two strategies for solving the problem of loss of activity of enzymes dissolved in ILs: (i) the design of enzyme-compatible ILs, or (ii) modification of the enzyme, using either protein engineering techniques or immobilisation to render it more resistant to deactivation by the IL.

The first example of deliberate design and use of an enzyme compatible IL was, to our knowledge, the morphine dehydrogenase catalysed oxidation of codeine described by Walker and Bruce.[109] The NADP cofactor was regenerated using glucose dehydrogenase coupled with gluconolactone (see Figure 8).The low solubility of codeine in both water and common organic solvents induced the authors to use an IL as the reaction medium. They surmised that an enzyme would function better in an IL if the latter closely resembled an aquatic environment. Hence, they designed an IL containing hydroxyl functionalities in both the cation and the anion. Indeed, an IL comprising the 3-hydroxypropyl-1-methylimidazolium cation and the glyoxylate anion dissolved the substrate, product, cofactor and the enzyme. Moreover, the dissolved enzyme was more active, even at a water content of 100 ppm, than as a suspension in other ILs.

Figure 8. Enzymatic oxidation of codeine in an ILs

Building further on the seminal work of Walker and Bruce, a wide variety of designer ILs and PILs have been described that contain hydroxyl[110,111,112] or alkoxyl[76,113,114,115,116,117,118] groups to enable sustainable biocatalysis. More recently, some basic principles were proposed by the group of Ou[119] as a guide for the successful design of biocompatible ILs in which enzymes dissolve and maintain their activity.

Water is a strongly amphoteric solvent, with a high dielectric constant (ε_r=81) and high donor (DN=33.0) and acceptor (AN=54.8) properties, and readily dissolves polyelectrolytes such as proteins. Hence, in order to be biocompatible, an IL needs to have good, water-like ionizing/dissociating abilities by having a high dielectric constant and consisting of both cations and anions with high DN and AN values. This is achieved by introducing hydroxyl groups into both the cations and anions of high dielectric constant ILs,[120] which is advantageous for dissolving and ionizing enzymes. Among common organic compounds they found three that meet this requirement: dimethyl sulfone (εr=47), triethanolamine (εr=29) and imidazole (εr=23). Furthermore, the IL should be a salt of a strong acid and a strong base in order to create a pH neutral environment.

Two series of biocompatible ILs - hydroxyalkyl imidazolium hydroxyalkyl sulfonates, and 3-hydroxypropyl-tri-(2-hydroxyethyl) ammonium hydroxylalkyl sulfonates (see Figure 9 for structures) were designed and synthesized based on these principles. They were subsequently tested for compatibility with lipases from *C. Antarctica* and *P. cepacia* in the transesterification of 1-butanol at 50^0C.[119] The lipases dissolved in these ILs and the resulting activities were comparable to those observed in water under the same conditions.

Figure 9. Structures of designer biocompatible liquids

Gomes and co-workers[121] have recently reviewed the status of the design of biocompatible ILs for use in biocatalysis.

Protein engineering for increased enzyme stability in ILs

Two strategies can be distinguished for modifying enzymes to increase activity and/or stability in ILs: protein engineering or biocatalyst engineering through immobilisation. In 2013 Nordwald and Kaar[122] studied the effect of the ratio of positive (protonated) amino groups to negatively charged carboxylate groups in the enzyme on the stability of chymotrypsin, papain and a lipase in [bmim][Cl] and [emim][$EtSO_4$]. They found that chemical modifications that reduce the ratio of positive to negative charges increase the enzyme's stability towards IL-induced denaturation.[123] More recently, the same group identified variants of an industrially relevant endoglucanase from *Acidothermus cellulolyticus*, with improved activity and stability, using directed mutagenesis of active site residues, including the introduction of a disulfide bridge.[124]

Similarly, the activity of a laccase from *trametes versicolor* in the degradation of lignin dissolved in [emim][$EtSO_4$] was increased using computational assisted protein engineering involving saturation mutagenesis at four amino acid residues in the so-called L1 loop.[125] Interestingly, a highly thermostable, high redox potential laccase was recently designed by incorporating 27 mutations that had been identified in directed evolution campaigns.[126] It would be interesting to test this laccase variant for activity and stability in ILs.

Enzyme immobilisation for increased stability in ILs

An alternative strategy for improving stability in ILs is to immobilise the enzyme. This would simultaneously improve commercial viability by facilitating enzyme isolation and reuse and enabling application in continuous flow reactors.[127, 128] The most widely known immobilised enzyme is undoubtedly Novozym 435, formed by adsorbing *C. antarctica* lipase B on a poly(methyl methacrylate) resin which is cross-linked with divinylbenzene.[129] It was used, for example, to catalyse the synthesis of mannosyl myristate by transesterification of mannose in 1-butyl-3-methyl pyrrolidinium trifluoromethane-

sulfonate, [BMpyrr] [CF_3SO_3], with a lower activity loss after 5 recycles than that observed in tert-butanol.[130]

Immobilisation as cross-linked enzyme aggregates (CLEAs)[131] is a useful method for stabilising enzymes in ILs. For example, CaLB-CLEA was active in the transesterification of ethyl butanoate with 1-butanol in [bmim][$N(CN)_2$]. In contrast, the free enzyme dissolved with complete loss of activity.[132] Similarly, CaLB-CLEA catalysed the enantioselective hydrolysis of phenylglycine methyl ester in [bmim][BF_4][133] and the lipase-driven chemo-enzymatic epoxidation of olefins and Baeyer-Villiger oxidation of ketones in hydrogen bond donating (HBD) ILs (Figure 10).[134] In contrast, when Novozym 435 was used in the latter reaction, catalysis was attributed to soluble CaLB that was leached from the polymer surface by the IL.

Figure 10. CaLB-CLEA catalysed chemo-enzymatic Baeyer-Villiger oxidation.

Penicillin expansum lipase CLEA, prepared directly from fermentation broth, catalysed biodiesel production by transesterification of microalgal oil in [bmim][PF_6].[135] Similarly, feruloyl esterase CLEA catalysed the esterification of sinapic acid with glycerol in the amphiphilic (hydrophilic cation and hydrophobic anion) IL, [$HOCH_2CH_2$mim][PF_6].[136]

Interestingly, a CLEA of *Pseudomonas stutzeri* lipase catalysed esterification of glycerol with benzoic acid in the analogous ChCL/glycerol DES whereas the free enzyme was inactive under the same conditions.[137]

Another method to immobilise enzymes for reactions is the use of solid IL-coated enzymes (ILCEs), first introduced by Lee and Kim[138] in 2002. It involved the use of an IL, 1-methyl-3-(3-phenylpropyl) imidazolium hexafluorophosphate, [ppmim][PF_6], which is solid at room temperature and becomes a liquid above 53 °C. The enzyme, *Pseudomonas cepacia lipase,* was coated by mixing with the IL at a temperatures above 53^{0}C and then cooling to ambient temperature. The resulting ILCE was used successfully in the enantioselective transesterification of chiral secondary alcohols. The methodology was subsequently, modified and optimized by Itoh and co-workers.[139]

An interesting application of this methodology is the co-immobilisation of a transaminase and the pyridoxal phosphate co-factor for multiple recycling in organic solvents, such as MTBE, by coating with an IL, such as [emim][Br}, with a melting point above both ambient and the operating temperature.[140] The coated TA exhibited good activity and operating stability in a variety of transaminations and is suitable for carrying out these transformations in continuous flow operation.

In the same vein, Lozano and co-workers[141] developed ILs based on tetraalkylammonium or dialkylimidazolium cations containing a hydrophobic alkyl group, such as dodecyl, hexadecyl or octadecyl, and perfluorinated anions, that are solid and sponge-like at ambient temperature but form a homogeneous liquid phase above 50^{0}C.

These sponge-like ILs (SLILs) can, in principle, be used in conjunction with free or immobilised enzymes, whereby the reaction is conducted at, e.g. 60^{0}C, and subsequently cooled to ambient temperature. The enzyme and IL are separated as a solid phase from the liquid products, like wringing out a sponge, at ambient temperature. The methodology was successfully applied, for example, in the synthesis of liquid flavour esters by Nov 435 catalysed esterification (Figure 11)[142]

Figure 11. Flavour ester synthesis in sponge-like ILs

SLILs comprising [C_{18}tma] or [C_{18}mim] cations and [$CF_3SO_2]_2N$] anion were also used in the Nov 435 catalysed production of oxygenated biofuels by lipase-catalyzed transesterification of various vegetable oils, including waste cooking oil, and/or direct esterification of free fatty acids with methanol or solketal.[143] Similarly, highly selective (up to 100%) production of monoacyl glycerides (MAGs), non-ionic emulsifying agents with widespread applications in food, pharmaceuticals and cosmetics, was observed in [C_{12}mim][BF_4].[144] For industrial viability it would be interesting to repeat these impressive studies with SLILs comprising the same cations, but with more acceptable hydrophobic anions such as sulfonate or phosphonate. Yet another possibility would be to develop analogous hydrophobic PILs.

Enzyme-surfactant nanoconstructs soluble and stable in ILs

Alternatively, the surface of enzymes can be modified to render them soluble and stable in anhydrous ILs. This is enabled by adding a surfactant, such as ethylene glycol ethoxylate lauryl ether, to a surface-cationised enzyme.[145] The resulting nanoconstructs combine miscibility with both hydrophobic and hydrophilic, anhydrous ILs with dramatically increased thermal stability, thus significantly broadening the scope of enzymes in ILs. The surfactant corona provides a protective environment with the conformational freedom necessary for effective enzyme activity.

The technique enabled the solubilisation of β-glucosidase in hydrophilic, [bmpyrr][OAc], and hydrophobic, [bmpyrr][($CF_3SO_2)_2N$] ILs with increased thermal stability up to 137 °C and an activity 30 times greater than that observed in aqueous media.[146] The glucosidase nanoconstructs catalysed the hydrolysis of cellulose in [emim][$EtSO_4$] at temperatures up to 110^{0}C.

Biocatalysis in ionic liquids

Biocatalysis in ILs can involve either whole microbial cells or isolated enzymes as the catalysts, both of which have their advantages and limitations.

Whole cell biocatalysis in ILs

Whole cell biocatalysis, employing either naturally occurring or genetically engineered microorganisms, is used on an industrial scale for the production of a broad spectrum of chemicals. It has the advantage that it avoids costly isolation and purification of the enzyme(s) involved and expensive cofactors, e.g. NAD(P) and NAD(P)H with oxidoreductases, are regenerated *in situ.* These days it is quite commonplace to express multiple heterologous enzymes in an engineered microbial host. This means that complex multi-enzyme cascade processes can, in principle, be performed *in vivo* in an IL.

Whole-cell biocatalysis in ILs has been less studied compared with isolated enzymes and was mainly limited to

aqueous biphasic catalysis using hydrophobic ILs[147,148] as an alternative for volatile organic solvents such as toluene. The biocatalytic reaction takes place in the water phase and the IL phase acts as a reservoir for the substrate and product. It has the advantage that substrate and product inhibition are minimised. The IL phase can generally be recycled and reused thus rendering the use of an IL economically viable.

The microbial cells provide a natural environment for the enzymes thus preventing denaturation and conformational changes in the protein structure that may lead to loss of activity in nonconventional reaction media such as ILs. In most of the early studies 2nd generation hydrophobic ILs containing perfluorinated anions such as [bmim][PF_6] and [bmim][$(CF_3SO_2)_2N$] were generally used for proof of concept. Unfortunately, they are not viable for large scale applications owing to their high cost combined with ecotoxicity and lack of biodegradability. Moreover, the toxicity of 2nd generation ILs towards the microbial cells used as catalyst can be an issue.[149] In a recent comprehensive review, Egerova and Ananikov [150] concluded that more detailed studies on the biological activity and toxic effects of ILs towards microorganisms are needed to stimulate the application of whole-cell biocatalysis in ILs. Another disadvantage is the high viscosity of most ILs compared with common organic solvents and there is a definite need for designing less viscous ILs for use in aqueous biphasic systems.

Most studies of whole cells in ILs were concerned with the enantioselective reduction of prochiral ketones catalysed by microbial whole cells containing ketoreductases (KREDs). For example, in 2001 Howarth and coworkers reported the enantioselective reduction of a range of prochiral, aliphatic and cyclic ketones, in [bmim][PF_6]/water mixtures containing added methanol as an energy source, catalysed by *S. cerevisiae* (baker's yeast) cells immobilised in calcium alginate gel.[151] In some cases higher enantioselectivities were obtained than those observed in organic solvents.

Weuster-Botz and co-workers[152] investigated the enantioselective reduction of 4-chloroacetophenone to (R)-1-(4-chlorophenyl)ethanol with *Lactobacillus kefir* cells in [bmim][$CF_3SO_2]_2N$] /water in 93% yield and 98.6% ee. The same group subsequently investigated 21 ILs, based on seven different cations and three perrfluorinated anions, in the biphasic enantioselective reduction of 2-octanone and 4-choroacetophenone, catalysed by recombinant *E. coli* cells overexpressing a *Lactobacillus brevis* KRED and a *Candida boidinii* formate dehydrogenase[153] Excellent yields and enantioselectivities were obtained (Figure 12) but the use of ILs consisting of perfluorinated anions has questionable large scale viability.

Figure 12. Biphasic microbial reduction of ketones in ILs

More recent studies have been devoted to the use of more biocompatible ILs with low toxicity towards microbial cells. For example, Zong and coworkers[154] reported that ILs based on cholinium cations and/or amino acid anions combine low toxicity with good biodegradability and, hence, are "promising candidates for use as environmentally friendly solvents in large-scale applications." However, such ILs are generally hydrophilic and are, therefore, not applicable to aqueous biphasic systems. Recycling of a hydrophobic IL in aqueous biphasic systems is a relatively simple matter[155] but recycling of a hydrophilic IL is more of a challenge.

Nevertheless, Du and co-workers[156] successfully developed the enantioselective reduction of ethyl-4-chloro-3-oxobutanoate to the corresponding *S*-alcohol, mediated by recombinant *E.coli* cells in water containing 2.5% 2-hydroxyethyltrimethylammonium fluoroborate, [$HOCH_2CH_2N(CH_3)_3$][BF_4], and 20 vol% isopropanol for cofactor regeneration (Figure 13a). The chiral alcohol product, a key intermediate in the synthesis of statin drugs such as atorvastatin, was produced on a 5L scale, with 500g/L substrate, in 97% yield and >99% ee. Similarly, the same system was used to produce (*R*)-3,5-bis(trifluoromethyl)-1-phenylethanol, a key intermediate in the synthesis of the drug, aprepitant, by enantioselective reduction of 3,5-bis(trifluoromethyl)acetophenone on a 250g/L scale (Figure 9). Recycling of the IL was not mentioned.

Microbial reduction of ketones can also be conducted in DESs. For example, good results were obtained in the synthesis of the key atorvastatin intermediate by performing the microbial ketone reduction in a mixture of water and the hydrophilic DES, ChCl/Gly (see Figure 13b)[157]

Figure 13. Monophasic microbial ketone reductions in (a) IL-H_2O; (b) DES-H_2O

We expect that the further development of new IL-tolerant microorganisms through metagenome mining and/or genetic engineering of existing strains will stimulate a broader application of whole-cell biocatalysis in ILs. Hydrophilic ILs are also eminently suitable for use in one-pot aqueous pre-treatment, saccharification and fermentation of lignocellulosic biomass to produce biofuels and commodity chemicals[158] (see later).

Biocatalysis with isolated enzymes

Biocatalysis with isolated enzymes in ILs has been widely investigated in the last two decades. In common with whole cell biocatalysis the reactions can be conducted in mono- or bi-phasic systems.[159]

Hydrolases

More than half of the published papers concern lipases[160] but this is rapidly changing as more and more attention is focused on the processing of biomass in ILs (see later). In particular, lipase catalysed transesterifications in ILs have been extensively studied in connection with the transesterification of triglycerides and carbohydrates for the production of biodiesel and biosurfactants, respectively (see later). They have also been successfully used in organic synthesis. An early example of this is the dynamic kinetic resolution (DKR) of chiral benzylic alcohols, using a lipase in combination with a Ru catalyst, in

[bmim][PF_6] reported by Kim and co-workers in 2004 (Figure14).[161]

Figure 14. Lipase catalysed DKR of benzylic alcohols in an IL

Similarly, proteases and esterases have been widely used for transesterifications in ILs.[162] However, almost all of these studies were conducted in 2nd generation, dialkyl imidazolium salts with perfluorinated or halide anions. It is also worth pointing out that the use of ILs as solvents for these reactions has to compete with green organic solvents which these days are often derived from renewable biomass.

Glycosidases have also been widely studied because of their importance in carbohydrate conversions and, in particular, biomass conversion (see later).

Oxidoreductases

Enantioselective reduction of ketones to chiral secondary alcohols, catalysed by KREDs, can be performed in ILs.[163] Kroutil and co-workers, for example, reported the enantioselective reduction of various prochiral ketones catalysed by a KRED from *Rhodococcus ruber* in both mono- and bi-phasic systems.[164] In particular, excellent results were obtained in a monophasic system using hydroxyl functionalised ILs (see Figure 15 for examples).

Figure 15. Enantioselective reduction of ketones in a mono-phasic system with biocompatible IL.

Lignin degrading enzymes, exemplified by various lignin peroxidases and particularly laccases, have been extensively studied[165] in ILs because of their importance in lignocellulose valorisation (see Bioconversions of Polysaccharides). Other enzymes successfully applied in ILs include hydroxynitrile lyases (HNIs)[166]

Biocatalysis and the bio-based economy: the defossilisation of chemicals manufacture.

The decarbonisation of the energy sector and defossilisation of chemicals manufacture are crucial factors underpinning the ongoing global transition to a circular green and sustainable economy. It encompasses the valorisation of waste polysaccharides[167] to afford simple carbohydrates, such as glucose, as the basic feedstocks of biorefineries to replace the lower olefin and aromatic feedstocks of the petrochemical industry. It constitutes an opportunity for the widespread application of biocatalysis in the conversion of bio-renewables - carbohydrates, triglycerides and proteins - to biofuels and platform chemicals.[168,169]

Biocatalytic conversions of highly polar substrates.

For highly polar substrates, including carbohydrates and nucleosides, water is the solvent of choice for enzymatic conversions but conducting certain reactions, e.g. (trans)esterifications and amidations, in aqueous media is problematical because of equilibrium limitations and/or competing product hydrolysis. On the other hand, reactions in common organic solvents are generally not a viable option owing to the low solubilities of such substrates in these media. Polar aprotic solvents such as dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) and ethers, such as tetrahydrofuran (THF), are an option but are generally environmentally unacceptable and/or incompatible with enzymes.

In contrast, ILs can form a viable option. Moreover, their use as the solvent can afford very different chemo-selectivities. For example, CaLB catalyses the acetylation of glucose suspended in THF to afford the 3,6-diacetate in 53 % selectivity at 99% glucose conversion (see Figure 16). In stark contrast, Park and Kazlauskas[170] reported in 2001, that the same reaction in 1-methoxyethyl-3-methylimidazolium tetrafluoroborate, [$MeOCH_2CH_2$mim][BF_4], afforded the desired 6-O-acetyl derivative in 93% selectivity at 99% conversion. This dramatic improvement in chemo-selectivity was attributed to the relative solubilities of substrate and product(s) in the IL vs THF. Glucose is only sparingly soluble in THF but the mono-acetate, is much more soluble and hence undergoes preferential further acetylation to the di-acetate. In contrast, glucose is readily soluble in the IL and the high selectivity to the mono-ester is a truer reflection of the selectivity of the enzyme for mono- vs di-acetylation.

Figure 16. Enzymatic esterification of glucose in ILs

Fatty acid esters of sucrose are commercially important bio-surfactants with numerous applications in food, cosmetics and pharmaceuticals. In addition to being derived from renewable resources, they are tasteless, odourless, non-toxic, non-irritant, and biodegradable. They are currently manufactured by a chemical process at elevated temperatures, resulting in low selectivities and coloured impurities. There is mounting interest,[171] therefore, in enzymatic alternatives that proceed under milder conditions and afford higher selectivities and higher product qualities. Nov 435 catalyses the selective monoacylation of sucrose with ethyl dodecanoate in refluxing tert-butanol but rates are too low for commercial viability.[172]

Solubilities of carbohydrates in ILs are primarily determined by the anion. Those exhibiting strong H-bond acceptor properties, e.g. chloride and acetate, facilitate dissolution by rupturing intermolecular H-bonds. Macfarlane and co-workers[173] showed that ILs comprising the dicyanamide anion (dca), $N(CN)_2^-$, dissolved large amounts of carbohydrates. We showed that [bmim][$N(CN)_2$] dissolved substantial amounts of, e.g. sucrose and lactose, and was a convenient solvent for enzymatic esterification of sucrose.[174] Subsequently, the solubilities of carbohydrates in ILs[175] and their enzymatic esterification in ILs[176,177,178] have been extensively studied.

Bioconversions of polysaccharides: lignocellulose deconstruction in ILs

Deconstruction of lignocellulosic biomass and further conversion of the carbohydrate building blocks into biofuels and commodity chemicals is at the very heart of a sustainable bio-based economy. [179]

The current status of the technology was recently reviewed by Scowan and co-workers.[180] A pretreatment step is needed in order to reduce the recalcitrance of lignocellulosic polysaccharides towards enzymatic hydrolysis. It was shown more than a decade ago that ILs and PILs containing imidazolium cations and anions with hydrogen-bond basicity, such as chloride, acetate, phosphate, phosphonate and alkylsulfate, readily dissolve polysaccharides, including lignocellulosic materials such as wood.[181] This makes them suitable solvents, alone or in mixtures with water,[182] for lignocellulose pretreatment and in the past decade pretreatment with ILs has emerged as a potentially viable method for industrial scale biomass conversion.[183,184,185,186,187] However, these ILs are not biocompatible which presents problems in the subsequent biocatalytic conversion of the lignocellulose to biofuels and platform chemicals.

In order to rectify this shortcoming, Kuroda and co-workers[188] designed biocompatible zwitter-ionic liquids (ZILs) that not only dissolve substantial amounts of lignocellulose (see Figure 17) but are also biocompatible and can enable the bioconversion of the polysaccharides to ethanol analogous to starch based processes. For example, the ZIL, OE_2imC_3C, containing an oligoether side chain, dissolved cellulose, hemicellulose and lignin. Moreover, it exhibited low toxicity towards *E. coli*. Thus, treatment of bagasse for 8h at 120^{o}C followed by addition of acetate buffer and cellulose-catalysed hydrolysis at 50^{o}C and fermentation with *E. coli* at 37^{o}C was used to produce ethanol in a one-pot process. The authors noted that this method could be applied to the production of other advanced biofuels and platform chemicals.

Figure 17. Zwitterionic liquids (ZILs) that dissolve lignocellulose

Depending on the specific IL used, pretreatment can result in complete dissolution of the lignocellulose or selective dissolution of the lignin and hemicellulose.[189,190] In the ionoSolv process,[191] deconstruction of lignocellulose is conducted in low-cost (ca. $ 1-2 / kg) PILs, exemplified by 1-methylimidazolium chloride, [hmim][Cl],[192] $[Et_3NH][HSO_4]$,[193,194,195,196] $[Me_2BuNH][HSO_4]$[197] and $[HOCH_2CH_2NH_3][HSO_4]$[198] at elevated temperatures, affording a cellulose rich pulp. In contrast, when low-cost tetramethylguanidinium (TMG) hydrogen sulfate was used, the biomass completely dissolved and the cellulose was precipitated with ethanol.[199] Subsequent enzymatic hydrolysis in aqueous medium followed by fermentation afforded ethanol. Alternatively, hybrid pretreatments involving a combination of an inexpensive PIL and an environmentally acceptable commodity organic solvent, such as ethanol or glycerol, have been described.[200]

ILs containing carboxylate anions, such as 1-ethyl-3-methyl imidazolium acetate, [emim][OAc],[201] and choline acetate,[202,203] have also been widely used in lignocellulose pretreatment. However, industrial viability depends very much on efficient recycling and reuse of the IL for several consecutive cycles. This has been achieved with, for example, [emim][OAc][204,205,206] following evaporation of any anti-solvents, such as water or ethanol. However. [emim][OAc] is thermally unstable and can decompose through the intermediate formation of an N-heterocyclic carbene (NHC).[178] Recently, trialkyltriazolium salts have been identified as a novel class of cellulose-dissolving ILs. They have improved properties compared with the corresponding dialkylimidazolium salts, e.g. the undesirable formation of N-heterocyclic carbenes (NHCs) by reaction of the cation with acetate anion is significantly suppressed.[207] However, the cost effectiveness of such ILs could be a problem.

Following the pretreatment, the biomass needs to be washed with water to remove the IL as it inactivates the cellulolytic enzymes.[208] A major challenge in IL pretreatment technology is, therefore, reduction of the overall complexity of the process, from lignocellulose feedstock to, for example, ethanol. Streamlining of the three steps in a one-pot, pretreatment and simultaneous saccharification and fermentation (SSF) is hindered by the above mentioned inactivation of the cellulase enzyme cocktail at relatively low IL concentrations, and pH incompatibility of the three steps. In practice this means that extensive water washing and pH adjustment is required following the pretreatment.

The latter problem can be avoided by taking advantage of the self-buffering effect of PILs and the former problem by incorporating a hydroxyl functionality in the cation of the PIL. Thus, Singh and coworkers[209] conducted the pretreatment of lignocellulose followed by SSF of the cellulose and hemicellulose fractions as an integrated one-pot process in ethanolamine acetate, $[HOCH_2CH_2NH_3][OAc\}$, as an inexpensive (estimated 0.7-1.4 $/kg), biocompatible PIL. A high biomass loading was achieved in a water-wash free process with no requirement for pH adjustment or solid/liquid separation.[210]

The biocompatible cholinium lysinate, [Ch][Lys] was successfully used in the delignification of rice and wheat straw.[211] Similarly, good results were obtained in woody biomass processing by conducting the integrated pretreatment, saccharification and fermentation in cholinium lysinate, [Ch][Lys].[212,213] The cholinium cation and the amino acid anion render this IL biocompatible and self-buffering, respectively.[214,215] DESs have also been used in lignocellulose deconstruction[216]

Biocatalytic lignin valorization in ILs.

In current strategies for the valorisation of waste lignocellulose as a renewable feedstock, the lignin is generally separated and used as a source of energy. However, a potentially attractive alternative for cellulosic bio-refineries is to use lignin degrading enzymes (LDEs), such as laccases and lignin peroxidases, to degrade lignin to value added chemicals.[217]

This will require the use of biocompatible and LDE-tolerant ILs, to catalyse the selective deconstruction of the lignin fraction.[152,218] Stevens and co-workers[219] obtained promising results using a thermally stable laccase (T_{opt} > 90^{0}C) from a hyperthermophilic bacterium, *Thermus thermophilis*, in [emim][OAc]. However, significant (50%) loss of activity was observed in the laccase in only 2% aqueous IL and no improvements were observed using protein engineering. This strongly suggests that a more enzyme compatible IL is needed, possibly through introduction of hydroxyl functionalities in the cation and/or anion. Indeed, Galai and coworkers[220] showed that $[Ch][H_2PO_4]$ was very effective in enhancing and stabilising the activity of *T.versicolor* laccase for use in the decolourisation of anthraquinone dyes.

Chitin deconstruction in ILs

Chitin is is a linear polymer of 2-(acetylamino)-2-deoxy-D-glucose (see Figure 18). It is the second most abundant polysaccharide after

cellulose and is a component of exoskeletons of shellfish and insects and cell walls of fungi. Processing of shell food and mushroom waste streams in chitin bio-refineries is a potentially interesting source of commodity chemicals.[221] There is a definite incentive, therefore, to substitute environmentally unattractive chemical processing of chitin waste with more sustainable enzymatic hydrolysis with chitinase.

However, In common with lignocellulose, chitin is a recalcitrant biopolymer that is insoluble in water and common organic solvents. Consequently, a pretreatment step is needed to reduce the crystallinity of the chitin and render it accessible to the chitinase. This can be achieved using ILs such as [emim][OAc][222] that render the chitin accessible for enzymes.

Figure 18. Structure of chitin

Enzymatic conversion of triglycerides: biodiesel production in ILs

The industrial production of biodiesel by transesterification of renewable triglycerides from dedicated crops, such as rapeseed, or waste streams has undergone spectacular growth in the last decade.[223] The EU leads the pack with 34% of the global biodiesel production of 41.2 mio tonnes in 2018.

Industrial production of biodiesel generally involves the transesterification of triglycerides with methanol in the presence of alkaline catalysts, to afford fatty acid methyl esters (FAMEs). However, this process has several disadvantages: relatively high energy consumption and waste formation coupled with problems with recovery of the glycerol by-product in acceptable purity and rigorous waste water treatment required. Furthermore, the chemical process has strict requirements regarding the free fatty acid content of the feedstock since they deactivate the catalyst.

In contrast, lipase-catalysed transesterification proceeds under mild conditions, with low waste production and facile recovery of the biodiesel and glycerol byproduct in high purity.[224] In addition, inexpensive low quality feedstocks, such as waste cooking oil and animal fat, with high free fatty acid and water contents, can be used as the feedstock as the free fatty acids are also converted by the lipase into FAMEs. Nonetheless, the enzyme costs represent a significant portion of the total manufacturing costs and, hence, much effort is devoted to reducing the cost-contribution of the lipase. This can be achieved by appropriate immobilisation of the lipase to enable multiple recycling and continuous processing in flow reactors. It is clearly only a matter of time before the enzyme and processing costs are sufficiently reduced and the enzymatic method becomes the major, or only process for biodiesel production.

The use of ILs as solvents can overcome the inactivation of the lipase catalyst by methanol[225] and increase its stability and activity through an appropriate choice of cation and anion. Most studies of enzymatic biodiesel production in ILs have involved 2nd generation hydrophobic ILs, e.g. the earlier mentioned SLILs consisting of dialkylimidazolium cations containing a long chain alkyl group and PF_6^- or $[CF_3SO_2]_2N^-$ as the anions,[226,227] which are probably not economically viable for large scale production.

More recently, emphasis has switched to the use of more cost-effective, biocompatible and environmentally acceptable ILs. A striking example is the recently reported[228] use of Nov 435, in a biocompatible, hydrophilic IL, $[Ch][H_2PO_4]$ for the production of biodiesel from triolein, sunflower oil and non-edible castor oil, in yields up to 96%. After completion of the reaction two phases were formed: an upper layer of biodiesel and a lower layer of IL which could be readily separated and recycled together with the solid enzyme.

Interestingly, a DES comprising ChCl/glycerol in a 1:2 molar ratio was used as an inexpensive, non-toxic, biodegradable and biocompatible solvent for the Nov 435 catalysed methanolysis of soybean oil.[229] 88% conversion was observed in 24h at 50^0C and the immobilised lipase was recycled four times with little loss of activity. Similarly, Ch/Glycerol was used for the transesterification of refined rapeseed oil or waste cooking oil with ethanol in a two-enzyme-one-pot system combining Thermomyces Lanuginosus lipase (TLL) with CalB.[230]

Downstream processing

As with all solvents, recovery and reuse of the IL is an important part of the downstream processing (DSP) of biocatalytic conversions in ILs. Indeed, for an industrially viable process, a smart and effective integration of the chemical conversion, product separation and recovery and reuse of the biocatalyst and the IL is of paramount importance.[231] The fusion of biocatalysis and continuous flow operation is an important enabling technology in this respect.[127,128,232,233,234] The key to designing smart DSP is to take full advantage of the unique properties of ILs. Since an important motivation for using ILs was to replace volatile organic solvents, product extraction with an organic solvent would, *a priori*, not appear to be an attractive option. However, if it is an environmentally attractive, bio-renewable solvent, such as 2-methylTHF, it would constitute an improvement.

An attractive alternative is to use supercritical carbon dioxide, $scCO_2$, to extract the product from the IL phase. ILs and $scCO_2$ form biphasic systems whereby $scCO_2$ is highly soluble in the IL but the IL is not measurably soluble in the $scCO_2$ phase.[235] This phase behaviour creates the possibility of using IL/$scCO_2$ to integrate a biphasic reaction with product extraction and recovery of the catalytic IL-phase in a single-process. It provided the basis for the development of biphasic biocatalysis in IL/scCO2 mixtures[236] in which the $scCO_2$ acts as a mobile phase for the continuous extraction of products from the IL phase. The product is subsequently isolated by decompression of the $scCO_2$ and the latter is recycled. The enzyme remains dissolved in the IL phase and can be recycled.

This concept was applied, for example, in the highly enantioselective (>99.9% ee) kinetic resolution of 1-phenylethanol with good operational stability using CaLB dissolved in $[bmim][Tf_2N]$ (Figure 19a).[237,238] IL/scCO2 mixtures can be mono- or biphasic depending on the pressure and temperature. Hence, a 'miscibility switch', by adjusting the pressure, can be used to enable reaction in a single homogeneous phase followed by product separation in the $scCO_2$ phase and recycling of the IL phase to the reactor.[239]

ILs/$scCO_2$ mixtures can also be used with immobilised enzymes in continuous operation, for example in the chemo-enzymatic dynamic kinetic resolution of 1-phenylethanol over a combination of Nov 435 and an acidic zeolite (US-Y) catalyst in butyltrimethylammonium bis-triflamide, $[btma][Tf_2N]$ (Figure 19b).[240]

Figure 19. Enzymatic resolution of 1-phenylethanol in IL-$scCO_2$

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In another variation on this theme, the IL is immobilised as a supported ionic liquid phase (SILP) by simple adsorption on a porous solid[241] or covalent attachment to e.g. polymethacrylate resins.[242] The enzyme is subsequently dissolved in the SILP and used in a continuous flow bioreactor. Alternatively, the SILP can be formed by entrapment in an ionogel formed by mixing, for example, an IL and PVC.[243]

Conclusions & Prospects

Biocatalysis in ILs, both with isolated enzymes and whole microbial cells, has evolved over the last two decades from conception to a flourishing area of research with broad scope and potential for industrial application. As mentioned earlier, an important driver in the latter is the imminent switch from hydrocarbons to carbohydrates and triglycerides as the base chemicals. ILs are ideal media for conducting biocatalytic processes with such substrates. Indeed, the ILs may be derived from renewable biomass.[244,245]

By a suitable choice of cation and anion the IL can be designed to be hydrophobic or hydrophilic, for use in mono- or bi-phasic conditions with water. Polar hydrophobic ILs, analogous to chlorinated hydrocarbons are also possible. The IL can be designed for biocompatibility with the dissolved or immobilised enzyme using inexpensive, hydroxyl-functionalised cations. There still remains a need for the development of ILs with improved tolerance for living microbial cells.

Unfortunately, most studies have been performed with ILs based on perfluorinated anions that are expensive, and environmentally less acceptable. Such studies need to be up-dated using e.g. dialkylphosphate[63,246] and phosphonate[247] anions. As mentioned earlier, Ils comprising such anions presumably have more biocompatibility with whole microbial cells and isolated enzymes considering the high concentrations of phosphate present in microbial cells. Studies of biocatalysis in ILs were generally performed with quaternary ILs. However, we suggest that, in most cases, the equivalent PILs would probably have worked at least as well but would be much more cost-effective.

Finally, many envisaged applications of ILs will be in the production of commodity chemicals and biofuels from carbohydrates and triglycerides, possibly in biocatalytic cascade processes. For commercial viability such processes will involve continuous, in-flow operation using immobilised enzymes. Initial studies have demonstrated the feasibility of such processes. In conclusion, we expect that biocatalysis in ILs has a bright future.

"Science is magic that works" Kurt Vonnegut.

Conflicts of interest

"There are no conflicts to declare".

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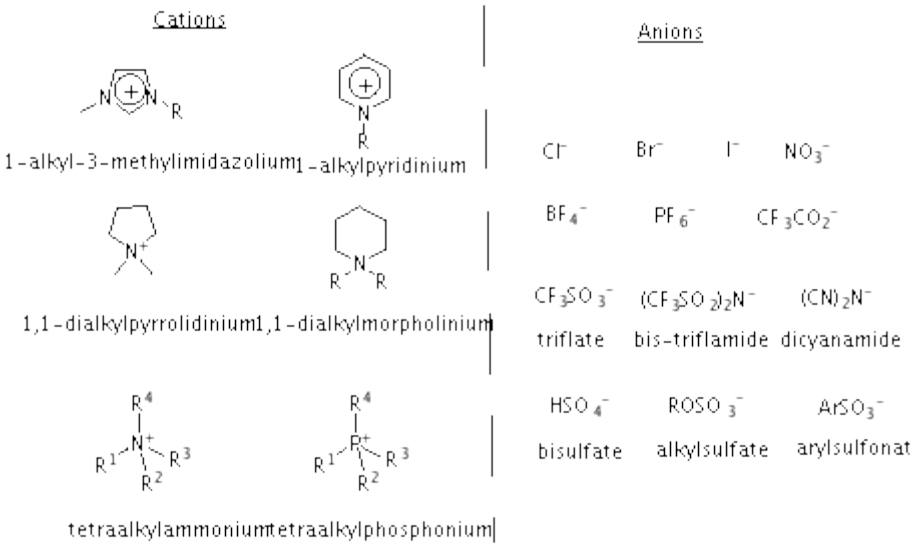


Figure 1. Structures of 2nd generation ILs

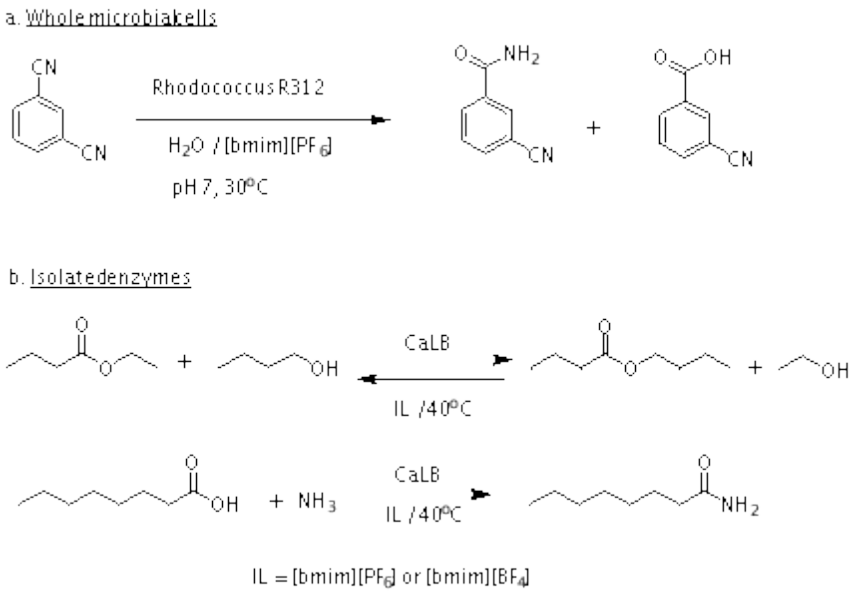


Figure 2. Early examples of biocatalysis in hydrophobic ILs.

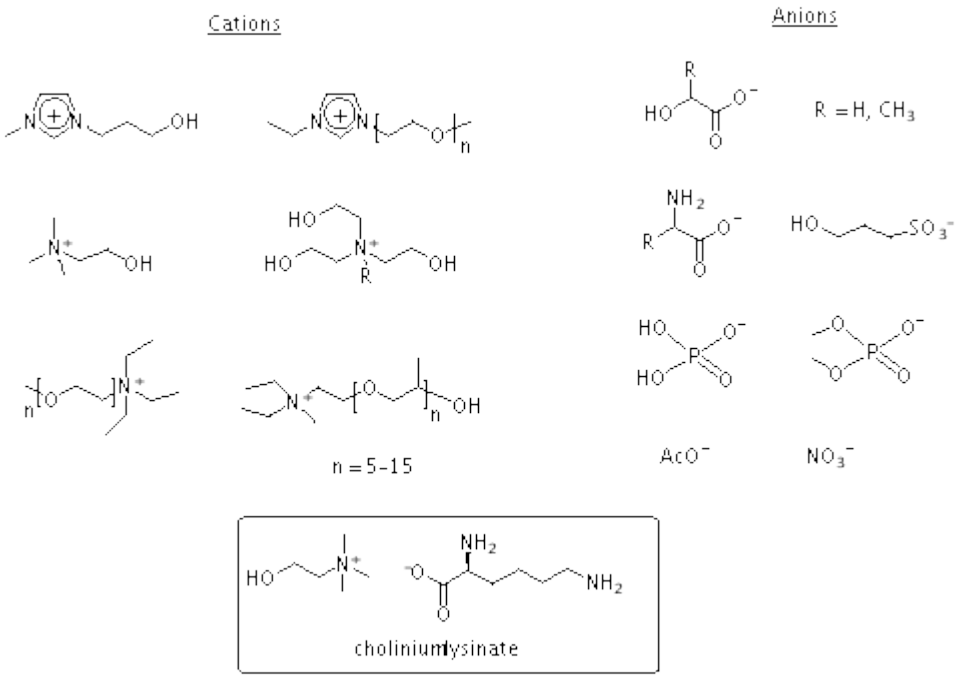


Figure 3. Structures of 3rd generation ILs

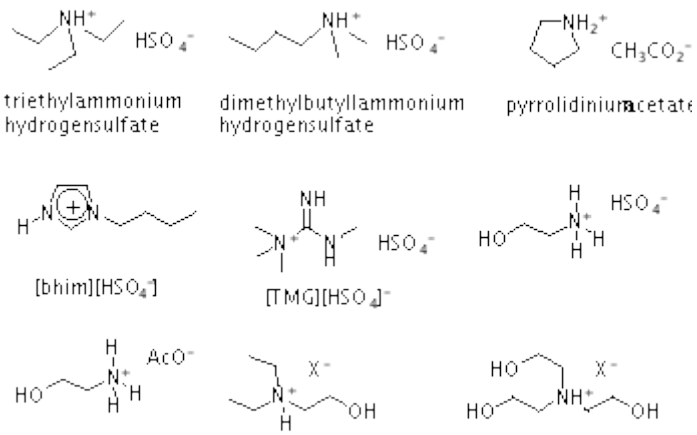


Figure 4. Structures of PILs

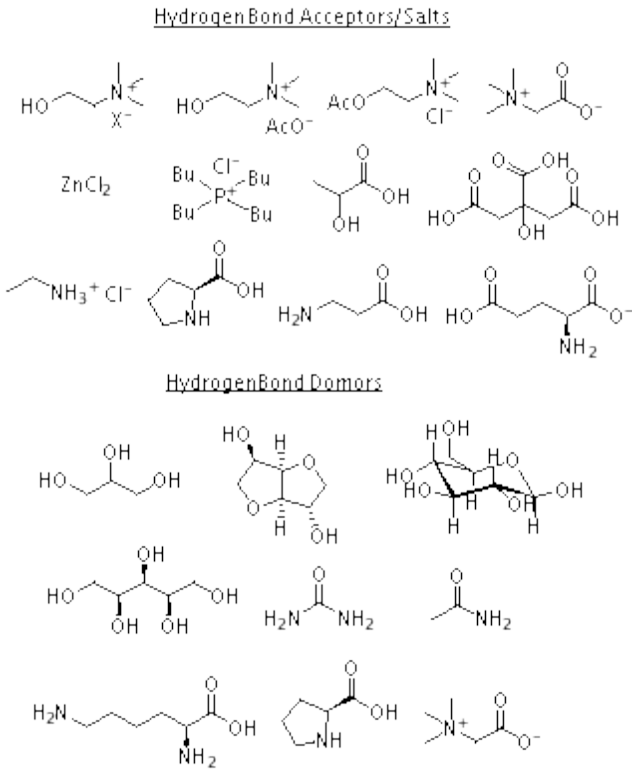


Figure 5. Examples of DESs

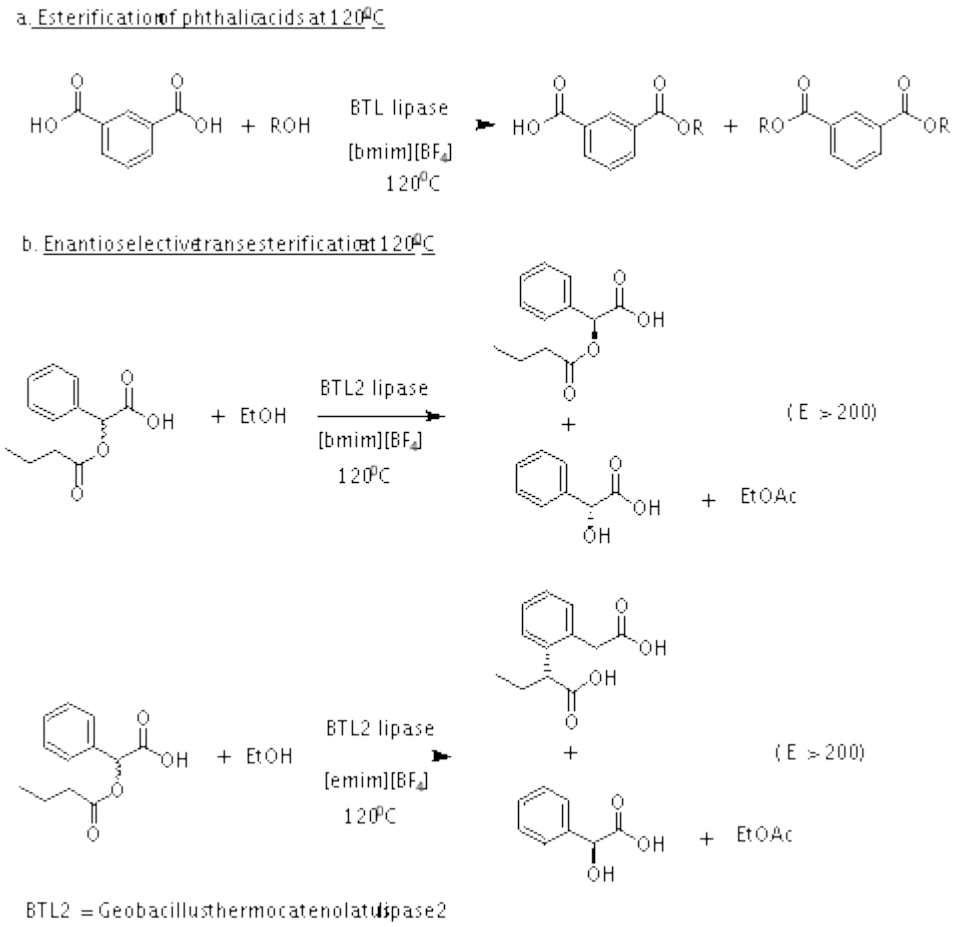


Figure 6. Lipase catalysed (trans)esterifications in ILs at 120°C

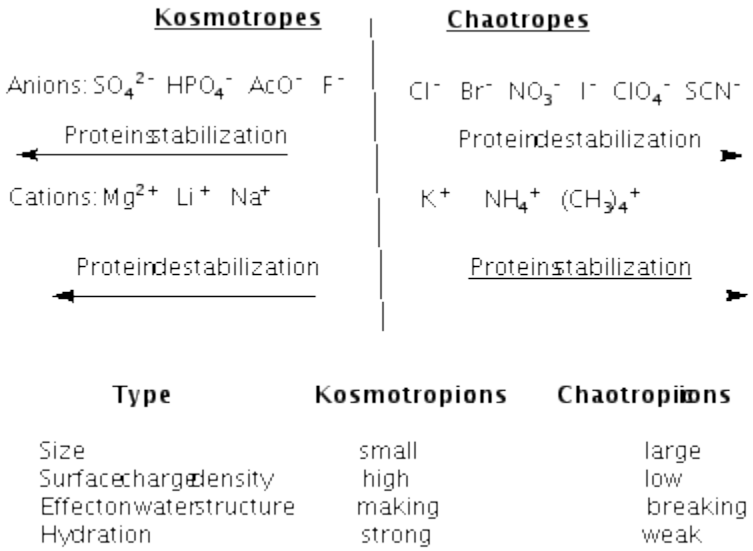


Figure 7. Hofmeister series

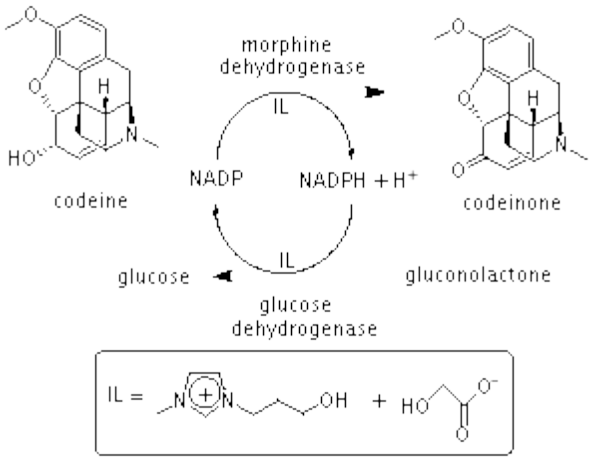


Figure 8 . Enzymatic oxidation of codeine in an IL .

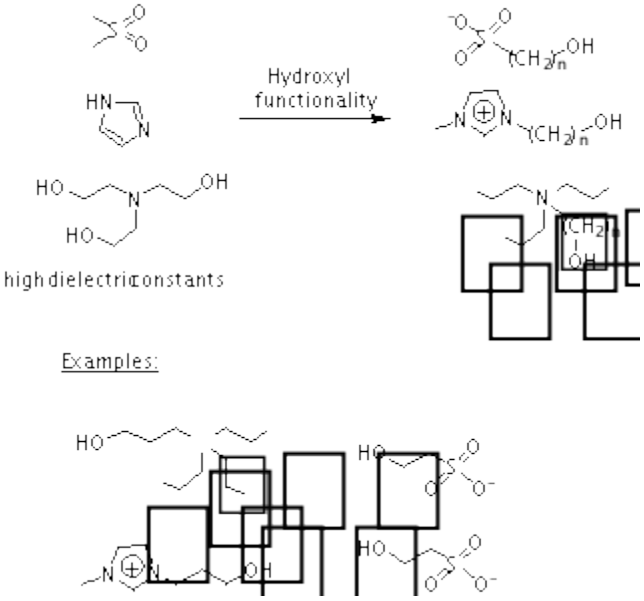


Figure 9. Structures of designer biocompatible liquids

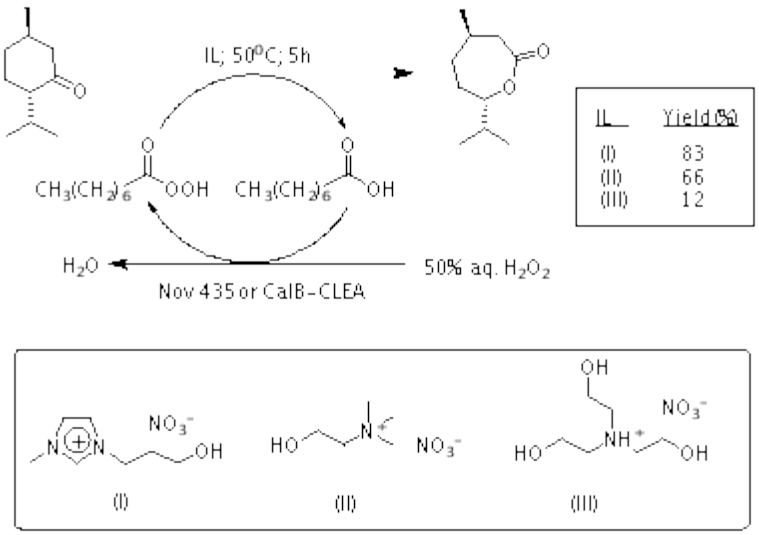


Figure 10. Chemoenzymatic Baeyer-Villiger oxidation in ILs

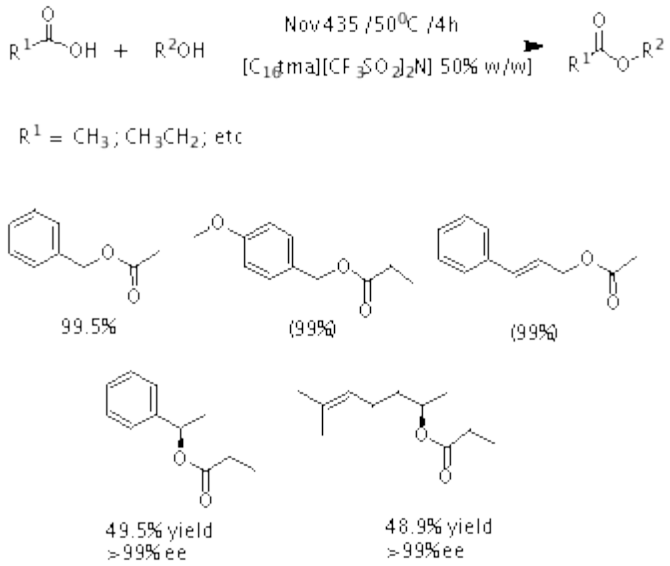


Figure 11. Flavour ester synthesis in sponge-like ILs

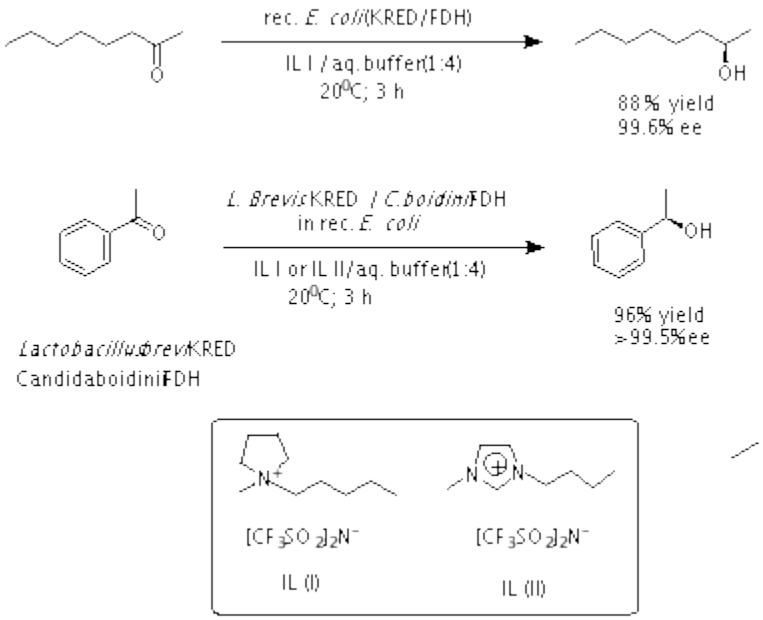


Figure 12. Biphasic microbial reduction of ketones in ILs

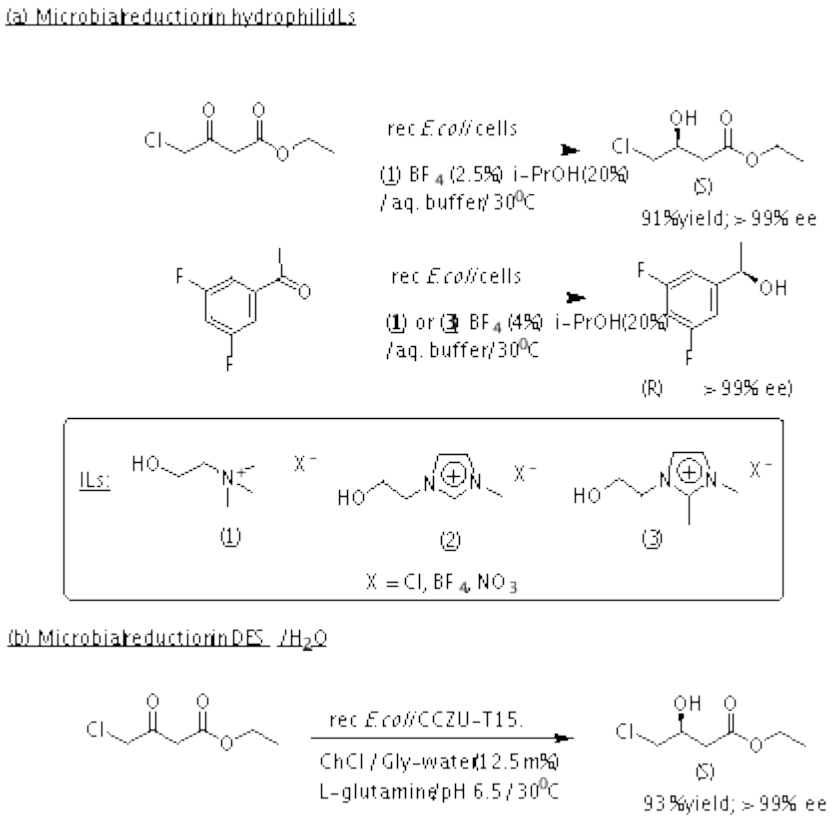
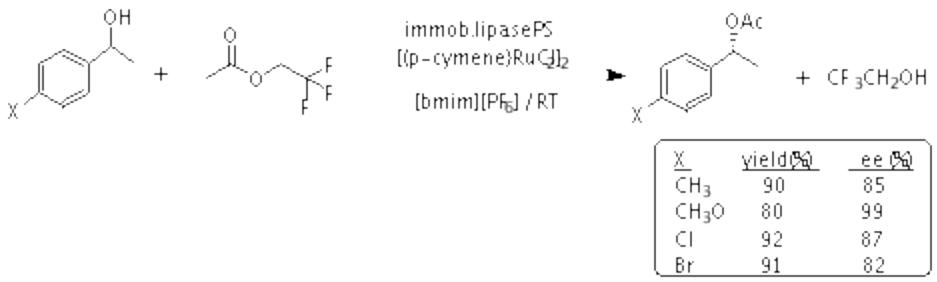


Figure 13. Monophasic microbial ketone reduction



X	yield(%)	ee (%)
CH_3	90	85
CH_3O	80	99
Cl	92	87
Br	91	82

Figure 14. Lipase catalysed DKR of 1-phenylethanol in an IL

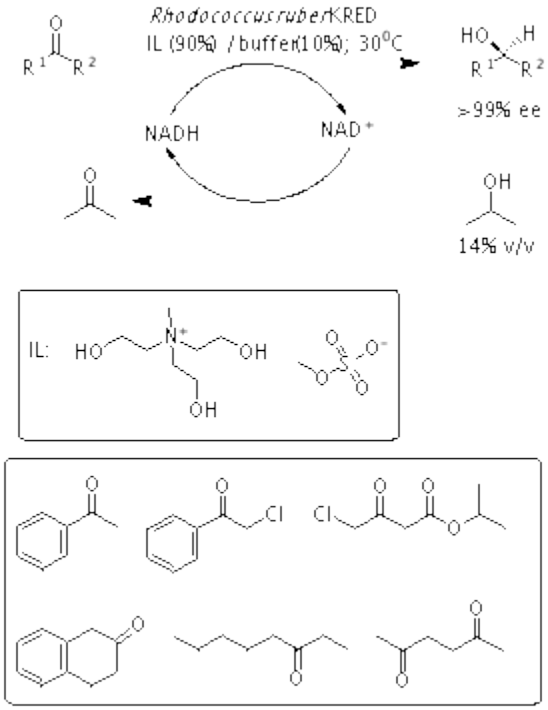
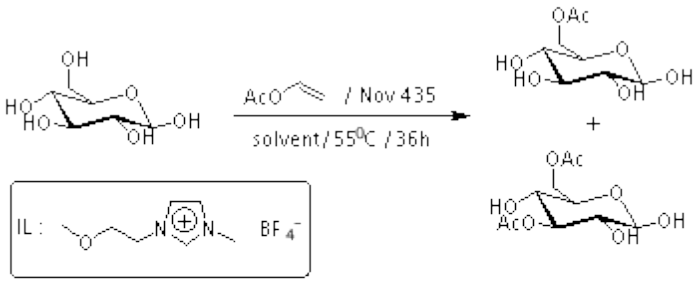


Figure 15. Enantioselective reduction of ketones in a monophasic system with biocompatible IL.



Solvent	Conv. (%)	Sel. (%)	
		mono-	di-
THF	99	53	47
IL	99	93	7

Figure 16. Enzymatic esterification of glucose in ILs

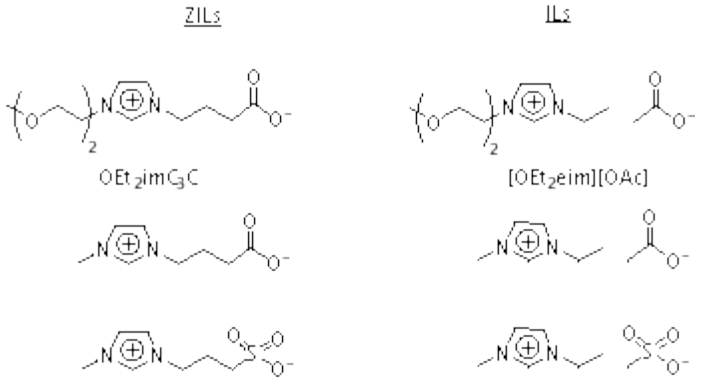
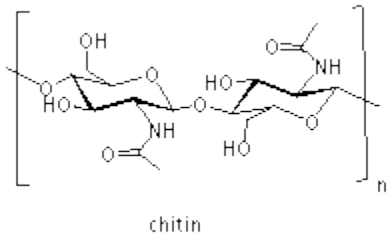


Figure 17. Zwitterionic liquids (ZILs) that dissolve lignocellulose



chitin

Figure 18. Structure of chitin

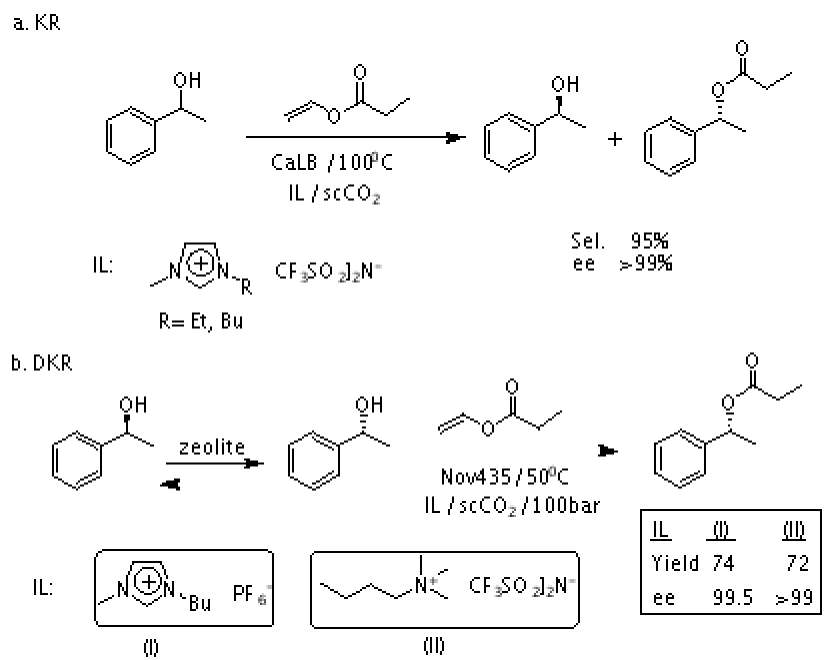


Figure 19. Enzymatic resolution of 1-phenylethanol in IL-scCO_2