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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

([C$_3$C$_1$Im]Cl) and 1-butyl-3-methylimidazolium chloride ([C$_4$C$_1$Im]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 ($[C_2C_1Im][OAc]$), 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% $[C_2C_1Im][OAc]$ (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% $[C_2C_1Im][OAc]$, and the secreted endoglucanase and xylanase enzymes continued to be active even in the presence of 20% $[C_2C_1Im][OAc]$ (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 ($[C_2C_1Im]Cl$) (Khudyakov et al. 2012). *Lactobacillus delbruekii* 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% $[C_2C_1Im][OAc]$ (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 ($[NMe_3(CH_2CH_2OH)][C_nH_{2n+1}CO_2]$ (n = 1-9)) (Petkovic et al. 2010). *Penicillium olsonii*, isolated from cork, grew in the presence of 0.375 M $[C_2C_1Im]Cl$ (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 $[N_{111}(EtOH)]Cl$, $[C_2C_1Im]Cl$, and $[C_2C_1Im][C_2SO_4]$ (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% $[C_2C_1Im][OAc]$ (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 ($[C_2C_1Im][DEP]$) and $[C_2C_1Im]Cl$, and in 100 mM $[C_2C_1Im][OAc]$ (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 $[C_2C_1Im]Cl$ 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 $[C_2C_1Im]Cl$ in *Enterobacter lignolyticus* strain SCF1 (Khudyakov et al. 2012). Their study revealed significant differential expression of 688 genes in response to 375 mM $[C_2C_1Im]Cl$, 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 $[C_2C_1Im]Cl$. 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 $[C_2C_1Im]Cl$ 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 (Davin-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-Ercek 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 [C$_2$C$_1$Im][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 [C$_2$C$_1$Im]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 [C$_2$C$_1$Im]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 (P*marR'*, P*ydfO'*, and P*ydfA'*) were evaluated for their ability to provide a dynamic control system for regulating *eilA* gene expression in response to [C$_2$C$_1$Im]Cl, potentially providing systems for regulating the expression of heterologous genes in host engineering. An [C$_2$C$_1$Im][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 [C$_2$C$_1$Im]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 [C$_2$C$_1$Im]Cl tolerance. This study also demonstrated an engineered *Saccharomyces cerevisiae* strain Y133-IIL with deletion of *PTK2* could convert glucose and xylose to ethanol in the presence of 1% [C$_4$C$_1$Im]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.

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