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Ionic liquid tolerant microorganisms and microbial communities for lignocellulose conversion to bioproducts

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Abstract

Chemical and physical pretreatment of biomass is a critical step in the conversion of lignocellulose to biofuels and bioproducts. Ionic liquid (IL) pretreatment has attracted significant attention due to the unique ability of certain ILs to solubilize some or all components of the plant cell wall. However, these ILs not only inhibit enzyme activities, but also the growth and productivity of microorganisms used in downstream hydrolysis and fermentation processes. While pretreated biomass can be washed to remove residual IL and reduce inhibition, extensive washing is costly and not feasible in large-scale processes. IL tolerant microorganisms and microbial communities have been discovered from environmental samples and studies begun to elucidate mechanisms of IL tolerance. The discovery of IL tolerance in environmental microbial communities and individual microbes has lead to the proposal of molecular mechanisms of resistance. In this article, we review recent progress on discovering IL tolerant microorganisms, identifying metabolic pathways and mechanisms of tolerance, and engineering microorganisms for IL tolerance. Research in these areas will yield new approaches to overcome inhibition in lignocellulosic biomass bioconversion processes and increase opportunities for the use of ILs in biomass pretreatment.

Keywords:

Ionic liquid tolerance, microbial community enrichment, ionic liquid pretreatment, engineering ionic liquid tolerance

Introduction

Lignocellulosic biomass, such as agricultural residues and dedicated energy crops, have the potential to provide low cost and renewable feedstocks for biofuels and bioproducts (Gallezot 2008; Gomez et al. 2008). Lignocellulosic feedstocks are primarily composed of complex matrices of polysaccharides (cellulose and hemicellulose) and lignin

which form plant cell walls (Buchanan et al. 2015). These durable cell walls are recalcitrant to biomass pretreatment processes due to the crystallinity of the cellulose (protecting cellulose from chemical and biological degradation), and the presence of covalent cross-linkages between lignin and hemicellulose (Blanch and Wilke 1982; Chandra et al. 2007; Li et al. 2010), resulting in a high cost for pretreatment (Chandra et al. 2007; Merino and Cherry 2007; Simmons et al. 2008; Yang and Wyman 2008).

A variety of physical, chemical, and physicochemical pretreatment methods have been tested to overcome the recalcitrance of lignocellulose, improve enzyme efficiency, and increase the yield of sugars as described in several recent reviews (Kim et al. 2016; Silveira et al. 2015; Singh et al. 2015; Zhang et al. 2016). These pretreatments include dilute acid (Lloyd and Wyman 2005; Saha et al. 2005; Schell et al. 2003), hot water (Liu and Wyman 2004; Ruiz et al. 2013), ammonia fiber expansion (Hoover et al. 2014; Lau et al. 2008; Murnen et al. 2007), steam explosion (Grous et al. 1986; Kaar et al. 1998), lime (Chang et al. 1997; Kim and Holtzapple 2005), organic solvent (Zhang et al. 2007; Zhao et al. 2009b), and pyrolysis and mechanical disruption (Mosier et al. 2005). In all these treatments, the substantial degradation of lignin is accompanied by considerable reduction in fermentable sugar content of the feedstock, resulting in a loss of 20-35% of the mass of lignocellulose (Galbe and Zacchi 2007; Lee et al. 2009). In contrast, recent reports give ample evidence that pretreating biomass with ionic liquids can disrupt the interactions between plant cell wall polymers, resulting in significant improvements in enzymatic hydrolysis kinetics while preventing the loss of fermentable sugars and facilitating the fractionation of biomass (Cheng et al. 2011; da Costa Lopes et al. 2013; Li et al. 2010; Li et al. 2009; Mora-Pale et al. 2011; Singh et al. 2009; Zakrzewska et al. 2010; Zavrel et al. 2009).

The properties of ILs are unique, and certain ones appear to be ideally suited for the purpose of biomass pretreatment. As molten organic salts, ILs typically stay in a liquid state below 100°C. Most ILs have excellent chemical stability, high thermal stability up to approximately 300°C, and are almost completely non-volatile and non-flammable (Dadi et al. 2006; Lee et al. 2009; Yang and Pan 2005). ILs have high polarity and usually fall in the range of 0.6-0.7 on the normalized polarity scale (E_T^N), similar to lower alcohols and formamide (van Rantwijk et al. 2003). Therefore, ILs have the ability to dissolve polar and non-polar organic, inorganic, and polymeric compounds in chemical and biochemical processes. Furthermore, the combination of anions and cations that can be used to synthesize ILs is nearly unlimited (Freemantle 1998; Lee and Lee 2005; Yang and Pan 2005).

Used as a pretreatment regime, ILs have been demonstrated to be effective on a variety of biomass sources, such as switchgrass, corn stalk, rice straw, bagasse, sugarcane, pine wood, maple wood, hardwood red oak, and mixtures of softwoods (Fort et al. 2007; Kilpelainen et al. 2007; Lee et al. 2009; Li et al. 2010; Li et al. 2008; Singh et al. 2009; Sun et al. 2009; Tan et al. 2009; Trinh et al. 2015). They are also becoming more cost-effective as compared to other pretreatment options (George et al. 2015). ILs are efficient for biomass dissolution, which is typically reported as a change in crystallinity index (CrI) (Kumar et al. 2009; Segal et al. 1959; Thygesen et al. 2005). For example, the degrees of crystallinity in cellulose reconstituted after being dissolved in 1-allyl-3-methylimidazolium chloride

([C₃C₁Im]Cl) and 1-butyl-3-methylimidazolium chloride ([C₄C₁Im]Cl) were lower than untreated cellulose, resulting in an increased accessibility of the polysaccharide chains to cellulases (Dadi et al. 2007; Dadi et al. 2006; Liu and Chen 2006). A reduction in crystallinity was observed after IL pretreatment of switchgrass, maple wood, yellow pine, and hardwood red oak (Lee et al. 2009; Li et al. 2010; Sun et al. 2009). Moreover, in IL pretreated biomass samples, cellulose can be easily recovered with the addition of anti-solvents, such as water, methanol, ethanol, and acetone, and the enzymatic hydrolysis of the recovered cellulose is much greater than that of untreated cellulose (da Costa Lopes et al. 2013; Dadi et al. 2006; Lee et al. 2009; Li et al. 2009; Singh et al. 2009; Sun et al. 2009; Zhao et al. 2009a).

Although biomass pretreatment using IL can be very effective, the toxicity of low IL levels (0.2 – 5% w/w) to microorganisms used in downstream fermentation processes is a serious problem for obtaining high yields of biofuels and chemicals (Docherty and Kulpa 2005; Pham et al. 2010; Quijano et al. 2010; Romero et al. 2008). ILs have similar structures to detergents, pesticides and antibiotics which attack lipid structure, resulting in an increase in osmotic pressure and disruption of the membrane of microorganisms (Matsumoto et al. 2004; Pernak et al. 2001; Pernak et al. 2003; Pham et al. 2010). Moreover, one study suggested that some ILs are similar to cationic surfactants which induce polar narcosis due to their interfacial properties, causing membrane-bound protein disruption (Bernot et al. 2005). The toxicity of certain ILs can also inhibit enzymatic activity of microorganisms due to accumulation in cell membranes (Matsumoto et al. 2004; Pham et al. 2010; Romero et al. 2008). Some ILs also have been shown to inhibit cell growth and cell viability for various microorganisms (Ganske and Bornscheuer 2006; Jing et al. 2014; Pernak et al. 2003; Pfruender et al. 2006; Sendovski et al. 2010). Many challenges associated with IL toxicity must be overcome before ILs can be utilized at the commercial scale (Egorova and Ananikov 2014). Others have reviewed IL toxicity ranges and mechanisms of toxicity to microorganisms (Pham et al. 2010; Quijano et al. 2010; Santos et al. 2014), as well as the impact of ILs on enzymes relevant to biomass conversion (Portillo and Saadeddin 2015). Here we present processing alternatives to overcome IL toxicity, and review the discovery of IL tolerant microorganisms from environmental samples and their metabolic pathways and mechanisms of tolerance reported in the literature.

Approaches to Manage IL Toxicity

Management of toxicity requires an understanding of how certain ILs interfere with cell function and how microorganisms respond to them. Potential approaches to address toxicity may include: 1) processing the biomass to remove the IL, 2) altering the chemistry of the IL, and 3) modifying the organisms and enzymes used downstream of pretreatment. In bench scale experiments, washing the reconstructed cellulose after the IL pretreatment can remove residual ILs, reducing the inhibition of cellulolytic enzymes and microbes in the subsequent fermentation steps (Datta et al. 2010; Ouellet et al. 2011). However, in large scale processes, extensive washing would be too costly and therefore not feasible (Klein-Marcuschamer et al. 2011).

In general, the toxicity of ILs appears to be directly proportional to the length of the alkyl chain as well as the number of alkyl groups substituted on the cation (Pernak et al. 2003; Romero et al. 2008; Sendovski et al. 2010). Increasing the length of the alkyl group in an IL structure also increases hydrophobicity, causing accumulation of toxicity in bacteria (Pham et al. 2010). However, ionic liquid physical and chemical properties, including polarity, hydrophobicity, viscosity, and solvent miscibility, can be adjusted by altering the cation, anion, and attached substituents (Yang and Pan 2005). These features can be managed to reduce toxicity to microorganisms and enzymes. For instance, ILs containing alkyl groups between 10 and 14 carbon atoms are considered toxic toward bacteria and fungi. Designing ILs with alkyl groups shorter than 10 carbon atoms may avoid microbial toxicity (Pernak et al. 2003). In addition, enzymes are typically inactive in ILs containing the anions NO_3^- , CH_3CO_2^- , CF_3CO_2^- , and CF_3SO_3^- , but active with the anions BF_4^- , PF_6^- , and Tf_2N^- (Kaar et al. 2003; Lozano et al. 2001; Yang and Pan 2005). For example, in a study of 1-ethyl-3-methylimidazolium acetate ($[\text{C}_2\text{C}_1\text{Im}][\text{OAc}]$), interaction of the CH_3CO_2^- ion with hydrogen bonds in proteins was suggested to result in protein denaturation (Kaar et al. 2003). The conclusion from all of these studies is that simple alterations in the chemistry of ILs can greatly reduce toxicity of IL to microorganisms and enzymes used in the downstream conversion steps.

Discovering and Screening for IL Tolerant Microorganisms Relevant to Biomass Conversion

Microbial communities in nature have evolved over 3.5 billion years to survive under a variety of harsh living conditions, and for this reason represent a valuable resource for genetic information and metabolic pathways for tolerance to extreme environments (Armstrong et al. 2015). However, ecosystems are often too complex for direct identification of microorganisms that possess unique tolerance traits. Thus, engineered culture conditions are used to enrich for desirable traits and lead to the discovery of functional microbial consortia or specific microorganisms. For example, starting with microbial communities collected from compost and soil environments, a selection of simplified communities have been found that can deconstruct lignocellulosic biomass in extreme conditions, such as high temperature, high-solid loading, and low moisture (DeAngelis et al. 2010; Jimenez et al. 2014; Reddy et al. 2013; Simmons et al. 2014; Yu et al. 2015). The enrichment process generates less complex lignocellulolytic microbial communities that can facilitate targeted discovery of potential enzymes and microorganisms for biomass deconstruction (Allgaier et al. 2010).

Using similar approaches, several studies have discovered IL-tolerant microorganisms and evaluated their tolerance levels under a variety of growth conditions (Table 1). The major focus was on IL tolerance in aqueous systems, however, to minimize water usage in industrial conversion processes, IL tolerant microorganisms that thrive in high solid, thermophilic environments must be considered. Enrichment processes have been used identify microorganisms from green waste compost that can deconstruct switchgrass in the presence of ILs, and these IL-tolerant microorganisms can potentially be applied in lignocellulosic bioconversion processes (Reddy et al. 2012; Simmons et al. 2014). For example, three genera (*Geobacillus*, *Bacillus*, and *Ureibacillus*) affiliated with the phylum *Firmicutes* were able to decompose switchgrass enriched under high-solids and thermophilic conditions in the presence of 6% $[\text{C}_2\text{C}_1\text{Im}][\text{OAc}]$ (Reddy et al. 2012). Similarly, *Chelatococcus* and *Bacillaceae* were found to

decompose green waste under high-solids and thermophilic conditions in the presence of 75 mM tetrabutylphosphonium chloride and tributylethylphosphonium diethylphosphate, respectively (Pace et al. 2016).

The fungus *Aspergillus fumigatus*, isolated from a switchgrass adapted community, could grow in the presence of 10% [C₂C₁Im][OAc], and the secreted endoglucanase and xylanase enzymes continued to be active even in the presence of 20% [C₂C₁Im][OAc] (Singer et al. 2011). Several bacteria that are naturally resistant to ILs have been reported. For example, *Enterobacter lignolyticus* strain SCF1, a lignocellulolytic bacterium from tropical rain forest soil, was able to grow in 0.5 M 1-ethyl-3-methylimidazolium chloride ([C₂C₁Im]Cl) (Khudyakov et al. 2012). *Lactobacillus delbrueckii* subsp. *Lactic* NRIC 1683 could grow in the presence of 5% imidazolium-based ionic liquids (Matsumoto et al. 2004). Also, the thermophilic bacterium *Bacillus coagulans*, isolated from a switchgrass adapted community, tolerated up to 4% [C₂C₁Im][OAc] (Simmons et al. 2014).

Several fungi isolated from cork and soil samples were reported to have high tolerance towards ionic liquids. For example, four species from the genus *Penicillium* (*brevicompactum*, *glandicola*, *corylophilum*, and *diversum*) were isolated from cork samples and able to grow in the presence of 2.5mM to 1.5M cholinium alkanoate ionic liquids ([NMe₃(CH₂CH₂OH)][C_nH_{2n+1}CO₂] (n = 1-9)) (Petkovic et al. 2010). *Penicillium olsonii*, isolated from cork, grew in the presence of 0.375 M [C₂C₁Im]Cl (Petkovic et al. 2009). Various fungi and bacteria isolated from soils (*Penicillium svalbardense*, *Penicillium roseopurpureum*, *Paecilomyces lilacinus*, *Metarhizium anisopliae*, *Beauveria bassiana*, *Rhodococcus erythropolis*, *Sporosarcina luteola*, *Virgibacillus* sp. jx15, *Bacillus aquimaris*, and *Pseudomonas* spp) were able to grow in the presence of 1M [N₁₁₁(EtOH)]Cl, [C₂C₁Im]Cl, and [C₂C₁Im][C₂SO₄] (Deive et al. 2011). In addition, several yeast species (from the genera *Clavispora*, *Debaryomyces*, *Galactomyces*, *Hyphopichia*, *Kazachstania*, *Meyerozyma*, *Naumovozyma*, *Wickerhamomyces*, *Yarrowia*, and *Zygoascus*) known to degrade plant cell walls were found to be tolerant to 5% [C₂C₁Im][OAc] (Sitepu et al. 2014). Any of these organisms could prove to be a useful in fermentation of IL-pretreated biomass to ethanol and other biofuels.

Some strains of the widely used *Saccharomyces cerevisiae* have been found tolerant to ILs (Docherty and Kulpa 2005; Nakashima et al. 2011; Ouellet et al. 2011), and one *S. cerevisiae* strain can maintain its original viability in the presence of 200 mM 1-ethyl-3-methylimidazolium diethyl phosphate ([C₂C₁Im][DEP]) and [C₂C₁Im]Cl, and in 100 mM [C₂C₁Im][OAc] (Nakashima et al. 2011). The maximum non-toxic concentrations (MNTC), the concentration at which it is possible to observe microbial growth and viability, was reported for various microorganisms in the presence of ILs (Santos et al. 2014). In this study, the bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa*, yeasts *Yarrowia lipolytica*, and filamentous fungi *Aspergillus brasiliensis* and *Rhizopus oryzae* were grown in the presence of imidazolium-based and cholinium-based ionic liquids. In addition, the growth of yeast *Trichosporon fermentans* was found to be stimulated in the presence of 30 mM cholinium lysine and 30 mM cholinium serine but was inhibited in cholinium acetate (Liu et al. 2015).

Microorganisms displaying IL tolerance have been screened using direct plating, growth in liquid culture and phenotypic microarrays (Deive et al. 2011; Khudyakov et al. 2012; Liu et al. 2015; Nakashima et al. 2011; Petkovic et al. 2009; Petkovic et al. 2010; Santos et al. 2014; Simmons et al. 2014; Singer et al. 2011; Sitepu et al. 2014). Phenotypic microarrays provide a method for rapid assessment of the phenotype of an organism under a wide variety of growth conditions such as different substrates, stressors, or nutrients (Bochner et al. 2001; Borglin et al. 2012). A commercially available OmniLog phenotypic microarray (Biolog, Inc. Hayward, CA) can be used to collect kinetic data allowing the discovery of fundamental differences in the respiration behavior of distinct organisms and substrates (Vaas et al. 2012). In general, a colorimetric reagent, Redox Dye A (proprietary tetrazolium-based reagent from Biolog, Inc. Hayward, CA) is added to multi-well plates along with samples and incubated in the Omnilog instrument. Growth in each well is measured in Omnilog (OL) units, which calculate the change in tetrazolium redox dye color intensity attributed to dye reduction during cell respiration (Bochner and Savageau 1977; Khudyakov et al. 2012). A few studies have utilized the OmniLog phenotypic microarray to screen for IL-tolerance (Khudyakov et al. 2012; Ruegg et al. 2014). For instance, in the study by Khudyakov et al., the assay was used in the empty plate mode to measure the growth of *Enterobacter lignolyticus* strain SCF1 in the presence of a range of [C₂C₁Im]Cl concentrations throughout a culture period of 72 h (Khudyakov et al. 2012). The data collected were used to assess the length of the lag phase of growth and magnitude of the specific growth rate in response to increasing IL concentration.

Elucidating Mechanisms of IL Tolerance

While the majority of studies to date have focused on discovering microorganisms that can grow in the presence of ILs, a few studies have attempted to identify potential mechanisms of IL tolerance. In one approach, a combination of phenotypic assays, phospholipid fatty acid analysis, and transcriptome analysis were used to investigate the molecular mechanisms of tolerance to [C₂C₁Im]Cl in *Enterobacter lignolyticus* strain SCF1 (Khudyakov et al. 2012). Their study revealed significant differential expression of 688 genes in response to 375 mM [C₂C₁Im]Cl, including up-regulation of osmoprotectant transporters and other efflux pumps, and down-regulation of certain membrane porins (Khudyakov et al. 2012). In response to osmotic stress and high extracellular solute concentration, microorganisms can accumulate compatible solutes by uptake from the medium (Empadinhas and da Costa 2008) (da Costa et al. 1998). Khudyakov et al. found that a group of transporters belonging to an ATP-binding cassette (ABC) superfamily transporter were highly up-regulated in *E. lignolyticus* cells exposed to [C₂C₁Im]Cl. ABC superfamily transporters can transport various substrates, including ions, amino acids, peptides, sugars, and other hydrophilic molecules across cellular membranes (Davidson et al. 2008). Khudyakov et al. suggested that these ABC transporters in SCF1 may use glutamate, glycine betaine, ectoine, and proline as compatible solutes to reduce adverse osmotic pressure effects of residual ionic liquid influx. Similarly, *Aspergillus nidulans* and *Neurospora crassa* may use betaine and glycerol, respectively, in response to the osmotic stress induced by [C₂C₁Im]Cl and choline (Martins et al. 2013).

Efflux pumps are included in a class of membrane transporters that export toxins from the cell using the proton motive force (Dunlop et al. 2011; Nikaido and Takatsuka 2009; Putman et al. 2000) and have been shown to facilitate microbial resistance to toxic compounds including hydrocarbons (Delcour 2009; Dunlop et al. 2011). Unlike efflux pumps, porins are involved in the influx of compounds across the bacterial outer membrane by passive diffusion. Both efflux pumps and porins contribute to microbial resistance to toxic compounds, such as antibiotics (Davín-Regli et al. 2008; Delcour 2009; Pages et al. 2008). In the study by Khudyakov et al., some of the efflux pumps and porins were up-regulated and down-regulated, respectively, in *E. lignolyticus* cells exposed to $[C_2C_1Im]Cl$, suggesting that the combination of these two mechanisms limits the intracellular IL concentration that is toxic to these cells. Similarly, the genes encoding several efflux pumps belonging to the ATP-binding cassette superfamily and the Major Facilitator Superfamily (MFS) were up-regulated in *Aspergillus nidulans* exposed to both cholinium chloride and 0.7 M $[C_2C_1Im]Cl$ (Alves et al. 2016).

After $[C_2C_1Im]Cl$ treatment, bacterial enterobactin biosynthesis, a variety of Fe^{2+} , Fe^{3+} , heme, porins, and siderophore transporters were down-regulated (Khudyakov et al. 2012). The mechanisms for these changes are not clear, but it was suggested that the toxicity of $[C_2C_1Im]Cl$ releases intracellular iron by disrupting formation or increasing degradation of iron-containing proteins. Another study using *Daphnia magna* demonstrated that, in response to the oxidative stress induced by imidazolium-based ILs ($[C_nC_1Im]Br$, where $n = 4, 6, 8, 10$, or 12), an increase was observed in the activities of antioxidant defense enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase (Pham et al. 2010). This reinforces the notion that IL pretreatment may indirectly induce oxidative damage, for example by causing the release of reactive oxygen species or altering the intracellular balance of Fe^{2+} and Fe^{3+} .

Exposure of *E. lignolyticus* to IL resulted in an up-regulation of metabolic pathways for cyclopropane fatty acid synthesis and increased production of cyclopropane fatty acids in the cell membrane. These small but significant changes in phospholipid content may act as a cellular defense against $[C_2C_1Im]Cl$ toxicity by reducing membrane fluidity and decreasing permeability (Khudyakov et al. 2012). A similar finding was reported for *Aspergillus nidulans* when exposed to alkyltributylphosphonium chlorides ($[P_{4.4.4.n}]Cl$, where $n = 1, 4, 8$ or 12), indicating that genes involved in the synthesis of saturated fatty acids were up-regulated in the presence of these ILs (Hartmann and Pereira 2013).

Engineering IL Tolerance in Bacteria

One approach to overcome the inhibition of microbial growth in the presence of ILs is to genetically modify microorganisms for IL tolerance (Ouellet et al. 2011). Microorganisms have been modified to address other challenges in biofuel production, including biofuel toxicity, which has been summarized in several reviews (Boyarskiy and Tullman-Ereck 2015; Dunlop et al. 2011; Jones et al. 2015; Mukhopadhyay 2015; Nicolaou et al. 2010; Ramos et al. 2002). With respect to engineering IL tolerance, Bokinsky et al. demonstrated that an *E. coli*

strain was able to convert [C₂C₁Im][OAc] treated biomass into advanced biofuels in a consolidated bioprocess at the laboratory scale (Bokinsky et al. 2011).

Unlike constitutive expression systems, which continually express the gene of interest, a dynamic expression system adjusts to the reaction environment (Holtz and Keasling 2010; Ruegg et al. 2014). Such a dynamic system can be developed through a transcriptional regulator that is induced by the efflux pump substrate to increase expression of the pump (Grkovic et al. 2001; Harrison and Dunlop 2012; Ruegg et al. 2014). The study by Khudyakov et al. revealed that [C₂C₁Im]Cl caused the differential expression of several transporter genes, including the highly expressed *mfs1* (*E. lignolyticus* locus tag Entcl_2352), a gene of unknown function predicted to be an MFS membrane transporter (Khudyakov et al. 2012). The gene for MFS1 was independently identified using a screen of *E. lignolyticus* genomic fragments in an *E. coli* host (Ruegg et al. 2014). This *mfs* gene alone was shown to be sufficient for *E. coli* growth in up to 0.4 M [C₂C₁Im]Cl, and so named *eilA*, for *Enterobacter* IL tolerant. With the identification of an adjacent gene, *eilR* (locus tag Entcl_2353), encoding a repressor protein, an autoregulation mechanism of the IL efflux pump was demonstrated (Ruegg et al. 2014). The concept of condition-dependent regulation of IL tolerance genes was later demonstrated (Frederix et al. 2014). Native *E. coli* IL-inducible promoters (*PmarR'*, *PydfO'*, and *Pydfa'*) were evaluated for their ability to provide a dynamic control system for regulating *eilA* gene expression in response to [C₂C₁Im]Cl, potentially providing systems for regulating the expression of heterologous genes in host engineering. An [C₂C₁Im][OAc] tolerant *E. coli* strain was later identified by a point mutation in the transcriptional regulator encoded by *rcdA*. This mutation derepresses a predicted MFS transporter, YbjJ, which appears to encode another efflux pump (Frederix et al. 2016).

A recent study reported the effect of deletion of genes encoding mitochondrial proteins in *Saccharomyces cerevisiae* exposed to [C₂C₁Im]Cl (Dickinson et al. 2016). The deletion of *PTK2* (a putative serine/threonine protein kinase), which is involved in regulation of polyamine cation transport across the plasma membrane (Erez and Kahana 2002), was shown to exhibit higher [C₂C₁Im]Cl tolerance. This study also demonstrated an engineered *Saccharomyces cerevisiae* strain Y133-III with deletion of *PTK2* could convert glucose and xylose to ethanol in the presence of 1% [C₄C₁Im]Cl.

Conclusions and Future Directions

Significant progress has been made in understanding the properties of ILs and their mechanisms of toxicity toward microorganisms. However, to date there are only few studies that have investigated IL tolerant microorganisms found in nature and identified metabolic pathways responsible for IL tolerance. Recently, enrichment cultures have been applied for discovering IL tolerant microorganisms from environmental samples by generating less complex lignocellulolytic microbial communities and facilitating the discovery of potential enzymes and microorganisms for biomass deconstruction (Pace et al. 2016; Reddy et al. 2012; Simmons et al. 2014; Singer et al. 2011). However, further studies on IL tolerant microbial communities using metagenomic and metatranscriptomic analyses are needed to understand how organisms in a community work synergistically to tolerate ILs. Also, to minimize water

and solvent use in industrial biofuel production processes, IL tolerant microorganisms that function in high solid systems must be considered. Moreover, due to the lack of well-mixed conditions in high solid systems, the mechanisms and pathways that microorganisms use to tolerate IL may be different between aqueous and high solid systems (Simmons et al. 2014). Although IL tolerant mechanisms have not been fully explored, a few studies have applied transcriptomic analyses to begin to elucidate the molecular mechanisms of IL tolerance (Khudyakov et al. 2012), and have successfully demonstrated an engineered IL-tolerant microorganism with potential applications in converting IL treated biomass into advanced biofuels (Bokinsky et al. 2011; Dickinson et al. 2016; Frederix et al. 2014; Ruegg et al. 2014). These recent studies demonstrate the potential opportunities for discovering genes from environmental communities that confer IL tolerance, and engineering industrial microorganisms for bioprocesses that include IL pretreatment. A combined approach that includes ILs designed for reduced toxicity and robust IL-tolerant microorganisms will ultimately result in more efficient and economical IL-pretreatment based bioconversion processes.

Compliance with Ethical Standards

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Conflict of Interest: Chaowei Yu, Blake A. Simmons, Steven W. Singer, Michael P. Thelen, and Jean S. VanderGheynst declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References:

- Allgaier M, Reddy A, Park JI, Ivanova N, D'Haeseleer P, Lowry S, Sapra R, Hazen TC, Simmons BA, VanderGheynst JS, Hugenholtz P (2010) Targeted Discovery of Glycoside Hydrolases from a Switchgrass-Adapted Compost Community. *PLoS One* 5(1) doi:10.1371/journal.pone.0008812
- Alves PC, Hartmann DO, Nunez O, Martins I, Gomes TL, Garcia H, Galceran MT, Hampson R, Becker JD, Pereira CS (2016) Transcriptomic and metabolomic profiling of ionic liquid stimuli unveils enhanced secondary metabolism in *Aspergillus nidulans*. *Bmc Genomics* 17 doi:10.1186/s12864-016-2577-6
- Armstrong Z, Mewis K, Strachan C, Hallam SJ (2015) Biocatalysts for biomass deconstruction from environmental genomics. *Curr Opin Chem Biol* 29:18-25 doi:10.1016/j.cbpa.2015.06.032
- Bernot RJ, Brueske MA, Evans-White MA, Lamberti GA (2005) Acute and chronic toxicity of imidazolium-based ionic liquids on *Daphnia magna*. *Environ Toxicol Chem* 24(1):87-92 doi:10.1897/03-635.1
- Blanch HW, Wilke CR (1982) Sugars and chemicals from cellulose. *Rev Chem Eng*:71-119
- Bochner BR, Gadzinski P, Panomitros E (2001) Phenotype MicroArrays for high-throughput phenotypic testing and assay of gene function. *Genome Res* 11(7):1246-1255 doi:10.1101/gr.186501
- Bochner BR, Savageau MA (1977) Generalized indicator plate for genetic, metabolic, and taxonomic students with microorganisms *Appl Environ Microbiol* 33(2):434-444
- Bokinsky G, Peralta-Yahya PP, George A, Holmes BM, Steen EJ, Dietrich J, Lee TS, Tullman-Ercek D, Voigt CA, Simmons BA, Keasling JD (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. *Proc Natl Acad Sci U S A* 108(50):19949-19954 doi:10.1073/pnas.1106958108
- Borglin S, Joyner D, DeAngelis KM, Khudyakov J, D'Haeseleer P, Joachimiak MP, Hazen T (2012) Application of phenotypic microarrays to environmental microbiology. *Curr Opin Biotechnol* 23(1):41-48 doi:10.1016/j.copbio.2011.12.006
- Boyarskiy S, Tullman-Ercek D (2015) Getting pumped: membrane efflux transporters for enhanced biomolecule production. *Curr Opin Chem Biol* 28:15-19 doi:10.1016/j.cbpa.2015.05.019
- Buchanan BB, Gruissem W, Jones RL (eds)(2015) *Biochemistry and Molecular Biology of Plants*. American Society of Plant Biologists and Wiley Blackwell
- Chandra RP, Bura R, Mabee WE, Berlin A, Pan X, Saddler JN (2007) Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? In: Olsson L (ed) *Biofuels*. Advances in Biochemical Engineering-Biotechnology, vol 108, pp 67-93
- Chang VS, Burr B, Holtzapple MT (1997) Lime pretreatment of switchgrass. *Appl Biochem Biotechnol* 63-5:3-19 doi:10.1007/bf02920408
- Cheng G, Varanasi P, Li CL, Liu HB, Menichenko YB, Simmons BA, Kent MS, Singh S (2011) Transition of Cellulose Crystalline Structure and Surface Morphology of Biomass as a Function of Ionic Liquid Pretreatment and Its Relation to Enzymatic Hydrolysis. *Biomacromolecules* 12(4):933-941 doi:10.1021/bm101240z
- da Costa Lopes AM, João KG, Morais ARC, Bogel-Lukasik E, Bogel-Lukasik R (2013) Ionic liquids as a tool for lignocellulosic biomass fractionation. *Sustain Chem Process* 1(1):1 %@ 2043-7129
- da Costa MS, Santos H, Galinski EA (1998) An overview of the role and diversity of compatible solutes in Bacteria and Archaea *Adv Biochem Eng Biotechnol*. vol 61,
- Dadi AP, Schall CA, Varanasi S (2007) Mitigation of cellulose recalcitrance to enzymatic hydrolysis by ionic liquid pretreatment. *Appl Biochem Biotechnol* 137:407-421 doi:10.1007/s12010-007-9068-9
- Dadi AP, Varanasi S, Schall CA (2006) Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. *Biotechnol Bioeng* 95(5):904-910 doi:10.1002/bit.21047
- Datta S, Holmes B, Park JI, Chen ZW, Dibble DC, Hadi M, Blanch HW, Simmons BA, Sapra R (2010) Ionic liquid tolerant hyperthermophilic cellulases for biomass pretreatment and hydrolysis. *Green Chem* 12(2):338-345 doi:10.1039/b916564a

- Davidson AL, Dassa E, Orelle C, Chen J (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* 72(2):317-364 doi:10.1128/mmbr.00031-07
- Davin-Regli A, Bolla JM, James CE, Lavigne JP, Chevalier J, Garnotel E, Molitor A, Pages JM (2008) Membrane Permeability and Regulation of Drug "Influx and Efflux" in Enterobacterial Pathogens. *Curr Drug Targets* 9(9):750-759 doi:10.2174/138945008785747824
- DeAngelis KM, Gladden JM, Allgaier M, D'Haeseleer P, Fortney JL, Reddy A, Hugenholtz P, Singer SW, Vander Gheynst JS, Silver WL, Simmons BA, Hazen TC (2010) Strategies for Enhancing the Effectiveness of Metagenomic-based Enzyme Discovery in Lignocellulolytic Microbial Communities. *Bioenergy Res* 3(2):146-158 doi:10.1007/s12155-010-9089-z
- Deive FJ, Rodriguez A, Varela A, Rodrigues C, Leitao MC, Houbraeken J, Pereiro AB, Longo MA, Sanroman MA, Samson RA, Rebelo LPN, Pereira CS (2011) Impact of ionic liquids on extreme microbial biotypes from soil. *Green Chem* 13(3):687-696 doi:10.1039/c0gc00369g
- Delcour AH (2009) Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta-Proteins and Proteomics* 1794(5):808-816 doi:10.1016/j.bbapap.2008.11.005
- Dickinson Q, Bottoms S, Hinchman L, McIlwain S, Li S, Myers CL, Boone C, Coon JJ, Hebert A, Sato TK, Landick R, Piotrowski JS (2016) Mechanism of imidazolium ionic liquids toxicity in *Saccharomyces cerevisiae* and rational engineering of a tolerant, xylose-fermenting strain. *Microb Cell Fact* 15 doi:10.1186/s12934-016-0417-7
- Docherty KM, Kulpa CF (2005) Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. *Green Chem* 7(4):185-189 doi:10.1039/b419172b
- Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. *Mol Syst Biol* 7 doi:10.1038/msb.2011.21
- Egorova KS, Ananikov VP (2014) Toxicity of Ionic Liquids: Eco(cyto)activity as Complicated, but Unavoidable Parameter for Task-Specific Optimization. *ChemSusChem* 7(2):336-360 doi:10.1002/cssc.201300459
- Empadinhas N, da Costa MS (2008) Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. *Int Microbiol* 11(3):151-161 doi:10.2436/20.1501.01.55
- Erez O, Kahana C (2002) Deletions of SKY1 or PTK2 in the *Saccharomyces cerevisiae* trk1 Delta trk2 Delta mutant cells exert dual effect on ion homeostasis. *Biochem Biophys Res Commun* 295(5):1142-1149 doi:10.1016/s0006-291x(02)00823-9
- Fort DA, Remsing RC, Swatloski RP, Moyna P, Moyna G, Rogers RD (2007) Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-n-butyl-3-methylimidazolium chloride. *Green Chem* 9(1):63-69 doi:10.1039/b607614a
- Frederix M, Hutter K, Leu J, Batth TS, Turner WJ, Ruegg TL, Blanch HW, Simmons BA, Adams PD, Keasling JD, Thelen MP, Dunlop MJ, Petzold CJ, Mukhopadhyay A (2014) Development of a Native *Escherichia coli* Induction System for Ionic Liquid Tolerance. *PLoS One* 9(7) doi:10.1371/journal.pone.0101115
- Frederix M, Mingardon F, Hu M, Sun N, Pray T, Singh S, Simmons BA, Keasling JD, Mukhopadhyay A (2016) Development of an *E. coli* strain for one-pot biofuel production from ionic liquid pretreated cellulose and switchgrass. *Green Chem* doi:10.1039/C6GC00642F
- Freemantle M (1998) Designer solvents - Ionic liquids may boost clean technology development. *Chem Eng News* 76(13):32-37 doi:10.1021/cen-v076n013.p032
- Galbe M, Zacchi G (2007) Pretreatment of lignocellulosic materials for efficient bioethanol production. In: Olsson L (ed) *Biofuels. Advances in Biochemical Engineering-Biotechnology*, vol 108, pp 41-65
- Gallezot P (2008) Catalytic Conversion of Biomass: Challenges and Issues. *Chemsuschem* 1(8-9):734-737 doi:10.1002/cssc.200800091
- Ganske F, Bornscheuer UT (2006) Growth of *Escherichia coli*, *Pichia pastoris* and *Bacillus cereus* in the presence of the ionic liquids BMIM BF₄ and BMIM PF₆ and organic solvents. *Biotechnol Lett* 28(7):465-469 doi:10.1007/s10529-006-0006-7

- George A, Brandt A, Tran K, Zahari SMSNS, Klein-Marcuschamer D, Sun N, Sathitsuksanoh N, Shi J, Stavila V, Parthasarathi R (2015) Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. *Green Chem* 17(3):1728-1734
- Gomez LD, Steele-King CG, McQueen-Mason SJ (2008) Sustainable liquid biofuels from biomass: the writing's on the walls. *New Phytol* 178(3):473-485 doi:10.1111/j.1469-8137.2008.02422.x
- Grkovic S, Brown MH, Skurray RA (2001) Transcription regulation of multidrug efflux pumps in bacteria. *Semin Cell Dev Biol* 12(3):225-237 doi:10.1006/scdb.2000.0248
- Grous WR, Converse AO, Grethlein HE (1986) Effect of steam explosion pretreatment on pore-size and enzymatic-hydrolysis of poplar. *Enzyme Microb Technol* 8(5):274-280 doi:10.1016/0141-0229(86)90021-9
- Harrison ME, Dunlop MJ (2012) Synthetic feedback loop model for increasing microbial biofuel production using a biosensor. *Front Microbiol* 3 doi:10.3389/fmicb.2012.00360
- Hartmann DO, Pereira CS (2013) A molecular analysis of the toxicity of alkyltributylphosphonium chlorides in *Aspergillus nidulans*. *New J Chem* 37(5):1569-1577 doi:10.1039/c3nj00167a
- Holtz WJ, Keasling JD (2010) Engineering Static and Dynamic Control of Synthetic Pathways. *Cell* 140(1):19-23 doi:10.1016/j.cell.2009.12.029
- Hoover AN, Tumuluru JS, Teymouri F, Moore J, Gresham G (2014) Effect of pelleting process variables on physical properties and sugar yields of ammonia fiber expansion pretreated corn stover. *Bioresour Technol* 164:128-135 doi:10.1016/j.biortech.2014.02.005
- Jimenez DJ, Dini-Andreote F, van Elsas JD (2014) Metataxonomic profiling and prediction of functional behaviour of wheat straw degrading microbial consortia. *Biotechnol Biofuels* 7 doi:10.1186/1754-6834-7-92
- Jing CQ, Mu LM, Ren TF, Li BN, Chen SJ, Nan WB (2014) Effect of 1-Octyl-3-methylimidazolium Chloride on Cell Replication and Membrane Permeability of *Escherichia coli* DH5 alpha. *Bull Environ Contam Toxicol* 93(1):60-63 doi:10.1007/s00128-014-1269-7
- Jones CM, Lozada NJH, Pfleger BF (2015) Efflux systems in bacteria and their metabolic engineering applications. *Appl Microbiol Biotechnol* 99(22):9381-9393 doi:10.1007/s00253-015-6963-9
- Kaar JL, Jesionowski AM, Berberich JA, Moulton R, Russell AJ (2003) Impact of ionic liquid physical properties on lipase activity and stability. *J Am Chem Soc* 125(14):4125-4131 doi:10.1021/ja028557x
- Kaar WE, Gutierrez CV, Kinoshita CM (1998) Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. *Biomass Bioenergy* 14(3):277-287 doi:10.1016/s0961-9534(97)10038-1
- Khudyakov JI, D'Haeseleer P, Borglin SE, DeAngelis KM, Woo H, Lindquist EA, Hazen TC, Simmons BA, Thelen MP (2012) Global transcriptome response to ionic liquid by a tropical rain forest soil bacterium, *Enterobacter lignolyticus*. *Proc Natl Acad Sci U S A* 109(32):E2173-E2182 doi:10.1073/pnas.1112750109
- Kilpelainen I, Xie H, King A, Granstrom M, Heikkinen S, Argyropoulos DS (2007) Dissolution of wood in ionic liquids. *J Agric Food Chem* 55(22):9142-9148 doi:10.1021/jf071692e
- Kim JS, Lee YY, Kim TH (2016) A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour Technol* 199:42-48 doi:<http://dx.doi.org/10.1016/j.biortech.2015.08.085>
- Kim S, Holtzaple MT (2005) Lime pretreatment and enzymatic hydrolysis of corn stover. *Bioresour Technol* 96(18):1994-2006 doi:10.1016/j.biortech.2005.01.014
- Klein-Marcuschamer D, Simmons BA, Blanch HW (2011) Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. *Biofuels Bioproducts & Biorefining-Biofpr* 5(5):562-569 doi:10.1002/bbb.303
- Kumar R, Mago G, Balan V, Wyman CE (2009) Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresour Technol* 100(17):3948-3962 doi:10.1016/j.biortech.2009.01.075

- Lau MW, Dale BE, Balan V (2008) Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation. *Biotechnol Bioeng* 99(3):529-539 doi:10.1002/bit.21609
- Lee SH, Doherty TV, Linhardt RJ, Dordick JS (2009) Ionic Liquid-Mediated Selective Extraction of Lignin From Wood Leading to Enhanced Enzymatic Cellulose Hydrolysis. *Biotechnol Bioeng* 102(5):1368-1376 doi:10.1002/bit.22179
- Lee SH, Lee SB (2005) The Hildebrand solubility parameters, cohesive energy densities and internal energies of 1-alkyl-3-methylimidazolium-based room temperature ionic liquids. *Chem Commun*(27):3469-3471 doi:10.1039/b503740a
- Li CL, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S (2010) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification. *Bioresour Technol* 101(13):4900-4906 doi:10.1016/j.biortech.2009.10.066
- Li CZ, Wang Q, Zhao ZK (2008) Acid in ionic liquid: An efficient system for hydrolysis of lignocellulose. *Green Chem* 10(2):177-182 doi:10.1039/b711512a
- Li Q, He YC, Xian M, Jun G, Xu X, Yang JM, Li LZ (2009) Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment. *Bioresour Technol* 100(14):3570-3575 doi:10.1016/j.biortech.2009.02.040
- Liu CG, Wyman CE (2004) Impact of fluid velocity on hot water only pretreatment of corn stover in a flowthrough reactor. *Appl Biochem Biotechnol* 113:977-987
- Liu LP, Hu Y, Wen P, Li N, Zong MH, Ou-Yang BN, Wu H (2015) Evaluating the effects of biocompatible cholinium ionic liquids on microbial lipid production by *Trichosporon fermentans*. *Biotechnol Biofuels* 8 doi:10.1186/s13068-015-0299-7
- Liu LY, Chen HZ (2006) Enzymatic hydrolysis of cellulose materials treated with ionic liquid BMIM Cl. *Chin Sci Bull* 51(20):2432-2436 doi:10.1007/s11434-006-2134-9
- Lloyd TA, Wyman CE (2005) Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour Technol* 96(18):1967-1977 doi:10.1016/j.biortech.2005.01.011
- Lozano P, de Diego T, Guegan JP, Vaultier M, Iborra JL (2001) Stabilization of alpha-chymotrypsin by ionic liquids in transesterification reactions. *Biotechnol Bioeng* 75(5):563-569 doi:10.1002/bit.10089
- Martins I, Hartmann DO, Alves PC, Planchon S, Renaut J, Leitao MC, Rebelo LPN, Pereira CS (2013) Proteomic alterations induced by ionic liquids in *Aspergillus nidulans* and *Neurospora crassa*. *J Proteomics* 94:262-278 doi:10.1016/j.jprot.2013.09.015
- Matsumoto M, Mochiduki K, Kondo K (2004) Toxicity of ionic liquids and organic solvents to lactic acid-producing bacteria. *J Biosci Bioeng* 98(5):344-347 doi:10.1263/jbb.98.344
- Merino ST, Cherry J (2007) Progress and challenges in enzyme development for Biomass utilization. In: Olsson L (ed) *Biofuels. Advances in Biochemical Engineering-Biotechnology*, vol 108, pp 95-120
- Mora-Pale M, Meli L, Doherty TV, Linhardt RJ, Dordick JS (2011) Room Temperature Ionic Liquids as Emerging Solvents for the Pretreatment of Lignocellulosic Biomass. *Biotechnol Bioeng* 108(6):1229-1245 doi:10.1002/bit.23108
- Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol* 96(6):673-686 doi:10.1016/j.biortech.2004.06.025
- Mukhopadhyay A (2015) Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. *Trends Microbiol* 23(8):498-508 doi:10.1016/j.tim.2015.04.008
- Murnen HK, Balan V, Chundawat SPS, Bals B, Sousa LD, Dale BE (2007) Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of *Miscanthus x giganteus* to fermentable sugars. *Biotechnol Prog* 23(4):846-850 doi:10.1021/bp070098m

- Nakashima K, Yamaguchi K, Taniguchi N, Arai S, Yamada R, Katahira S, Ishida N, Takahashi H, Ogino C, Kondo A (2011) Direct bioethanol production from cellulose by the combination of cellulase-displaying yeast and ionic liquid pretreatment. *Green Chem* 13(10):2948-2953 doi:10.1039/c1gc15688h
- Nicolaou SA, Gaida SM, Papoutsakis ET (2010) A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. *Metab Eng* 12(4):307-331 doi:10.1016/j.ymben.2010.03.004
- Nikaido H, Takatsuka Y (2009) Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta-Proteins and Proteomics* 1794(5):769-781 doi:10.1016/j.bbapap.2008.10.004
- Ouellet M, Datta S, Dibble DC, Tamrakar PR, Benke PI, Li CL, Singh S, Sale KL, Adams PD, Keasling JD, Simmons BA, Holmes BM, Mukhopadhyay A (2011) Impact of ionic liquid pretreated plant biomass on *Saccharomyces cerevisiae* growth and biofuel production. *Green Chem* 13(10):2743-2749 doi:10.1039/c1gc15327g
- Pace S, Ceballos SJ, Harrold D, Stannard W, Simmons B, Singer SW, Thelen MP, Vander Gheynst JS (2016) Enrichment of microbial communities tolerant to the ionic liquids tetrabutylphosphonium chloride and tributylethylphosphonium diethylphosphate. *Appl Microbiol Biotechnol*:1-14 doi:10.1007/s00253-016-7525-5
- Pages JM, James CE, Winterhalter M (2008) The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nat Rev Microbiol* 6(12):893-903 doi:10.1038/nrmicro1994
- Pernak J, Rogoza J, Mirska I (2001) Synthesis and antimicrobial activities of new pyridinium and benzimidazolium chlorides. *Eur J Med Chem* 36(4):313-320 doi:10.1016/s0223-5234(01)01226-0
- Pernak J, Sobaszekiewicz K, Mirska I (2003) Anti-microbial activities of ionic liquids. *Green Chem* 5(1):52-56 doi:10.1039/b207543c
- Petkovic M, Ferguson J, Bohn A, Trindade J, Martins I, Carvalho MB, Leitao MC, Rodrigues C, Garcia H, Ferreira R, Seddon KR, Rebelo LPN, Pereira CS (2009) Exploring fungal activity in the presence of ionic liquids. *Green Chem* 11(6):889-894 doi:10.1039/b823225c
- Petkovic M, Ferguson JL, Gunaratne HQN, Ferreira R, Leitao MC, Seddon KR, Rebelo LPN, Pereira CS (2010) Novel biocompatible cholinium-based ionic liquids-toxicity and biodegradability. *Green Chem* 12(4):643-649 doi:10.1039/b922247b
- Pfruender H, Jones R, Weuster-Botz D (2006) Water immiscible ionic liquids as solvents for whole cell biocatalysis. *J Biotechnol* 124(1):182-190 doi:10.1016/j.jbiotec.2005.12.004
- Pham TPT, Cho CW, Yun YS (2010) Environmental fate and toxicity of ionic liquids: A review. *Water Res* 44(2):352-372 doi:10.1016/j.watres.2009.09.030
- Portillo MD, Saadeddin A (2015) Recent trends in ionic liquid (IL) tolerant enzymes and microorganisms for biomass conversion. *Crit Rev Biotechnol* 35(3):294-301 doi:10.3109/07388551.2013.843069
- Putman M, van Veen HW, Konings WN (2000) Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 64(4):672-+ doi:10.1128/mmbr.64.4.672-693.2000
- Quijano G, Couvert A, Amrane A (2010) Ionic liquids: Applications and future trends in bioreactor technology. *Bioresour Technol* 101(23):8923-8930 doi:10.1016/j.biortech.2010.06.161
- Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. *Annu Rev Microbiol* 56:743-768 doi:10.1146/annurev.micro.56.012302.161038
- Reddy AP, Simmons CW, Claypool J, Jabusch L, Burd H, Hadi MZ, Simmons BA, Singer SW, VanderGheynst JS (2012) Thermophilic enrichment of microbial communities in the presence of the ionic liquid 1-ethyl-3-methylimidazolium acetate. *J Appl Microbiol* 113(6):1362-1370 doi:10.1111/jam.12002
- Reddy AP, Simmons CW, D'Haeseleer P, Khudyakov J, Burd H, Hadi M, Simmons BA, Singer SW, Thelen MP, VanderGheynst JS (2013) Discovery of Microorganisms and Enzymes Involved in High-Solids Decomposition of Rice Straw Using Metagenomic Analyses. *PLoS One* 8(10):12 doi:10.1371/journal.pone.0077985

- Romero A, Santos A, Tojo J, Rodriguez A (2008) Toxicity and biodegradability of imidazolium ionic liquids. *J Hazard Mater* 151(1):268-273 doi:10.1016/j.jhazmat.2007.10.079
- Ruegg TL, Kim EM, Simmons BA, Keasling JD, Singer SW, Lee TS, Thelen MP (2014) An auto-inducible mechanism for ionic liquid resistance in microbial biofuel production. *Nat Commun* 5 doi:10.1038/ncomms4490
- Ruiz HA, Rodriguez-Jasso RM, Fernandes BD, Vicente AA, Teixeira JA (2013) Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: A review. *Renewable Sustainable Energy Rev* 21:35-51 doi:10.1016/j.rser.2012.11.069
- Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochem* 40(12):3693-3700 doi:10.1016/j.procbio.2005.04.006
- Santos AG, Ribeiro BD, Alviano DS, Coelho MAZ (2014) Toxicity of ionic liquids toward microorganisms interesting to the food industry. *Rsc Advances* 4(70):37157-37163 doi:10.1039/c4ra05295a
- Schell DJ, Farmer J, Newman M, McMillan JD (2003) Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor - Investigation of yields, kinetics, and enzymatic digestibilities of solids. *Appl Biochem Biotechnol* 105:69-85 doi:10.1385/abab:105:1-3:69
- Segal L, Creely JJ, Martin Jr AE, Conrad CM (1959) An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Text Res J* 29(10):786-794 doi:10.1177/004051755902901003
- Sendovski M, Nir N, Fishman A (2010) Bioproduction of 2-Phenylethanol in a Biphasic Ionic Liquid Aqueous System. *J Agric Food Chem* 58(4):2260-2265 doi:10.1021/jf903879x
- Silveira MHL, Moraes ARC, da Costa Lopes AM, Oleksyszcz DN, Bogel-Lukasik R, Andreus J, Pereira Ramos L (2015) Current Pretreatment Technologies for the Development of Cellulosic Ethanol and Biorefineries. *ChemSusChem* 8(20):3366-3390 doi:10.1002/cssc.201500282
- Simmons BA, Loque D, Blanch HW (2008) Next-generation biomass feedstocks for biofuel production. *Genome Biol* 9(12)
- Simmons CW, Reddy AP, VanderGheynst JS, Simmons BA, Singer SW (2014) *Bacillus coagulans* tolerance to 1-ethyl-3-methylimidazolium-based ionic liquids in aqueous and solid-state thermophilic culture. *Biotechnol Prog* 30(2):311-316 doi:10.1002/btpr.1859
- Singer SW, Reddy AP, Gladden JM, Guo H, Hazen TC, Simmons BA, VanderGheynst JS (2011) Enrichment, isolation and characterization of fungi tolerant to 1-ethyl-3-methylimidazolium acetate. *J Appl Microbiol* 110(4):1023-1031 doi:10.1111/j.1365-2672.2011.04959.x
- Singh J, Suhag M, Dhaka A (2015) Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review. *Carbohydr Polym* 117:624-631 doi:<http://dx.doi.org/10.1016/j.carbpol.2014.10.012>
- Singh S, Simmons BA, Vogel KP (2009) Visualization of Biomass Solubilization and Cellulose Regeneration During Ionic Liquid Pretreatment of Switchgrass. *Biotechnol Bioeng* 104(1):68-75 doi:10.1002/bit.22386
- Sitepu IR, Shi S, Simmons BA, Singer SW, Boundy-Mills K, Simmons CW (2014) Yeast tolerance to the ionic liquid 1-ethyl-3-methylimidazolium acetate. *FEMS Yeast Res* 14(8):1286-1294 doi:10.1111/1567-1364.12224
- Sun N, Rahman M, Qin Y, Maxim ML, Rodriguez H, Rogers RD (2009) Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate. *Green Chem* 11(5):646-655 doi:10.1039/b822702k
- Tan SSY, MacFarlane DR, Upfal J, Edye LA, Doherty WOS, Patti AF, Pringle JM, Scott JL (2009) Extraction of lignin from lignocellulose at atmospheric pressure using alkylbenzenesulfonate ionic liquid. *Green Chem* 11(3):339-345 doi:10.1039/b815310h
- Thygesen A, Oddershede J, Lilholt H, Thomsen AB, Stahl K (2005) On the determination of crystallinity and cellulose content in plant fibres. *Cellulose* 12(6):563-576 doi:10.1007/s10570-005-9001-8

- Trinh LTP, Lee YJ, Lee J-W, Lee H-J (2015) Characterization of ionic liquid pretreatment and the bioconversion of pretreated mixed softwood biomass. *Biomass Bioenergy* 81:1-8
doi:<http://dx.doi.org/10.1016/j.biombioe.2015.05.005>
- Vaas LAI, Sikorski J, Michael V, Goker M, Klenk HP (2012) Visualization and Curve-Parameter Estimation Strategies for Efficient Exploration of Phenotype Microarray Kinetics. *PLoS One* 7(4)
doi:10.1371/journal.pone.0034846
- van Rantwijk F, Lau RM, Sheldon RA (2003) Biocatalytic transformations in ionic liquids. *Trends Biotechnol* 21(3):131-138 doi:10.1016/s0167-7799(03)00008-8
- Yang B, Wyman CE (2008) Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels Bioproducts & Biorefining-Biofpr* 2(1):26-40 doi:10.1002/bbb.49
- Yang Z, Pan WB (2005) Ionic liquids: Green solvents for nonaqueous biocatalysis. *Enzyme Microb Technol* 37(1):19-28 doi:10.1016/j.enzmictec.2005.02.014
- Yu CW, Reddy AP, Simmons CW, Simmons BA, Singer SW, VanderGheynst JS (2015) Preservation of microbial communities enriched on lignocellulose under thermophilic and high-solid conditions. *Biotechnol Biofuels* 8 doi:10.1186/s13068-015-0392-y
- Zakrzewska ME, Bogel-Lukasik E, Bogel-Lukasik R (2010) Solubility of Carbohydrates in Ionic Liquids. *Energy Fuels* 24:737-745 doi:10.1021/ef901215m
- Zavrel M, Bross D, Funke M, Buchs J, Spiess AC (2009) High-throughput screening for ionic liquids dissolving (ligno-)cellulose. *Bioresour Technol* 100(9):2580-2587
doi:10.1016/j.biortech.2008.11.052
- Zhang K, Pei Z, Wang D (2016) Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: A review. *Bioresour Technol* 199:21-33
doi:<http://dx.doi.org/10.1016/j.biortech.2015.08.102>
- Zhang YHP, Ding SY, Mielenz JR, Cui JB, Elander RT, Laser M, Himmel ME, McMillan JR, Lynd LR (2007) Fractionating recalcitrant lignocellulose at modest reaction conditions. *Biotechnol Bioeng* 97(2):214-223 doi:10.1002/bit.21386
- Zhao H, Jones CIL, Baker GA, Xia S, Olubajo O, Person VN (2009a) Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. *J Biotechnol* 139(1):47-54
doi:10.1016/j.jbiotec.2008.08.009
- Zhao XB, Cheng KK, Liu DH (2009b) Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. *Appl Microbiol Biotechnol* 82(5):815-827 doi:10.1007/s00253-009-1883-1