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Research review paper

Biocatalysis in ionic liquids for lignin valorization: Opportunities and recent developments

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ABSTRACT

Lignin holds tremendous potential as a renewable feedstock for upgrading to a number of high-value chemicals and products that are derived from the petroleum industry at present. Since lignin makes up a significant fraction of lignocellulosic biomass, co-utilization of lignin in addition to cellulose and hemicelluloses is vital to the economic viability of cellulosic biorefineries. The recalcitrant nature of lignin, originated from the molecule's compositional and structural heterogeneity, however, poses great challenges toward effective and selective lignin depolymerization and valorization. Ionic liquid (IL) is a powerful solvent that has demonstrated high efficiency in fractionating lignocellulosic biomass into sugar streams and a lignin stream of reduced molecular weight. Compared to thermochemical methods, biological lignin deconstruction takes place at mild temperature and pressure while product selectivity can be potentially improved *via* the specificity of biocatalysts (lignin degrading enzymes, LDEs). This review focuses on a lignin valorization strategy by harnessing the biomass fractionating capabilities of ILs and the substrate and product selectivity of LDEs. Recent advances in elucidating enzyme-IL interactions as well as strategies for improving enzyme activity in IL are discussed, with specific emphases on biocompatible ILs, thermostable and IL-tolerant enzymes, enzyme immobilization, and surface charge engineering. Also reviewed is the protein engineering toolsets (directed evolution and rational design) to improve the biocatalysts' activity, stability and product selectivity in IL systems. The alliance between IL and LDEs offers a great opportunity for developing a biocatalytic route for lignin valorization.

1. Introduction

Lignin is synthesized biologically by plants and is one of the most abundant biopolymers on the planet. Besides serving as one of the primary building blocks for plant cell wall, lignin plays important roles such as facilitating water transport and protecting the plant from pathogenic attack. As a result of human's practice in utilizing plant materials (*i.e.*, paper and pulping industry), lignin is extracted from the cellulose portion and commonly treated as a low-value/waste stream. Due to the recent surge in cellulosic biofuel production, the volume of lignin from cellulosic biorefineries is predicted to reach 60 million tons annually by 2022, which will add on top of the lignin produced by the paper industry (Tilman et al., 2009). Currently, lignin is primarily burned in boiler to generate heat and steam that power upstream processes (Mottiar et al., 2016; Pettersson, 2011). Converting lignin to value-added chemicals will add revenue to the biorefineries and reduce emitted CO_2 when burned (Mottiar et al., 2016; Ragauskas et al., 2014). Despite its great potential, lignin valorization is still challenging.

Lignin depolymerization is the first step toward chemical production since lignin in its polymeric form has limited uses. The breakdown of lignin to low molecular weight compounds helps to channel desirable functions and make suitable intermediates for upgrading to fuels and chemicals. Lignin deploymerization can be categorized into thermochemical routes such as high temperature pyrolysis, hydrogenolysis, and catalytic oxidation and biological routes using lignin degrading enzymes (LDEs) and/or microorganisms (Beckham et al., 2016; De Wild et al., 2014; Laskar et al., 2013; Mu et al., 2013). Tremendous efforts have been focused on improving the catalyst efficiency and selectivity in a thermochemical conversion process (Chatel and Rogers, 2013; Zakzeski et al., 2010a). However, the effectiveness and selectivity of the catalyst as developed on lignin model compounds often get compromised when applied to plant derived lignin due to its intrinsic structural and compositional complexity (Rinaldi et al., 2016).

Biological lignin deconstruction can be performed at lower temperature and pressure than thermochemical methods while product selectivity can be potentially improved *via* the specificity of biocatalysts (Linger et al., 2014). The dominant lignolytic enzymes produced by white and brown rot fungi or some Actinobacteria are lignin

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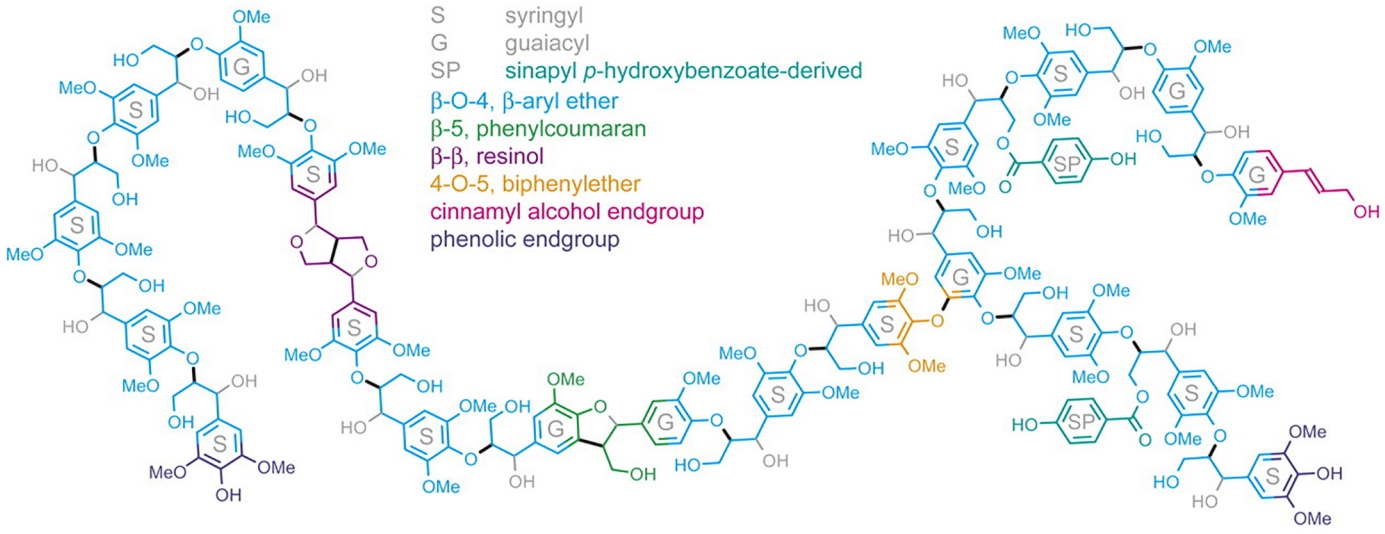

Fig. 1. Representative lignin structure consisting of syringyl and guaiacyl units (adapted from (Stewart et al., 2009)).

peroxidases, versatile peroxidases, manganese peroxidases, and laccases. In addition, several unique pathways for the cleavage of lignin linkages by nicotinamide adenine dinucleotide (NAD) or glutathione dependent enzymes have been discovered in certain soil bacteria such as *Sphingobium* sp. *SYK-6* (Munk et al., 2015; Shi et al., 2012; Varman et al., 2016). Application of these lignolytic enzymes in an industrial relevant lignin valorization process is still hindered due to the high cost of enzyme production and by the very low lignin solubility (for many industrial relevant lignin streams) in the aqueous environment compatible with enzyme activity (Brown and Chang, 2014; Bugg et al., 2011a). However, most of the known effective lignin solvents such as hexane, DMSO, acetone, and alkalis are detrimental to enzyme activity in their pristine form or aqueous solutions (Klibanov, 2001; Mozhaev et al., 1989) and often require high temperature for complete lignin dissolution especially when dealing with plant derived native lignins (Park and Kazlauskas, 2003). Thus, a better solvent system with both high lignin solubility and enzyme compatibility is essential for developing a better biological lignin depolymerization process.

As alternatives to common organic solvents, certain ionic liquids (ILs) have demonstrated a remarkable ability to solubilize and depolymerize lignin during biomass pretreatment (Dutta et al., 2016; Liu et al., 2017; Xia et al., 2014). The cellulose portion recovered from IL pretreatment is highly digestible by using cellulolytic enzymes while lignin in the liquid stream can undergo additional processing and be converted to value-added chemicals (Liu et al., 2017). Taking advantage of IL solvent systems, a number of base, acid and metal catalysts have been tested previously for catalytically depolymerizing lignin in IL media (Behling et al., 2016; Chatel and Rogers, 2013; Das et al., 2017; Xu et al., 2014; Zakzeski et al., 2011; Zakzeski et al., 2010b). Meanwhile the use of biocatalysts in IL, *i.e.* LDEs has attracted increasing interest in recent years due to potential advantages such as better selectivity and affinity, in milder reaction conditions. The toxicity of certain ILs to enzymes and microbes however hinders direct product formation *via* biological lignin depolymerization in pure IL or its aqueous solution.

There have been several articles reviewing the effects of ILs on enzymes such as lipase, xylanase, cellulase, and laccase, *etc.* (Forsyth et al., 2002; Kaar et al., 2003; Klahn et al., 2011; Moniruzzaman et al., 2010; Naushad et al., 2012; Park and Kazlauskas, 2001; Weingartner et al., 2012; Zhao, 2010; Zhao et al., 2006). To the best of our knowledge, however, there has not been a review of the effects of ILs on LDEs in the context of lignin valorization. This paper emphasizes on the effects of IL on lignin characteristics, the relationships between LDE protein structure and function and ILs (enzyme-IL interactions), along with strategies for improving the efficiency and product selectivity of LDEs in ILs for lignin depolymerization. Finally, possible pathways toward a lignin valorization system using a biocompatible IL and LDE are proposed; challenges and perspectives associated with designing such a system are also discussed.

2. Lignin

2.1. Lignin structure and characteristics

Lignin is a heterogeneous biopolymer, responsible for providing plants with structural support, microbial resistance and channels for water and nutrient transport (Hofrichter, 2002). Lignin is constructed of three major subunits, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Boerjan et al., 2003). These monolignols are differentiated based on the degree of methoxylation of their phenyl rings; *p*-coumaryl alcohol has no methoxy group, coniferyl alcohol has one methoxy group (2-methoxyphenol), and sinapyl alcohol has two methoxy groups (2,6-dimethoxyphenol). These heterogeneous building blocks are the precursors to what will ultimately become lignin. During lignin biosynthesis, the monolignols undergo slight structural changes as they are converted to phenylpropanoids (Boerjan et al., 2003). The *p*-coumaryl alcohol is converted to hydroxyphenol (H-lignin), coniferyl alcohol is converted to guaiacyl (G-lignin), and sinapyl alcohol is converted to syringyl (S-lignin) (Vanholme et al., 2010; Weng and Chapple, 2010). Fig. 1 highlights the complex structure of lignin formed from coniferyl and sinapyl alcohol.

Monolignols are linked *via* a heterogeneous network of inter-unit linkages. The inter-unit linkages seen in lignin are largely dictated by the monolignols present. The most common linkage is the β-*O*-4′ linkage between H-, G-, or S-lignin (Boerjan et al., 2003; Sette et al., 2011; Vanholme et al., 2010). G- and H-lignin can be linked together *via* β-5′, 5–5'-*O*-4, or 4-*O*-5′ linkages. H-, G-, and S-lignin can further be linked together *via* β-β' or β-1′ linkages (Sette et al., 2011). Fig. 2 highlights the complexity of the most common inter-unit linkages and structural changes undergone by the monolignols during lignin biosynthesis. The combination of different monolignols alongside the network of inter-unit linkages, contributes to lignin's inherent heterogeneity.

The source of the biomass determines important properties of lignin. The fraction of lignin present in the biomass is dependent upon the source; softwoods contain 25–31% lignin, while hardwoods contain 16–24% lignin (Suhas and Ribeiro Carrott, 2007). Additionally, the ratio of monolignols present depends upon the source of biomass;

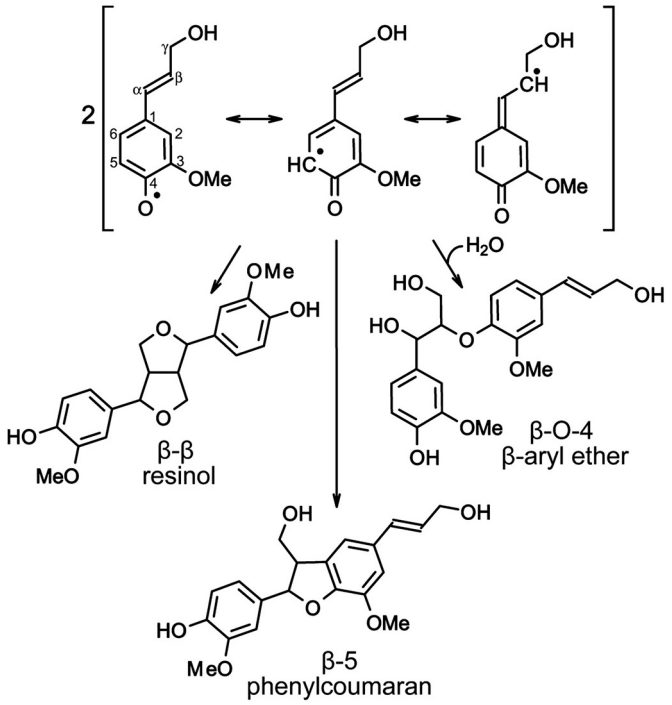

Fig. 2. Formation of inter-unit linkages during polymerization of coniferyl alcohol monomers (adapted from (Vanholme et al., 2010)).

softwoods primarily contain G-lignin, hardwoods primarily contain G- and S-lignin, and grasses contain different ratios of H-, G-, and S-lignin (Suhas and Ribeiro Carrott, 2007). It is this heterogeneity present at all levels of lignin that is responsible for the challenges in selectively depolymerizing these molecules (Zeng et al., 2013).

Plant cell wall engineering has attracted increasing interest in recent years. Genetic modification/engineering the monolignol composition and/or inter-unit linkages present in the native lignin, such as β-*O*-4′ linkages, could make lignin decomposition processes more efficient. For example, Wilkerson et al. genetically modified poplar trees to synthesize lignin from monolignol ferulate conjugates and demonstrated improved cell wall digestibility after mild alkaline pretreatment of the engineered plants (Wilkerson et al., 2014). In a follow-up study, Parsell et al. applied bimetallic Zn/Pd/C catalyst to breakdown engineered poplar tree that contain higher levels of S-lignin; results suggest that the ester linkages present in the genetically modified poplar resulted in higher levels of lignin digestion to a single product (dihydroeugenol) when compared to native poplar (Parsell et al., 2015).

Since lignin is such a vital component for plant growth, altering lignin biosynthesis may have deleterious effects. Repression of a gene responsible for lignin biosynthesis (hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase, HCT or *p*-coumaroyl shikimate 3′-hydroxylase, C3′H) in *Arabidopsis thaliana* led to stunted growth of the plant, a result of reduced auxin transport throughout the plant (Li et al., 2010). Bonawitz et al. were able to engineer an *Arabidopsis* mutant and simultaneously retain its growth and engineered lignin properties (Bonawitz et al., 2014). It was shown that disruption of a transcriptional regulatory complex, complex subunits MED5a and MED5b restored lignin levels to those found in wild type *Arabidopsis*. Interestingly, the resulting lignin was primarily made of H-lignin (~95%), with trace amounts of G- and S- lignin, a trait greatly improving enzymatic digestibility of this mutant after pretreatment (Shi et al., 2016). Engineering plants with lower lignin content, different lignin composition and inter-unit linkages, and lignin deposition creates opportunities to design better energy crops with traits suitable for bioconversion to biofuels and chemicals (Simmons et al., 2010; Weng and Chapple, 2010).

2.2. Lignin depolymerization methods

Lignin is often underutilized in its polymeric form. Most of the lignin is burned to produce heat and power; *i.e.* a small portion finds its applications as dispersants and binders (Lora and Glasser, 2002). The breakdown of lignin opens access to desirable functions that are not achievable when lignin is a polymer; while the resulting low molecular weight compounds become suitable for upgrading to fuel and chemicals. Several lignin depolymerization pathways are being developed, including catalytic oxidation, pyrolysis, gasification, and hydrogenolysis, *etc.* These methods utilize a suite of tools like high temperature, chemical catalysts and solvents to break down lignin. For example, Alkali processes typically use sodium hydroxide (NaOH) in combination with an oxidative agent for lignin decomposition, which are the most common methods in the paper industry. The two most common alkali pretreatment methods are the Soda and Kraft processes, both of which use NaOH along with other chemicals such as sodium sulfide and anthraquinone (Yang et al., 2016). Ammonium hydroxide has also been shown to be effective for lignin decomposition (Liu et al., 2017).

While use of high pH methods is effective, chemistry was shown to be effective in depolymerizing lignin in acid, alkaline, or organic solvents. Acid-catalyzed lignin decomposition uses a variety of catalysts including mineral acids, Lewis acids, and ILs (Li et al., 2015), most notably through the use of dilute sulfuric acid (H_2SO_4). Lignin extracted from dilute H_2SO_4 pretreated switchgrass was found to have a reduced molecular weight compared to untreated lignin (Liu et al., 2017). One marker of lignin degradation is the formation of aromatic products due to the aromatic nature of monolignols. Rahimi et al. (Rahimi et al., 2014) used formic acid for lignin decomposition, resulting in a yield of over 60 wt% aromatics under mild reaction conditions. While the oxidative chemistry shown to be effective to depolymerize lignin in acid, alkaline or solvents, reductive chemistry using hydrogen molecules or hydrogen donor provides another pathway.

Hydrogenolysis breaks down lignin using hydrogen with help from metal catalysts. During hydrogenolysis, reductive bond cleavage takes place within lignin and/or lignin model compounds in presence of hydrogen as a reducing agent (Molinari et al., 2016). A range of catalysts, including noble metals (such as platinum), transition metals and bimetallic catalyst, such as Zn/Pd/C and Pd/CeO_2, *etc.* were reported to facilitate the cleavage of lignin into aromatic products (Deng et al., 2015; Parsell et al., 2015). Alternatively, catalytic transfer hydrogenolysis (CTH) has shown great promise (Barta et al., 2010). In CTH reaction scheme, an equivalent of H_2 is transferred from a donor molecule to the acceptor molecule. Hydrogen donor molecules are often inexpensive organic alcohols capable of readily generating hydrogen molecules and the same time serving as solvents for lignin (Regmi et al., 2017). A variety of hydrogen donating agents have been tested including formic acid, methanol, ethanol, tetralin *etc.*, among which isopropyl alcohol remains a popular choice due to its relative low cost and easy subsequent separation from the reaction mixture (Kim et al., 2017).

Pyrolysis is the breakdown of large molecules into smaller ones by the application of heat in the absence of oxygen. During pyrolysis, lignin is heated to temperatures between 160 and 900 °C where cleavage of the ether (C—O) and C—C linkages takes place (Yang et al., 2007). Lignin pyrolysis produces a range of pyrolytic aromatic compounds in oil form in addition to gas products and residual char (Barekati-Goudarzi et al., 2017; Khachatryan et al., 2018). The yield and composition of pyrolytic oil are influenced by many factors, including lignin type and operation conditions (Mullen et al., 2010).

As opposed to thermochemical lignin depolymerization methods, which require intense energy input and/or harsh chemicals and costly catalysts, biological lignin depolymerization takes advantage of microorganisms and their enzymatic excreta to cleavage the inter-unit lignin linkages and lignin-carbohydrate linkages. The LDEs play key

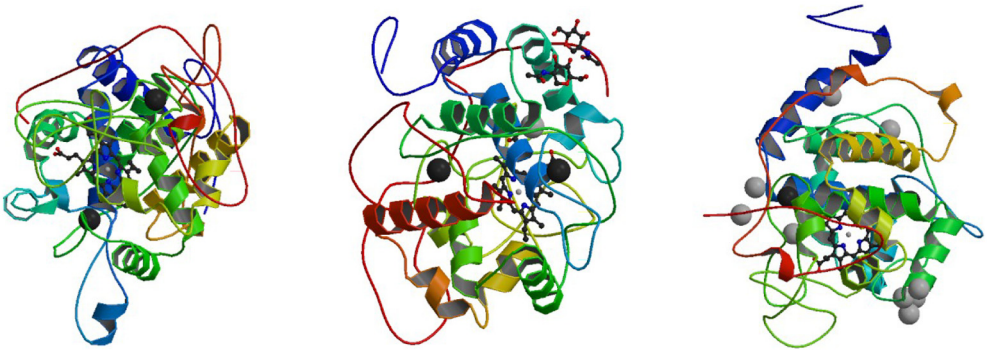

Fig. 3. a) Lignin peroxidase from *Phanerochaete chrysosporium* (PDBID: 1B82) (Blodig et al., 2001), b) Manganese peroxidase from *Phanerochaete chrysosporium* (PDBID: 1MNP) (Sundaramoorthy et al., 2010) and c) Versatile peroxidase from *Pleurotus eryngii* (PDBID: 2BOQ) (Perez-Boada et al., 2005). Figures downloaded from the Research Collaboratory for Structural Bioinformatics' Protein Data Bank (RCSB PDB) (Berman 2000).

roles in biological lignin depolymerization in nature. The next section summarizes the key LDEs with emphasis on their temperature and pH optima (parameters relevant to IL tolerance) and potential applications in lignin depolymerization.

3. Lignin degrading enzymes

3.1. Peroxidases

Lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) are a series of iron-based LDEs present in nature (Fig. 3) (Dong et al., 2014; Martínez, 2002; Nousiainen et al., 2014). Numerous forms of these LDEs have been isolated from a variety of lignolytic fungi and bacteria (Camarero et al., 1999; Glenn et al., 1983; Johansson and Nyman, 1993; Martínez, 2002; Sahadevan et al., 2016). Additionally, isozymes have been identified within individual species. For instance, 16 forms of LiP and 5 forms of MnP were isolated from liquid culture containing *Trametes versicolor* (Johansson and Nyman, 1993). While these enzymes serve a similar purpose in nature, many have been found to have unique properties.

Peroxidases can retain activity in a wide range of pH and temperature. The optima are dependent upon the enzyme's source and the oxidation substrate. It was found that LiP and MnP from a soil fungus Deuteromycete retain activity over a pH range of 4–11 and a temperature range of 30–55 °C (Sahadevan et al., 2016). Fourteen forms of MnP and LiP from *Pleurotus ostreatus* and *Ceriporiopsis subvermispora* were expressed in *Escherichia. coli*; remarkably, an isozyme of MnP from *C. subvermispora* retained over 80% activity at pH 2.0 (Fernández-Fueyo et al., 2012). Recombinant VP from *P. eryngii* expressed in *P. chrysosporium* was found to have optimal activity at pH 3.0, while retaining activity at temperatures up to 40 °C (Coconi-Linares et al., 2014). Fujii et al. observed different pH optima for the LDEs collected at different depths from the forest floor of regions in Japan, Thailand, and Indonesia. It was shown that MnP activity was detected at pH greater than 5.0, while LiP activity was detected at pH less than 5.0 (Fujii et al., 2013). The different pH optima suggest that LiP and MnP fulfill important roles in biodegradation of plant matter at different depths of the forest floor. The optimal temperature for LDE activity has also been observed to vary with respect to the substrate present. A peroxidase from *Bacillus subtilis* had an optimal pH of 3.0, while maximum substrate oxidation was observed at different temperatures from 30 to 50 °C (Min et al., 2015). LiP displayed optimal removal of a nonylphenol at pH range 4.0–7.0 and temperature range of 25–50 °C (Dong et al., 2014). Evidence has suggested that the difference in pH and temperature optima between LDEs serves important functions for the microbes to adapt to various environments in nature.

LiP, MnP, and VP have a wide range of applications beside biodegradation of lignin. This is due to the capability of LDEs to oxidize a wide variety of phenolic substrates (Dong et al., 2014; Glenn et al., 1983; Semba et al., 2015; Tien and Kirk, 1983). For example, LiP was shown to remove a nonylphenol from wastewater (Dong et al., 2014), while a peroxidase expressed in *B. subtilis* can be used in dye-decolorizing operations (Min et al., 2015). MnP from *Bjerkandera adusta* was used to depolymerize chemically-pretreated coal, forming aromatic compounds in the process (Huang et al., 2013). Also reported is a MnP from *Ganoderma lucidum* for use in reducing both color and turbidity of apple and orange juices (Bilal et al., 2016). The oxidation potential of peroxidases allows for applications besides oxidizing phenolic

Table 1
Applications of lignolytic enzymes.

Enzyme	Source	Application	References
Peroxidase	*B. subtilis*	Dye decolorization	Min et al. (2015)
MnP	*Bjerkandera adusta*	Coal treatment	Huang et al. (2013)
MnP	*G. lucidum*	Food industry	Bilal et al. (2016), Alper and Acar (2004), Couto et al. (2006) andSelinheimo et al. (2006)
Laccase	*Trametes hirsuta*		
Laccase	*T. versicolor*	Lignin removal from wood pulp	Gamelas et al. (2005)
Laccase	*T. hirsute*	Dye decolorizing in textiles	Campos et al. (2001), Pazarlıoğlu et al. (2005), Lantto et al. (2004) andBasto et al. (2007)
	Trametes villosa		
	Sclerotium rolfsii		
	T. versicolor		
Laccase	*Trametes hirsute*	Wastewater treatment	Dong et al. (2014), Novotný et al. (2004); Pointing (2001), Anastasi et al. (2009), Niku-Paavola and
LiP	*Various lignolytic microbes*		Viikari (2000), Alcalde et al. (2006) and Minussi et al. (2007)
	T. versicolor		
	T. villosa		
	Lentimula edodes		
	Botrytis cinerea		
Laccase	*Myceliophtora thermophyla*	Production of pharmaceuticals	Nicotra et al. (2004) and Harris et al. (2004)
	Trametes pubescens		
	Saccharomyces cerevisiae		
Laccase	*T. versicolor*	Biocathodes	Mani et al. (2017) and Le Goff et al. (2015)
	T. hirusta		

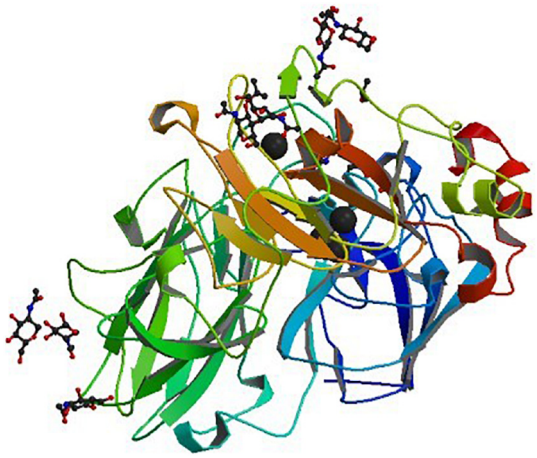

Fig. 4. Laccase from *Trametes versicolor* (PDBID: 1GYC) (Piontek et al., 2002). Figure downloaded from the Research Collaboratory for Structural Bioinformatics' Protein Data Bank (RCSB PDB) (Berman 2000).

substrates (Table 1).

3.2. Laccases

First discovered in 1883 in an extract from the tree *Rhus vernicifera*, laccases are a member of the superfamily of multicopper oxidases (An et al., 2015; Yoshida, 1883). Laccases utilize four coppers to reduce O_2 to H_2O and, in the process, oxidize the substrate (Piontek et al., 2002; Thurston, 1994). This oxidative mechanism stands in stark contrast to the mechanism of the peroxidase LDEs; the use of O_2 in place of H_2O_2 results in a milder chemical environment. This unique trait has led laccases to attract interests in the lignin valorization community. The most commonly investigated laccases are produced commercially from a polypore basidiomycete, *T. versicolor* (Fig. 4). The laccases secreted by *T. versicolor* have moderate degrees of stability at pH from 5 to 8 and at temperatures from 10 to 45 °C (Bourbonnais et al., 1995; Kurniawati and Nicell, 2008; Piontek et al., 2002).

3.2.1. Thermophilic laccases

Laccases from thermophilic microorganisms have received particular interest for use in lignin valorization and other applications because of the improved thermostability. These laccases have been shown to oxidize a wide variety of phenolic substrates. Kiiskinen et al. expressed a thermophilic laccase from *Melanocarpus albomyces* in *Trichoderma reesei*. This laccase is capable of oxidizing 2,6-dimethoxyphenol (DMP), syringaldazine, and guaiacol at temperatures up to 50 °C (Kiiskinen et al., 2004). Recently, An et al. expressed a thermostable fungal laccase (PPLCC2) from *Postia placenta* in *Pichia pastoris* (An et al., 2015). PPLCC2 displayed optimum oxidation of 2,2′-azinobis(3-ethylbenzthioazoline-6-sulphonic acid) (ABTS) and DMP at 60 °C. While continuing to search for thermophilic laccases in fungi, researchers have expanded their search to bacterial and archaeal laccases.

There have been several thermophilic laccases and laccase-like enzymes discovered in bacteria in the past decades. *B. subtilis* was found to produce a thermostable form of a laccase-like enzyme, CotA (Hullo et al., 2001), which has a maximal enzyme activity at 75 °C with ABTS as substrate (Martins et al., 2002). Laccase from the bacteria *Thermus thermophilus* (TtL) is the most thermostable laccase to date (Miyazaki, 2005); optimal substrate oxidation was observed at 92 °C. A recent work reported a thermostable laccase (LccA) isolated from the Dead Sea archaeon *Haloferax volcanii* (Uthandi et al., 2010). It was found that LccA oxidized the phenolic substrates DMP and syringaldazine along with the nonphenolic substrate ABTS. Remarkably, LccA retained significant activity at 50 °C and in salt concentrations up to 1.4 M.

These laccases have been observed to be reliably thermophilic; however, they have been observed to vary in pH optima. The pH optima of TtL and PPLCC2 change depending upon which oxidation substrate is present. PPLCC2 exhibits optimal oxidation of ABTS and DMP at pH 3.5 and 5.0, respectively (An et al., 2015). TtL exhibits optimal oxidation of ABTS and syringaldazine at pH 4.5 and 5.5, respectively (Miyazaki, 2005). The diverse temperature, pH, and substrate affinity exhibited by laccases enable their use in a number of occasions.

3.2.2. Laccase applications

Laccases have a wide variety of applications with a few examples listed in Table 1 (Couto et al., 2006; Muthukumarasamy and Murugan, 2014). In the pulp and paper industry, laccases have been used to reduce the lignin content of wood pulp (Gamelas et al., 2005). In the textile industry, laccases have been used to decolorize dyes and treat a number of fabrics (Basto et al., 2007; Campos et al., 2001; Lantto et al., 2004; Pazarlıoğlu et al., 2005). Laccases can also be used to remove chemicals from wastewater and contaminated soil (Alcalde et al., 2006; Anastasi et al., 2009; Niku-Paavola and Viikari, 2000; Novotný et al., 2004; Pointing, 2001). In food industry, laccase can be used in processing fruit juice and treating wastewater from various food processes (Alper and Acar, 2004; Couto et al., 2006; Minussi et al., 2007; Selinheimo et al., 2006). In addition, laccases have found use in the pharmaceutical industry, producing compounds that have various medical applications (Harris et al., 2004; Nicotra et al., 2004). The diverse list of laccase applications continues to expand with research into bioenergy.

The primary application of laccases for bioenergy is for biomass deconstruction. Laccase is capable of removing lignin from the biomass, resulting in increased digestibility of the cellulose and hemicellulose fractions (Chen et al., 2012). Laccase-mediator system has been reported to fractionate and modify lignin to produce quality carbon fibers (Li et al., 2017); such a system diverts lignin to a high molecular weight fraction suitable for carbon fiber and a soluble low molecular weight fraction suitable for biological conversion to lipids (Zhao et al., 2016). Laccases have also been used in microbial fuel cells (MFCs), in such cases laccases function as a biocathode, providing a cheaper alternative to platinum (Mani et al., 2017). Immobilization of laccase on carbon nanotubes further improves laccase activity in MFCs over time (Le Goff et al., 2015).

3.3. Other LDEs

In addition to the peroxidases and laccases, the search for LDEs led to the discovery of novel enzyme consortia in bacteria. Several classes of the bacterial dye-decolorizing peroxidases, multi-copper oxidases and β-etherases have been reviewed previously (Brown and Chang, 2014; Bugg et al., 2011b; de Gonzalo et al., 2016). The soil bacterium *Sphingobium* sp. SYK-6 is capable of growing on lignin-derived monoaryls and biaryls substrates *via* expression of several LDEs as shown in Fig. 5 (Masai et al., 2003; Masai et al., 2007; Pereira et al., 2016). SYK-6 has been shown to be extremely tolerant of high pH conditions, a desirable characteristic for lignin valorization (Varman et al., 2016). Additionally, SYK-6 was found to prefer vanillin when grown in the presence of several carbon sources; through further observations, it was determined that ligninolysis is a mechanism required by SYK-6 for survival (Varman et al., 2016). The vanillin catabolic pathway is responsible for providing SYK-6 with sufficient concentrations of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), important reducing species vital for microbial growth. These unique lignolytic characteristics of SYK-6 have made it an attractive target for designing lignin valorization strategies. The complete genome of SYK-6 has been sequenced, which consists of a 4,199,332-bp-long chromosome and a 148,801-bp-long plasmid (Masai et al., 2012).

The lignolytic capabilities of *Sphingobium* have been leveraged in plant engineering for lignin valorization. A family of LDEs from

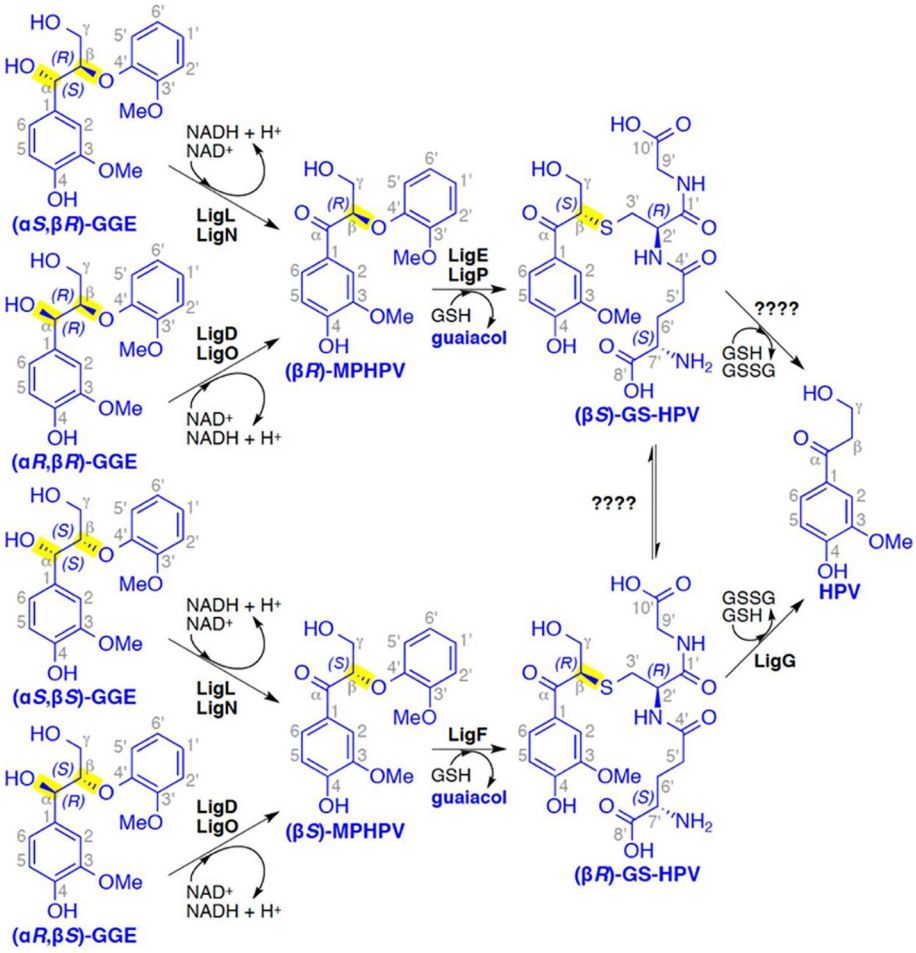

Fig. 5. Lignolytic pathways of *Sphingobium* sp. strain SYK-6 (Pereira et al., 2016).

Sphingobium, LigDFG, were expressed in *A. thaliana*, resulting in high levels of oxidized G- and S-lignin subunits in plants (Mnich et al., 2017). Furthermore, *E. coli* strain was engineered by combining vanillin degradation pathway (LigV, LigM) from SYK-6 and a protocheuate decarboxylase (aroY) and a catechol dioxygenase (CatA) from tobacco plant (Wu et al., 2017). In a follow-up study, an autoregulatory system was constructed into the engineered *E. coli* chassis and the new strategy demonstrated bioconversion of the central intermediates such as vanillin into *cis, cis*-muconic acid at high yield in *E. coli* (Wu et al., 2018). Such approaches highlight the opportunities of developing novel applications of bacterial LDEs for lignin depolymerization.

4. Ionic liquids

ILs are pairs of ions that exist in liquid form, often below 100 °C (also called room temperature ILs). Given the enormous selections of cations and anions, a large number (10^{18} theoretically) of ILs can be synthesized. For the same reason, ILs can be designed with fine-tuned properties to suit the needs of a process in a highly specific manner. For instance, by selecting environmentally friendly ion pairs, ILs can be synthesized to replace environmentally harmful volatile organic solvents commonly used in industry (Freemantle, 1998; Rogers and Seddon, 2003). Many ILs have little to no vapor pressure, minimizing harmful emissions that are associated with more common organic solvents (Brennecke and Maginn, 2001). There have been, however, some circumstances that challenge this notion (Cvjetko Bubalo et al., 2014). Many alkyl imidazolium ILs were shown to be flammable when exposed to a heat source (Smiglak et al., 2006). ILs have a wide range of applications, including biomass processing, CO_2 capture, compound extraction, thermostable lubricant, reaction medium/catalyst for catalysis, pharmaceuticals, *etc.* which can be seen from several review articles (Freemantle, 1998; Gutowski et al., 2003; van Rantwijk and Sheldon, 2007). The foci of this section are to review the properties of ILs that are relevant to lignin fractionation and enzyme activity/stability and the effect of IL treatment on lignin characteristics.

4.1. IL properties

Solvent properties, hydrophobicity, and biocompatibility/toxicity are important properties of ILs that determine their potential applications. Those properties are directly influenced by the cation and anion species. In other words, it is possible to tune the IL properties by changing the anion/cation combinations (Patel et al., 2014). The Kamlet-Taft system is commonly used to describe IL's solvent properties in three separate terms/parameters: polarizability (π*), hydrogen bond donator capacity (α) and hydrogen bond acceptor capacity (β) (Kamlet and Taft, 1976). Kamlet-Taft parameters were not only used for measuring solvent properties of a single IL but also applied to describing the average or bulk solvent properties of binary and ternary IL/H_2O mixtures (Brandt et al., 2011; Reichardt, 2004). Primarily determined by the anion, the β value has been used to predict ILs' capacity to disrupt cellulose (Ab Rani et al., 2011; Xu et al., 2010). As proposed by Doherty et al., higher β values indicate that ILs tend to form strong attractions with the hydroxyl protons of cellulose; this interaction leads to disruption of the crystalline lattice (Doherty et al., 2010). More computational-based evidence was reported by Sun and co-workers linking the predicted interaction energies with experimentally determined Kamlet-Taft parameters(Sun et al., 2014). A positive correlation was observed

between cellulose conversion and β values. ILs with larger differences between β and α, net basicity (β-α) tend to dissolve cellulose more efficiently(Hauru et al., 2012; Kahlen and Leonhard, 2010).

The hydrophobicity of IL is relevant to enzyme stability/activity in IL; some ILs have been observed to deactivate enzymes with decreasing hydrophobicity. When a series of alkyl imidazolium ILs were screened for hydrophobicity, the alkyl chain length of the cation was well correlated with the hydrophobicity of the ILs (Freire et al., 2007). In addition, hydrophobicity of the anion also contributes largely to the IL's hydrophobicity. For example, ILs with a bis(trifluoromethylsulfonyl) amide ([NTf_2]-) anion have been used to stabilize lithium electrochemistry due to the anion's hydrophobic nature (Bhatt et al., 2002; Hajime et al., 2000; Howlett et al., 2004; MacFarlane et al., 2014). It has been reported that alkylimidazolium ILs with alkyl sulfate anions, with decreasing anion chain length, resulted in higher *T. versicolor* laccase deactivation (Liu et al., 2018b; Rehmann et al., 2012). Another predictor of the interaction between an IL and an enzyme is the Hofmeister series (aka. Lyotropic series), a classification of ions in order of their ability to salt out or salt in proteins. It has been shown that kosmotropic anions and chaotropic cations are preferable for enzyme stability, with the anion's properties largely determining enzyme-IL interactions (Galai et al., 2015; Zhao et al., 2006). Molecular dynamics simulations of a lipase in the ILs 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4C_1Im][PF_6]) and 1-butyl-3-methylimidazolium nitrate ([C_4C_1Im][NO_3]) showed that IL-enzyme interactions are dominated by the anion (Burney and Pfaendtner, 2013). Collection of these properties leads to a more generic definition of IL's biocompatibility/toxicity.

Quantitative structure–property relationship (QSPR) modeling is a powerful tool predicting IL toxicity. Results from Rank et al. show strong correlation between lipophilicity of the cations and IL cytotoxicity by comparing experimental with computational data over a set of 74 ILs with imidazolium, pyrrolidinium, pyridinium, quinolinium, quaternary phosphonium and quaternary ammonium cations and the small anions such as Cl^-, Br^-, BF_4^- or PF_6^- (Ranke et al., 2007). Results from the same group suggest that anion's lipophilicity and/or vulnerability to hydrolytic cleavage also appeared to be a crucial structural feature leading to cytotoxicity (Stolte et al., 2006). Using a set of electronic, spatial, structural, thermodynamic, and topological descriptors, Couling et al. constructed QSPR models to predict IL toxicity; results show an increasing trend of toxicity based on cation type of ammonium, pyridinium, imidazolium, triazolium, tetrazolium and decreasing trend of toxicity with increasing number of negatively charged atoms and ring methylation on the cation (Couling et al., 2006). A more recent work from Yoo et al. (2016), adopted an integrated experimental and computational approach to explore the molecular-level details of IL's cytotoxicity. Molecular dynamics (MD) simulations based on 1-palmiltoyl-2-oleoyl-phosphatidylcholine lipid bilayer suggest that cytotoxicity increases with increasing alkyl chain length of the cation, due to the ability of the longer alkyl chain to insert in and disrupt the cell membrane (Yoo et al., 2016). Collectively, it is concluded that both the cation and anion play important roles in determining IL's toxicity/biocompatibility through their interactions with biomolecules.

4.2. Biomass pretreatment with ILs

The ability of certain ILs to solubilize one or all three major components of lignocellulose has made them attractive solvents for biomass pretreatment (Achinivu et al., 2014). This capability is largely attributed to IL's ability to form strong hydrogen bonds with the cellulose, hemicellulose, and lignin polymers present in biomass (Alvira et al., 2010; Haghighi Mood et al., 2013). A computational approach was used to predict the ability of 750 ILs to solubilize cellulose and lignin using *p*-coumaryl alcohol as a lignin model compound; it was predicted that a very wide range of anion/cation combinations can form ILs capable of solubilizing lignin (Casas et al., 2013). The factors affecting IL pretreatment performance and challenges facing this pretreatment technology are discussed below.

4.2.1. Effect of cation/anion on pretreatment

The properties of ILs are sensitive to the cation and anion species present in the IL. As a result, these species can significantly alter the products obtained from biomass after IL pretreatment. The ability of ILs to dissolve cellulose is dependent upon the anion and cation species. ILs with the 1-butyl-3-methylimidazolium ([C_4C_1Im]) cation are capable of dissolving cellulose when paired with a chloride anion ([Cl]) (Pinkert et al., 2010). When the same cation is paired with bis(trifluoromethanesulfonyl)amide ([Tf_2N]) anion, the IL can no longer dissolve cellulose. Furthermore, when [Cl] is paired with a pyrrolidinium or piperidinium cation, the IL can no longer dissolve cellulose (Kosan et al., 2010). The ability of choline amino acid ([Ch][AA]) ILs to solubilize the components of biomass have been shown to be sensitive to the choice of amino acid. Liu et al. screened a series of [Ch][AA] ILs for lignin, cellulose, and hemicellulose solubility and found hemicellulose solubility was heavily dependent upon the amino acid anions (Liu et al., 2012). When pretreating wheat straw with dilute [Ch][AA] ILs, it was found that glutamic acid significantly reduced lignin removal during pretreatment (Hou et al., 2013). In a follow up study, Hou et al. (2015) established a positive correlation between the pKa of the anion and the delignification observed during pretreatment.

Alkyl imidazolium ILs have served as benchmark solvents for pretreatment of lignocellulosic biomass (Fig. 6). These ILs have been shown to improve cellulose and hemicellulose digestibility after pretreatment, *via* lignin removal and reduction or change in the crystallinity of cellulose. Enzymatic hydrolysis sugar yields can be greatly improved after pretreating a variety of biomass feedstocks with alkyl imidazolium ILs (Fig. 7) (da Costa Lopes et al., 2013; Xia et al., 2014). In one example, it is shown that [C_2C_1Im][OAc] reduces the crystallinity of the cellulose in wood flour and removed 40% of lignin, leading to over 90% glucose yield from enzymatic hydrolysis of the pretreated biomass (Lee et al., 2009). Despite their effectiveness in pretreatment strategies, the high cost of alkyl imidazolium ILs has hindered their widespread adoption in biorefineries.

4.2.2. Cost reduction strategies to enable IL pretreatment

Three primary avenues for IL cost reduction are being explored: the use of inexpensive feedstocks for IL synthesis, use of aqueous ILs, and

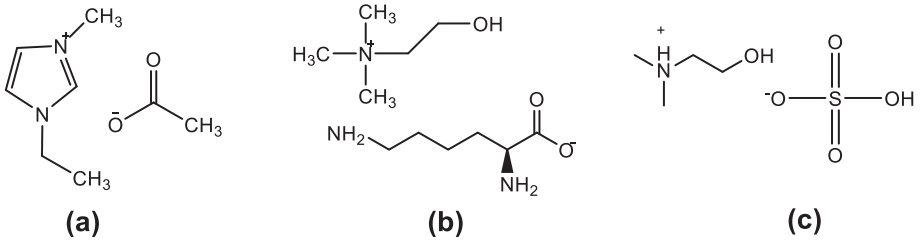

Fig. 6. a) [C_2C_1Im][OAc] has served as the benchmark IL for biomass pretreatment for several years. Recently, bioderived ILs like b) choline lysinate ([Ch][Lys]) and c) diethylamine hydrogen sulfate ([DEA][HSO_4]) have attracted interest due to their low cost.

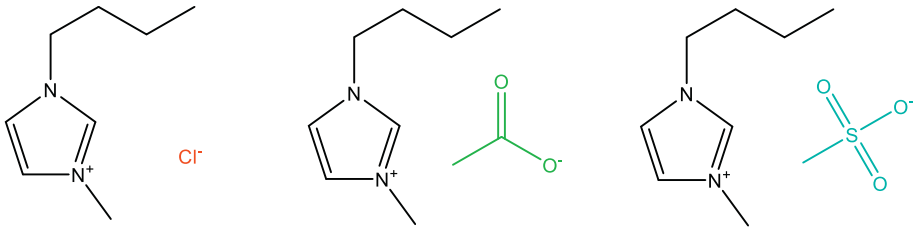

Fig. 7. Butylimidazolium ILs. These ILs have all been effectively utilized in biomass pretreatment strategies with different effects on downstream processing of biomass (adapted from (Xia et al., 2014)).

reusing or recovering ILs for repeating pretreatment cycles (Dutta et al., 2016).

4.2.2.1. Inexpensive feedstock for IL synthesis. There are a number of low-cost precursors that can be used for IL synthesis. To serve as viable replacements, however, these new ILs must match the pretreatment efficacy of alkyl imidazolium ILs. Cations and anions derived from renewable resources such as protein, sugars, and biomass-derived carboxylic acids are of peculiar interest. George et al. synthesized low-cost ILs from ammonium cations and the $[HSO_4]^-$ anion and tested their effectiveness in pretreating switchgrass; results show that sugar yields and lignin removal using the low-cost ILs were comparable to 80% $[C_2C_1Im][OAc]$ (George et al., 2015). ILs synthesized from choline chloride and bio-derived amino acids have been shown to be highly effective in solubilizing hemicellulose and lignin without solubilizing cellulose (Domínguez de María, 2014). Eight choline amino acid ([Ch][AA]) ILs were screened for their ability to remove lignin during pretreatment of rice straw. The majority of the ILs were shown to remove over 50% of lignin during pretreatment, with [Ch][Lys] removing 60.4% of lignin during pretreatment (Hou et al., 2012). These new ILs are potentially more biocompatible and cheaper compared to petroleum based alkyl imidazolium ILs (Sun et al., 2016). In addition to lowering the cost of IL itself, reducing the dosage of IL by using IL-water mixtures (aqueous IL) will further lower the chemical cost of pretreatment.

4.2.2.2. Aqueous ILs. Instead of using pure, or "neat" ILs, using IL-water mixtures reduces the amount of IL needed to achieve a desirable pretreatment outcome, thus reducing the overall cost. Elucidating the role of water on the IL pretreatment efficiency helps to design a better pretreatment process using aqueous IL. In a previous study, the effect of water on IL pretreatment was investigated by pretreating switchgrass with a series of $[C_2C_1Im][OAc]$-water mixtures. It was found that pretreatment with dilute $[C_2C_1Im][OAc]$ at 50–80% IL concentration led to comparable glucose yields and lignin removal to those obtained with 100% $[C_2C_1Im][OAc]$ (Shi et al., 2014). Pretreatment with dilute [Ch][AA] ILs was also shown effective in improving sugar yields and lignin removal when compared to pretreatment using pure ILs (Hou et al., 2013). These findings provide insights into the interplay of water as a co- and anti-solvent in aqueous IL system. Using aqueous IL as pretreatment agents offers additional benefits including reduced viscosity of the IL medium, less gel formation and lower energy requirements and costs associated with IL recycling (Liu et al., 2018a; Shi et al., 2014).

4.2.2.3. IL reuse and lignin recovery. Recovery and reuse of ILs following pretreatment can reduce the demand for purchasing or synthesizing nascent ILs. Oftentimes these ILs can be reused several times without a significant reduction in pretreatment efficacy. For instance, reuse of $[C_2C_1Im][OAc]$ for 5 times was possible without observing a significant change in the pretreatment maple wood flour (Lee et al., 2009). [Ch][Lys] can be recovered and reused for pretreatment of rice straw 5 times until a significant reduction in pretreatment efficacy was observed (Hou et al., 2012). Separation technologies such as evaporation/distillation, electrodialysis, reverse osmosis and pervaporation have been investigated for concentrating ILs for reuse (Mai et al., 2014; Sun et al., 2017; Wang et al., 2012). However, sugars and lignin need to be separated from the IL before sending the IL back to next batch; the cost associated with this step however can be prohibitive.

The use of IL-based solvents for biomass dissolution and lignin depolymerization is relatively new and much needs to be learned before an industrial scale process can be implemented. System complexities associated with IL recycling, biomass-solute separation and downstream processing remain the key challenges in the commercialization (Singh and Simmons, 2013). Recent studies have identified some key areas that influence the overall process economics of IL-based biorefinery processes (Klein-Marcuschamer et al., 2011; Konda et al., 2014; Sun et al., 2017). These areas include the use of low cost IL at high solid loading in the reaction, development of biocompatible ILs or low price IL-tolerant enzyme mixtures, development of efficient IL recycle and product recovery technologies, and system intensification and optimization. *In-situ* lignin valorization is one of the possible ways to lower the product recovery cost and in turn improve the overall economics.

4.3. Effect of IL treatment on lignin characteristics

The structure of lignin undergoes numerous changes during IL pretreatment. Molecular weight distribution can be quantified with gel permeation chromatography (GPC), while linkage frequency can be quantified with Heteronuclear Single Quantum Coherence Nuclear Magnetic Resonance (HSQC NMR). Changes in lignin structure as a result of pretreatment with $[C_2C_1Im][OAc]$ were investigated at different treatment temperatures and times. Changes in β-*O*-4′, β-5′, and β-β' linkage frequencies were observed, along with a reduction in the molecular weight distribution (Wen et al., 2014). GPC analysis of lignin extracted from switchgrass with dilute [Ch][Lys] found lignin in the liquid stream had a lower molecular weight than lignin found in the liquid stream after dilute acid and ammonium hydrolysis pretreatment (Liu et al., 2017). GPC and HSQC NMR provide helpful information regarding changes to the lignin structure. Due to the heterogeneous nature of lignin, it is challenging to elucidate lignin depolymerization mechanisms in ILs. β-*O*-4′ linked lignin model compounds have been used to elucidate probable depolymerization mechanisms of methylimidazolium ILs (Cox et al., 2011). Model lignin compounds enable researchers to better investigate the effects of IL treatment on common aryl ether or carbon-carbon linkages found in real lignin (Zakzeski et al., 2010b).

4.4. Rational for pairing LDE and IL for lignin depolymerization

Taken together, given the unique properties of certain ILs that provide for lignin solubility and biocompatibility to enzymes and microbes, there is a great opportunity to develop a new strategy for lignin extraction, depolymerization and upgrading *via* biocatalysis in ILs.

Instead of using harsh thermochemical processes, the relatively mild biochemical process facilitates the selective deconstruction of lignin and retains the functionality of the oligomeric lignols, thus helping to channel desirable aromatic products. Despite previous exploration of catalysis and enzyme activity in ILs, there is a gap in our fundamental understanding of the mechanisms determining the interactions of lignolytic enzyme and these solvents. Further exploration is needed into how the solvent properties (acidity, basicity and polarity), as determined by the cation and anion pairs impact the lignin solubility and enzyme activity in aqueous IL systems. Furthermore, in order to enable enzymatic reactions in IL, two aspects need further investigation: the development of biocompatible IL's with improved lignin solubility, and the selection of IL tolerant enzymes. These developments rely on a better understanding of the enzyme-IL interactions, such as the structural changes, surface charges, inhibition kinetics, enzyme stability and the structure-function relationship of enzyme in IL media. Based on that, the LDE-IL system can be tuned to improve the product yield and selectivity.

5. Enzyme-IL interactions

5.1. Enzyme activity and stability in ILs

Certain ILs, as part of their highly tunable nature, can provide an environment suitable for enzymatic activity. Cellulase (Bose et al., 2010), laccase (Alberto Domínguez et al., 2011; Galai et al., 2015; Harwardt et al., 2014), lipase (Lai et al., 2012; Lozano et al., 2001), α-chymotrypsin (Arai et al., 2010; Attri et al., 2011; Lai et al., 2012), tyrosinase (Lai et al., 2012), and lysozyme (Hekmat et al., 2007) activity have all been tested in the presence of IL (Table 2). Numerous studies have attempted to elucidate the IL characteristics affecting enzyme's activity and stability in IL. Efforts have also been made to identify enzymes with improved resistance to enzymatic inhibition caused by IL.

5.1.1. Enzyme activity in IL

A wide variety of factors are thought to play a role in enzyme activity in ILs. There is a clear trend that persists among studies performed: the higher the IL concentration, the more severe the inhibition observed. Laccase from *T. versicolor* was screened for activity and stability in a series of methylimidazolium ILs (Domínguez et al., 2011). Increasing concentrations of ILs resulted in increased levels of enzymatic inhibition; while 1-ethyl-3-methylimidazolum ethylsulfate ($[C_2C_1Im][EtSO_4]$) caused less inhibition to laccase activity as compared to $[C_4C_1Im][Cl^-]$. Similarly, as shown in another study, *T. versicolor* laccase activity decreased with increasing IL concentration of the two tested alkyl imidazolium ILs, $[C_2C_1Im][EtSO_4]$ and $[C_2C_1Im][OAc]$ (Harwardt et al., 2014). This trend was not always true. Improve laccase activity has been observed in some ILs. Galai et al. screened *T. versicolor* laccase activity in 56 ILs and found that 13 of the ILs improved laccase activity; the most notable improvement was seen in choline dihydrogenphosphate ($[Ch][H_2PO_4]$) which increased laccase activity 451% (Galai et al., 2015). In addition to the enzyme activity, which is determined by short-term inhibitory effect, the long-term effect of IL on enzyme, in other words, the enzyme stability in IL, is equally important.

5.1.2. Enzyme stability in IL

ILs have been shown to stabilize enzymatic activity over varying periods of time. The long-term effects vary with respect to the IL-enzyme pair and IL concentration. Some ILs, like $[Ch][H_2PO_4]$, continue positively impact enzymatic activity over time (Galai et al., 2015; Hekmat et al., 2007). However, the long-term effects of several alkyl imidazolium ILs stands in contrast to their short-term effects. *T. versicolor* laccase in $[C_4C_1Im][Cl]$, $[C_2C_1Im][OAc]$, 1-ethyl-3-methylimidazolium $MDEGSO_4$ ($[C_2C_1Im][MDEGSO_4]$), and $[C_2C_1Im][EtSO_4]$ exhibited improved stability over varying periods of time, however, all ILs caused inhibition to enzyme activity in short term (Domínguez et al., 2011).

Certain ILs have also been shown to improve enzymatic resistance to inhibition caused by pH and thermal deactivation. For example, *T. versicolor* laccase in 10 mM $[Ch][H_2PO_4]$ retained activity up to pH 12, compared to pH 9 without IL (Galai et al., 2015). Short alkyl chain ammonium ILs, triethyl ammonium acetate [TEA][OAc] and triethyl ammonium phosphate $[TEA][PO_4]$ were shown to improve the thermal stability of α-chymotrypsin when compared to enzyme activity in 0.05 M pH 8.2 Tris-HCl buffer (Attri et al., 2011). Not surprisingly, the ability of ILs to stabilize enzymes is sensitive to the cation and anion present; the thermostability of RNase A was improved in $[Ch][H_2PO_4]$ whereas choline chloride ([Ch][Cl]) did little to enhance thermostability (Constatinescu et al., 2010). This anion-specific effect can be partially explained by the Hofmeister series: $H_2PO_4^-$ has been observed to be less chaotropic than Cl^- when paired with the same cation (Liu et al., 2018b; Weingartner et al., 2012; Zhao and Song, 2007). In spite of the numerous studies of IL-enzyme interactions, the exact mechanisms by which ILs affect enzyme stability are still largely uncertain.

Table 2
Studies of enzyme activity and stability in ILs.

Enzyme	Source	Ionic liquid	Reference
Laccase	*T. versicolor*	Alkyl imidazolium	Alberto Domínguez et al. (2011) and Harwardt et al. (2014)
Laccase	*T. versicolor*	56 ILs	Galai et al., 2015
Cellulase	*T. reesei*	$[C_2C_1Im][OAc]$	Wang et al. (2011)
β-Glucosidase	*A. niger*	$[C_2C_1Im][OAc]$	Wang et al. (2011)
Laccase	*T. versicolor*	$[Ch][H_2PO_4]$	Galai et al., 2015
Laccase	*Aspergillus* (DeniLite Base)	$[C_2C_1Im][MDEGSO_4]$	Tavares et al. (2008)
Lysozyme	Chicken egg white	Ethanolammonium formate Ethylammonium nitrate Bis(2-methoxyethyl)ammonium acetate *N*,*N*-dimethylethanolammonium glycolate $[Ch][H_2PO_4]$	Hekmat et al. (2007)
Laccase	*T. versicolor*	$[C_2C_1Im][EtSO_4]$ $[C_2C_1Im][OAc]$	Harwardt et al. (2014)
Laccase	*T. versicolor*	$[Ch][H_2PO_4]$	Galai et al., 2015
α-Chymotrypsin	Bovine pancreas type II	Triethylammonium acetate Triethylammonium phosphate 1-benzyl-3-methylimidazolium chloride $[C_6C_1Im][Cl]$ 1-benzyl-3-methylimidazolium tetrafluoroborate $[C_6C_1Im][BF4]$ Tetra-butylphosphonium bromide	Attri et al. (2011)
RNase A	*N/A*	$[Ch][H_2PO_4]$ $[C_2C_1Im][DCA]$	Constatinescu et al. (2010)

5.2. Effect of IL on enzyme

Investigating the changes of enzyme structure provides important information about enzyme stability in different ILs with varying properties. The structure of an enzyme is critical for its function, whereas enzyme-IL interactions may cause structural changes of the enzyme that further impact its activity and stability. The enzyme-IL interactions are dependent on IL properties and the enzyme sources. Thus, an in-depth understanding of enzyme structure and enzyme-IL interactions is critical for designing future biocatalytic systems in ILs.

5.2.1. Enzyme structure

Circular Dichroism (CD) spectroscopy can help understand changes made to enzyme structure in ILs by highlighting secondary structures of the enzyme. CD spectroscopy of *T. versicolor* in [Ch][H_2PO_4] revealed that addition of IL resulted in a conformation change around the active site, along with a shift in the ratio of α/β structures (Galai et al., 2015). Enzyme crystallization in IL and resolving the structure could provide a more comprehensive view of structural changes; this approach will be discussed later in the review.

5.2.2. IL properties affecting enzymatic activity

There have been a myriad of in-depth reviews discussing the different IL properties responsible for enzymatic inhibition and stability (Forsyth et al., 2002; Kaar et al., 2003; Klahn et al., 2011; Moniruzzaman et al., 2010; Naushad et al., 2012; Park and Kazlauskas, 2001; Weingartner et al., 2012; Zhao, 2010; Zhao et al., 2006). Each of the reviews contains tables summarizing studies examining different IL properties and their effect on enzymatic activity and stability. Polarity, alkyl chain length of the cation, hydrophobicity, anion, and viscosity are common factors discussed in each of the reviews. Increased cation chain length, increased anion size, and decreased polarity (a consequence of chain length and anion size) were all factors associated with reduced α-chymotrypsin activity (Lozano et al., 2001). Thermostability of α-chymotrypsin in ILs revealed that ILs with imidazolium and phosphonium-based cations were weak stabilizers, while ILs with ammonium-based cations were strong stabilizers (Attri et al., 2011).

Increasing cation hydrophobicity and decreasing anion nucleophilicity has been shown to increase lipase activity (de los Ríos et al., 2011). Two enzymes, a lipase and a tyrosinase, were shown to be biocompatible with aqueous IL made of chaotropic cations and kosmotropic anions (Lai et al., 2011). Results indicate that in general ILs with choline or ammonium cations activated lipase while imidazolium ILs deactivated the enzyme. More specifically, ammonium ILs composed of chaotropic cations (favorably with H-bonding capability) and kosmotropic anions are favored for enzyme catalysis (Lai et al., 2011). The interactions between a lipase and several ILs were simulated with molecular dynamics (MD) simulations, revealing dominant Coulombic and van der Waals interactions (Klahn et al., 2011). The same simulations also showed some [C_4C_1Im] cations diffused into the active site, implicating competitive inhibition. There is no consensus as to which, if any, how IL property dominates the impact on enzyme activity and stability. Regardless, there have been numerous efforts focused on improving enzyme activity in ILs.

5.3. Methods improving enzyme stability in IL

In an effort to combat the inhibitory effects of ILs on enzymatic activity, a number of methods have been explored. Most predominant of these approaches are immobilization of the enzyme on a substrate, screening enzymes from thermophilic and halotolerant organisms, and engineering the enzyme surface residues. Each of these methods has been successfully demonstrated with different enzymes.

5.3.1. Immobilization

Enzymes can be attached to an inert, insoluble material *via* different mechanism such as adsorption, entrapment, cross-linking, *etc.* Enzyme immobilization has proven to be an effective method of improving enzyme activity and/or stability in less than optimal conditions (Bickerstaff, 1997). Furthermore, immobilized enzymes have better recyclability than free enzymes. Immobilization strategies have been successfully employed to improve enzyme activity in ILs. In one study, immobilization of lipase on acrylic resins improved yields of biodiesel in 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4C_1Im][PF_6]) (Lai et al., 2012). Laccase from *Myceliophthora thermophila* was immobilized on glyoxyl-agarose beads, improving activity and thermostability in [C_2C_1Im][$EtSO_4$] at 70 °C (Fernández-Fernández et al., 2014). Several other enzyme immobilization strategies (on solid support, sol–gel, and cross-linked enzyme aggregate, CLEA), have been reviewed previously and will not be discussed here (Zhao, 2010).

5.3.2. Thermophilic and halotolerant enzymes

Thermotolerant and halophilic organisms have evolved to produce enzymes with resistance to harsh environments. This selective pressure from the environment has enabled these enzymes to potentially resist the inhibitory effects of ILs. Cellulases from a hyperthermophilic bacterium and archaeon retained activity in two-fold higher [C_2C_1Im][OAc] concentration than their mesophilic counterparts (Datta et al., 2010). In a follow-up study, Zhang et al. identified a halophilic cellulase from a halophilic archaeon and found it can tolerate relatively high concentrations (20% *w*/w) of several alkylimdazolium ILs (Zhang et al., 2011). The halophilic and IL-tolerant cellulase identified by Zhang et al. was found to have a large number of negatively-charged surface residues (Zhang et al., 2011). Similarly, cellulases from the halophilic *Aspergillus terreus* strains displayed improved activity and stability in high salt conditions and a series of alkyl imidazolium ILs, most notably [C_2C_1Im][OAc] (Gunny et al., 2014). Although, limited number of structural evidences exist to explain what causes the improved resistance of those enzymes to IL, it's possible that the thermophilic cellulases evolved to have more negatively-charged surface residues than the mesophilic cellulase. Several studies have suggested that reducing the number of positively-charged surface residues and increasing the number of negatively-charged surface residues improve enzyme resistance to ILs (Burney et al., 2015; Nordwald et al., 2014). The surface characteristics enabling enzyme tolerance to IL have inspired artificial ways to modify enzyme surfaces.

5.3.3. Charge engineering

Altering the charged residues on the surface of the enzyme could affect the ability of the ILs to bind to the enzyme (Fig. 8). This reduction in binding affinity would reduce IL inhibition. Reducing the amine:acid ratio of surface residues has been shown to improve activity of several enzymes in [C_4C_1Im][Cl] (Nordwald et al., 2014). Later molecular dynamics simulations of modified lipase and α-chymotrypsin showed reduced anion binding and increased cation binding on the enzyme surface (Burney et al., 2015). Surface modification of enzyme by introducing protein–polymer surfactant nanoconstructs was shown to greatly improve enzyme solubility in hydrophilic and hydrophobic ILs (Brogan and Hallett, 2016). Applying this method, Brogan et al. (2018) recently demonstrated that chemically modified glucosidase was thermally stable at up to 137 °C and 30 times more active toward cellulose in [C_2C_1Im][$EtSO_4$] as compared to reactions in aqueous media due to solvent-induced substrate promiscuity of glucosidase.

6. Protein engineering to improve IL compatibility

6.1. Structure-function relationship of proteins

The structural hierarchy of proteins can be broken down into 4 levels: primary, secondary, tertiary, and quaternary. The primary structure of a protein is the sequence of amino acids. The secondary structure captures how those amino acids are folded into α-helices, β-sheets,

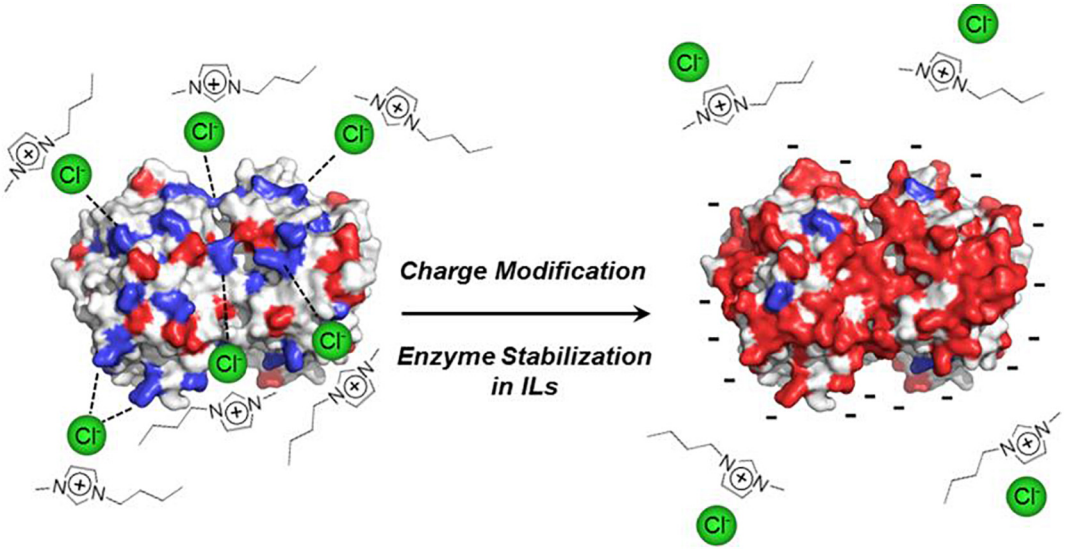

Fig. 8. Effect of surface charge modification of IL interaction with enzymes (Nordwald, 2015).

or loops. The tertiary structure captures how the secondary structures fold together to form the final protein structure; oftentimes this is accomplished by an intricate network of hydrogen bonds, salt bridges, and disulfide bonds (Dwivedi et al., 2011; Hildén et al., 2009). The quaternary structure is defined by tertiary structures forming higher-order structures such as dimers or trimers. Each of these structural levels plays a key role in the function of proteins.

6.1.1. Primary structure

Directed mutagenesis of key residues in a variety of enzymes has implicated the ubiquitous role primary structure plays in protein function. These residues have been shown to be responsible for a variety of key functions of the protein.

6.1.1.1. Substrate recognition and binding. In many proteins, key amino acids have been implicated in the binding of the substrate. Mutations of 2 residues near the heme access channel, H82A and Q222A, and 2 residues near the surface of the protein, W171A and F267L, revealed the importance of each residue for LiP substrate binding and function (Sollewijn Gelpke et al., 2002). Mutation of Asp376 to Asn, Glu, or Thr at the catalytic phosphorylation site of the α subunit leads to loss of ATPase activity (Ohtsubo et al., 1990). It was found that leptin binding to the leptin receptor of a variety of species is highly specific to the residues present on both the receptor and leptin (Prokop et al., 2012). Mutations of residues near the surface of Type III antifreeze protein (AFP) resulted in loss of activity (Chao et al., 1994). Oftentimes, the residues responsible for enzymatic activity have a net charge at physiological pH.

Altering the primary structure of an inactive enzyme can alter the enzyme activity and substrate affinity. A heme peroxidase from *Coprinus cinereus* was unable to catalyze the oxidation of veratryl oxidase, a common substrate used for screening lignolytic enzymes. The inactive heme peroxidase has a structure similar to LiP, so a number of mutations were made using the active LiP as a template (Smith et al., 2009). A Trp residue (Trp179) was introduced to interact with veratryl alcohol, while D179W, R258E, and R272D mutations created an environment mimicking that found around the catalytic Trp residue in the active LiP (Smith et al., 2009). All of these mutations enabled the previously inactive heme peroxidase to oxidize veratryl alcohol. This approach allows identification of primary structure that are crucial for proper enzyme function.

6.1.1.2. Charged residues. Charged residues on the surface of proteins can be implicated when binding to a charged substrate. For instance, alanine mutations of a series of positively-charged Lys and Arg residues resulted in decreased activity in proliferating cell nuclear antigen (PCNA) (Fukuda et al., 1995). Key mutations of charged residues of TRPV1 and TRPV4 dramatically reduce the permeability of calcium and magnesium ions (Owsianik et al., 2006). TRPC1 and TRPC5 channel permeation properties have been shown to be highly dependent upon negatively charged residues close to the pore region (Owsianik et al., 2006). As discussed in Section 5.3, modification to the charged residues on surface of the enzyme has proven effective to reduce IL inhibition by altering IL binding affinity to enzymes.

6.1.2. Secondary structure

As amino acids begin interacting with each other, they start forming higher-order structures. The presence of these secondary structures can impact the function and stability of a protein. *T. versicolor* laccase experienced a shift in the ratio of α-helices and β-sheets in the presence of [Ch][H_2PO_4], likely due to anion-peptide interactions, though the exact reason why this shift occurred was not confirmed (Galai et al., 2015). The β-sheet near the C-terminus of a Type III antifreeze protein is thought to be responsible for antifreeze activity (Chao et al., 1994). The secondary structure of a laccase is related to its thermostability. For instance, bacterial laccase has a coiled section connecting 2 domains, whereas plant and fungal laccases do not; for that reason bacterial laccase are typically more thermostable than fungal and plant laccases (Dwivedi et al., 2011; Hildén et al., 2009).

6.1.3. Tertiary and quaternary structure

Tertiary and quaternary structure have been shown to affect a wide variety of protein functions. Changes to the tertiary and quaternary structure of the Tobacco Mosaic Virus Coat Protein have been shown to significantly affect the immune system response of the host (Culver et al., 1994). An N-terminus domain of a protein from *Bacillus stearothermophilus* is responsible for anchoring the protein to the cell wall of the organism (Jarosch et al., 2001). The structure of G-protein coupled receptors (GPCRs) is critical in determining their conformation and, as a result, their function (Katritch et al., 2013). When a GPCR receptor is unbound, it adopts an inactive conformation; once it is bound by an agonist, the structure of the GPCR receptor changes to an active form (Katritch et al., 2013). Although few studies have examined IL effects on tertiary structure, it is reported that the tertiary structure of the immunoglobulin binding domain of streptococcal protein B disrupted in high concentrations (60% *v*/v) of [C_4C_1Im][Br] using high-resolution

magic-angle-spinning NMR spectroscopy (Warner et al., 2016). Knowledge of IL's impact on protein structure provides vital information for protein engineering strategies to improve enzymes' IL tolerance.

6.2. Protein engineering for improved function

To improve function of proteins under environmental stresses (high pH, high temperature, *etc.*), key residues can be identified and mutated. The library obtained from screening mutations of residues grows at an enormous rate as more sites are mutated, a consequence of the number of possible amino acids at each position (20^n, n = number of sites mutated). When screening mutations at 4 sites, the library will contain 20^4 = 160,000 entries. Addition of just 1 more site mutation increases the size of the library to over 3 million entries. It's for this reason that most mutation studies are limited to just a few sites. Despite this limitation, impressive results have been obtained by mutations of just a few sites on many proteins. Two protein engineering approaches are commonly used for improving protein function: directed evolution and rational design (Bornscheuer and Pohl, 2001). The ultimate goal of both approaches is the same, which is a protein with improved stability in a harsh environment. What sets the approaches apart is the size of the generated library of mutants. Directed evolution usually generates a much larger library of mutants than rational design.

6.2.1. Directed evolution

Directed evolution mimics the environmental stresses to an organism while is performed in lab using methods like error-prone PCR for diversity generation (random mutagenesis) and screenings in iterative cycles (Arnold, 1998). The organisms producing the best performing enzymes are subjected to another round of, and in most cases more severe, environmental stress. The screening procedure is repeated until a desired optimal protein is obtained. Throughout the screenings the proteins are sequenced and key residues that improve performance are identified. In one example, Martin et al. generated a thermostable mutant of a mesophilic cold shock protein through the use of directed evolution (Martin et al., 2001). Through application of high heat and high salt stresses, a mutant was produced that outperformed a thermophilic cold shock protein. By sequencing the mutants, key residues responsible for improving the thermostability of the protein can be identified. A thermophilic fungal laccase from *Myceliophthora thermophila* was subjected to repeated rounds of directed evolution in organic solvents, in the process developing an affinity for higher pH environments (Alcalde et al., 2006). Other directed evolution strategies involving laccases are primarily focused on improving expression in non-native organisms, *i.e.* fungal laccase expression in yeast (Mate and Alcalde, 2015).

6.2.2. Rational design

Rational design requires the knowledge of the residues thought to be responsible for improving a protein's tolerance to environmental stresses. These residues are often identified through the use of a homolog. The crystal structure of MnP from *C. subvermispora* was used as a template for directed mutagenesis of a *P. ostreatus* versatile peroxidase (VP), producing 2 variants with improved resistance to a low pH environment (Fernández-Fueyo et al., 2014). Identifying residues in ancestral forms of the enzyme, a triple mutant (H239F/T240L/I241L) LiP from *P. chrysosporium* was engineered for improved activity at high temperatures and stability in the presence of H_2O_2 (Semba et al., 2015). Threonine residues surrounding the type I copper in *B. subtilis* CotA, T415 and T418, were the focus of a rational design strategy by Khodakarami et al. (2018) with the goal of improving activity and substrate selectivity. The T415I mutation improved activity with ABTS 4-fold, whereas selectivity toward ABTS was 11 times higher than syringaldazine in the same mutant (Khodakarami et al., 2018). A fungal laccase from *T. versicolor* exhibited improved resistance to the IL $[C_2C_1Im][EtSO_4]$ when mutations were made to loop residues connecting domains 2 and 3 (Wallraf et al., 2018). While rational design can be a powerful tool for improving enzyme function in stressful environments, it can be limited by lack of knowledge about the protein's sequence and structure.

6.3. Protein crystallization in ILs

Crystallizing proteins in ILs could provide more detailed insight into the binding locations of ILs on the surface of proteins. Crystallization of proteins in ILs has been shown to, in some cases, improve crystal quality, size, and number. When trypsin and lysozyme were crystallized in ILs, crystal number and quality were improved; resolution in $[C_4C_1Im][BF_4]$ was improved from 2.8 Å to 2.1 Å (Judge et al., 2009). Addition of ILs resulted in formation of larger lysozyme crystals (Hekmat et al., 2007). When trypsin was crystallized in ILs, some fragments of the ILs were found to have been incorporated as part of the protein structure (Kennedy et al., 2011). Numerous studies have highlighted the benefits of using ILs for protein crystallization, though none have identified IL binding sites on the protein's surface. Docking simulations can be used to identify IL binding sites, as seen in the 2017 study by Anbarasan et al. with a thermophilic xylanase in biomass-dissolving ILs (Anbarasan et al., 2017). To get a clearer picture, however, crystallization of proteins in ILs is critical for elucidating the effects of enzyme-IL interactions.

7. Product selectivity

Due to the complex nature of lignin, controlling product formation from lignin depolymerization proves difficult. A biocatalysis system coupling the LDEs and ILs opens opportunities in directing product formation from lignin degradation. The selectivity is rooted from both the tunable properties of ILs and the specificity of LDEs. Additionally, use of a mediator alongside LDEs (*i.e.* laccase) can increase the oxidation window thus extend the suite of products obtained from lignin. Use of capping agents has also been shown to prevent condensation of lignin depolymerization products.

7.1. Controlling product formation via biocatalyst selectivity

The subject of directing formation of products from lignin has already been explored through the use of a number of base, acid and metal catalysts for catalytically depolymerizing lignin in IL media (Behling et al., 2016; Chatel and Rogers, 2013; Das et al., 2017; Xu et al., 2014; Zakzeski et al., 2011; Zakzeski et al., 2010b). The use of a biocatalyst, however, could potentially offer improved selectivity at less severe reaction conditions. Laccase uses O_2 as the final electron acceptor in its oxidative mechanism, and there is no need for harsh temperature/pressure and costly catalysts. In addition, a number of ways can be used to tune the biocatalyst for different products. One way is *via* the use of a mediator such as 1-hydroxybenzotriazole (HBT) and ABTS. Introduction of a mediator helps increase the oxidative potential of laccase, thus increasing the number of products obtained by enabling laccase to oxidize non-phenolic compounds (Arora and Sharma, 2010; d'Acunzo et al., 2006). Furthermore, the source of enzymes could potentially affect the products obtained from lignin as a consequence of the oxidative mechanisms. For instance, white rot fungi typically utilize several oxidative enzymes, such as LiP, MnP, and laccases (Bugg et al., 2011a) while brown rot fungi utilize a Fenton redox reaction when degrading lignin (Jensen et al., 2001; Vanden Wymelenberg et al., 2010). Recent discoveries of new classes of LDEs that selectively cleave C−C bonds, hold great promise since this capacity is unrivalled by common metal catalysts (Guengerich and Yoshimoto, 2018; McAndrew et al., 2016).

7.2. Controlling product formation via IL selectivity

ILs are capable of directly influencing the products obtained from lignin degradation. It has been demonstrated that acidic ILs are capable of cleaving the β-*O*-4′ linkage (Cox et al., 2011). Additionally, Dutta et al. (2017) demonstrated that product formation from lignin degradation was a function of the IL used and the treatment temperature. At high temperatures [Ch][Lys], [TEA][HSO_4], and [C_2C_1Im][OAc] were shown to favor formation of guaiacol, guaiacylacetone, and both guaiacol and guaiacylacetone, respectively. This product selectivity was only observed at high temperatures and high IL concentrations.

Several ILs have been shown to be capable of shifting product formation of biocatalysts. Increasing concentration of ILs was correlated with an increase in the monophenolase:diphenolase activity ratio of a tyrosinase (Goldfeder et al., 2013). A protic IL, triethylammonium mesylate increased the selectivity of lipase toward omega-3 fatty acids (Akanbi et al., 2012). Product selectivity of a sialidase was shown to be highly sensitive to the amount and the type of IL present in the system (Zeuner et al., 2014). Pairing the product selectivity offered by both the LDEs and ILs could potentially lead to more effective lignin depolymerization pathways.

7.3. Preventing lignin repolymerization and improving yield

One of the primary issues encountered when dealing with lignin decomposition, along with directing product formation, is that of lignin repolymerization. The intermediates formed during lignin decomposition are susceptible to repolymerizing rather than forming monomeric, dimeric, or oligomeric products. Under acid and/or high temperatures conditions commonly used for lignin extraction, stable carbon-carbon (C—C) bonds are formed once the lignin ether bonds are cleaved. This leads to severe and irreversible condensation that dramatically affects further upgrading of lignin. When examining depolymerization of organosolv lignin with base catalysts, it also was found that lignin repolymerization occurred; S-lignin was the primary subunit involved in the repolymerization reactions (Toledano et al., 2012). Condensation has also been observed in enzymatic depolymerization strategies. Lignin model compounds and dimers underwent condensation when treated with fungal laccases from *Melanocarpus albomyces* and *Trametes hirsuta* (Lahtinen et al., 2009). Interestingly, formation of some condensed lignin structures appeared at different times dependent upon the redox potential of the laccase. Preventing repolymerization could improve product yields from lignin depolymerization.

Perhaps the most promising and widely researched method for preventing lignin repolymerization is the use of capping agents. Several capping agents have been demonstrated to improve lignin product yield. Boric acid and phenol were used as capping agents, with phenol improving product formation from organosolv olive tree pruning lignin (Toledano et al., 2014). A phenol:lignin ratio of 2:1 resulted in the highest oil yield and lowest repolymerization. Phenol as a capping agent is not without its limitations, however; increasing the phenol:lignin ratio beyond 2:1 resulted in the formation of higher molecular weight lignin, which is likely a result of the excess phenol reacting with products and residual lignin. Formaldehyde has also been used as a capping agent for stabilizing lignin during biomass pretreatment, greatly improving yields of guaiacyl and syringyl monomers close to the their theoretical limits (Shuai et al., 2016). Using a capping agent alongside an enzyme-IL pair could improve product yields while enabling selective product formation.

The time and enzyme quantity required for significant biocatalytic lignin valorization will also need to be investigated as a potential roadblock toward improving yield. While most studies reported hours to days of reaction time used for enzymatic lignin depolymerization in ILs. The reaction times are generally comparable to enzymatic depolymerization of other biopolymers in IL, such as cellulose and hemicellulose. Studies investigating the sugar (xylose and glucose) yields from enzymatic saccharification after pretreatment of biomass with dilute [TEA][HSO_4] or [C_2C_1Im][OAc] required 24-72 h and several mg protein/g biomass to achieve the maximum sugar yields (Gschwend et al., 2018; Shi et al., 2014). Given the heterogeneous nature of lignin it's likely that the reaction times and enzyme quantities required for enzymatic lignin depolymerization, prior to enzyme engineering, will be similar or even greater. However, it is projected that the efficacy can be improved by enzyme engineering for better enzymes and/or by process intensification and optimization (*i.e.* immobilized enzymes) for better enzyme stability and recyclability.

8. Conclusions and perspectives

The great potential for lignin to be a renewable feedstock for commercial chemicals is hindered by its structural heterogeneity and recalcitrance. With such an intense research focus in recent years, great strides have been made in developing lignin valorization strategies. ILs have been proven to be a capable class of lignin solvents and guiding principles of designing biocompatible ILs have been proposed based on both experimental and computational studies. Thermophilic and IL-tolerant LDEs have been explored for increased resistance to harsh environments, highlighting their potential to be used alongside biocompatible ILs. However, further research is needed to elucidate the LDE-IL interactions for the purpose of lignin valorization. Toward this end, several research areas appear important: 1) Utilizing the suite of structural biology tools to explore the LDE-IL interactions and how they affect the lignolytic capabilities of the biocatalysts; 2) Protein engineering strategies can be applied to create novel LDEs that can withstand high concentrations of ILs while maintaining their lignolytic properties; 3) Understanding the interaction between IL and LDEs for better product selectivity and developing strategies to prevent lignin condensation in IL; 4) Test the effectiveness of lignin-IL-enzyme systems on industrial relevant polymeric lignin streams on top of model compounds. Collectively, this review highlights the tremendous potential of designing LDE-IL pairs for lignin valorization in the IL medium.

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