Modifying surface charges of a thermophilic laccase toward improving activity and stability in ionic liquid

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Abstract

The multicopper oxidase enzyme laccase holds great potential to be used for biological lignin valorization alongside a biocompatible ionic liquid (IL). However, the IL concentrations required for biomass pretreatment severely inhibit laccase activity. Due to their ability to function in extreme conditions, many thermophilic enzymes have found use in industrial applications. The thermophilic fungal laccase from *Myceliophthora thermophila* was found to retain high levels of activity in the IL, $[C_2C_1Im][EtSO_4]$, making it a desirable biocatalyst to be used for lignin valorization. In contrast to $[C_2C_1Im][EtSO_4]$, the biocompatibility of $[C_2C_1Im][OAC]$ with the laccase was markedly lower. Severe inhibition of laccase activity was observed in 15% $[C_2C_1Im][OAc]$. In this study, the enzyme surface charges were modified *via* acetylation, succinylation, cationization, or neutralization. However, these modifications did not show significant improvement on laccase activity or stability in $[C_2C_1Im][OAc]$. Docking simulations show that the IL docks close to the T1 catalytic copper, likely interfering with substrate binding. Although additional docking locations for $[OAc]^-$ are observed after making enzyme modifications, it does not appear that these locations play a role in the inhibition of enzyme activity. The results of this study could guide future enzyme engineering efforts by showing that the inhibition mechanism of $[C_2C_1Im][OAc]$ toward *M. thermophila* laccase is not dependent upon the IL interacting with the enzyme surface.

Introduction

Lignin is among the most abundant terrestrial biopolymers that makes up ~25% of the weight of plant biomass [1; 2]. It is primarily made of 3 phenylpropanoid subunits: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) that can be linked together *via* several condensed (C-C) or ether (C-O) bonds, with the phenylcoumaran (β – 5), β – aryl ether (β – O – 4), and pinoresinol (β – β) being the most common [3; 4; 5; 6]. Additionally, the ratio of subunits present in the lignin network is dependent upon the source of the biomass [7]. As a result of lignin's structural and monomeric heterogeneity, thermochemical and biological methods achieve poor selectivity and yield of breakdown products. Lignin is currently generated in high volumes as a waste product by the paper and pulping industry and during production of cellulosic biofuels [8; 9]. Converting lignin from a waste product to value added chemicals *via* new technologies will not only reduce waste lignin accumulation but also add value to the paper & pulping and future cellulosic biorefineries [10; 11]. While the applications of polymeric lignin are limited, the phenolic products obtained from lignin deconstruction can be used as fuels or precursors for chemicals in the food, pharmaceutical, or plastic industries; many of these fuels and chemicals are currently derived from petroleum [12; 13].

Current methods for lignin deconstruction can be divided into thermochemical (*e.g.* high temperature pyrolysis, catalytic oxidation, and hydrogenolysis) and biological (*e.g.* lignin degrading enzymes [LDEs] or microbes) [14; 15; 16; 17]. Much of the research into the thermochemical methods has focused on improving the catalyst performance at high temperatures and improving the selectivity of lignin-derived products [18; 19; 20]. The use of LDEs in biological lignin deconstruction strategies could enable lignin deconstruction at lower temperatures and improve product selectivity due to the inherent selectivity and efficiency of

biocatalysts [16]. Many LDEs such as the peroxidases (lignin, manganese, and versatile) and laccases have been discovered in bacteria, plant, and fungi [21; 22; 23]. Additionally, catabolic lignin pathways in soil bacteria, such as *Sphingobium* sp. SYK-6 that catabolizes lignin using NAD or glutathione dependent enzymes have recently been identified [24]. The application of LDEs for lignin deconstruction has been limited by a number of factors, chief among these being the high cost of enzyme production and the low yield of products due to the poor solubility of lignin in solvents biocompatible with LDE activity [25; 26].

Many of the known lignin solvents (*e.g.* dimethyl sulfoxide [DMSO] and alkaline solutions) reduce or altogether eliminate enzyme activity [27; 28]. Additionally, these solvents require high temperatures to facilitate the complete dissolution of plant derived lignin, leading to a further loss in enzyme activity [29]. Therefore, a solvent system that is capable of solubilizing lignin at low temperatures and mild pH is desirable. Ionic liquids (ILs) are molten, organic salts liquid at temperatures <100ºC, the properties of which can be tuned by selecting the appropriate cation and anion [30; 31; 32; 33]. The alkylimidazolium ILs, such as 1-ethyl-3-methylimidazolium acetate ($[C_2C_1Im][OAc]$) and 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$), have been the focus of numerous studies on biomass pretreatment at relatively low temperatures [34; 35; 36]. Due to the high cost associated with using these ILs, recent efforts have focused on developing low cost ILs from bioderived cations (*e.g.* choline and ammonium) and anions (*e.g.* carboxylic acids and amino acids) that can be used in low concentrations during pretreatment [37; 38; 39]. One example is cholinium lysinate ([Ch][Lys]) which, at only 10% (w/v) in water, was shown to remove 80% of lignin from genetically engineered switchgrass during pretreatment [40].

Laccases are a member of the multicopper oxidase superfamily of enzymes (E.C. 1.10.3.2). They were first discovered in extract from the Japanese lacquer tree (*Toxicodendron vernicifluum*)

in 1883 [41]. Laccases differ from the other group of lignolytic enzymes, the heme peroxidases, in that they are copper-containing enzymes that do not require the presence of a strong oxidant, such as hydrogen peroxide, for the reaction mechanism [42; 43]. The substrate is oxidized at the T1 catalytic copper *via* 4-electron removal, after which the electrons are shuttled 13Å to the trinuclear copper cluster where molecular oxygen is reduced to water [44]. The T1 catalytic copper and the trinuclear coppers (a T2 and 2 T3) are coordinated by 4 conserved HXH motifs; X = cysteine in the case of the T1 copper [42]. The T1 copper is also coordinated by a methionine or leucine/phenylalanine in bacterial/plant and fungal laccases, respectively [45].

Laccases are also capable of oxidizing nonphenolic lignin compounds when used alongside a small molecule mediator, such as 1-hydroxybenzotriazole (HBT) or 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [46; 47; 48]. The relatively mild reaction conditions required for laccase activity along with the oxidation of mediator compounds make laccases a desirable biocatalyst for use in many biotechnological applications, including biological lignin deconstruction strategies [49; 50]. Many ILs have been shown to be biocompatible with the activity of laccases at low concentrations in water. When Galai *et al.* investigated the effect of 56 ILs on the activity of *T. versicolor* laccase for dye decoloring applications, they found that 13 of the ILs increased the activity of the laccase; 10 mM choline dihydrogen phosphate ([Ch][H_2PO_4]) increased activity by 451% [51]. The initial activity of laccase from the white rot fungus *Trametes versicolor* was only reduced by 20% in a reaction mixture containing 50% (v/v) 1-ethyl-3-methylimidazolium ethylsulfate ([C_2C_1Im][EtSO4]) [52]. However, other alkylimidazolium ILs have been found to be less biocompatible with laccase activity. The initial activity of *T. versicolor* laccase was reduced by 50% in reaction mixtures containing just 3, 20, 40, and 5% (v/v) of

98 [C_2C_1Im][OAc], [C_4C_1Im][Cl], 1-hexyl-3-methylimidazolium bromide ([C_6C_1Im][Br]), and 1-
99 decyl-3-methylimidazolium chloride ([$C_{10}C_1$Im][Cl]), respectively [52; 53].

100 Recent studies have shown that ILs are more biocompatible with thermophilic enzymes
101 compared to mesophilic enzymes. Hydrolysis of carboxymethylcellulose (CMC) by
102 hyperthermophilic (T_{opt} > 95°C) and thermophilic (T_{opt} = 80°C) cellulases was minimally impacted
103 in 0 – 20% (v/v) [C_2C_1Im][OAc] while a mesophilic cellulase (T_{opt} = 37°C) was unable to
104 hydrolyze CMC in only 10% (v/v) [C_2C_1Im][OAc] [54]. Cellulases and xylanase secreted by
105 *Galactomyces sp.* showed improved tolerance to IL [Mmim][DMP] and effective hydrolysis of
106 chestnut shell [55]. Previous work also demonstrated integration of biocatalyst, e.g., ω-
107 transaminase and L-alanine dehydrogenase into a chemoenzymatic process to convert biomass to
108 valuable products such as furfurylamine [56; 57]. The activity of laccase from the thermophilic
109 fungi *Myceliophthora thermophila* (*MtL*) is increased 3-fold in 25% (v/v) [C_2C_1Im][$EtSO_4$] [58].
110 The bacteria *Bacillus subtilis* produces a laccase-like spore coat protein (CotA) that can oxidize
111 canonical laccase substrates ABTS and syringaldazine (SGZ) in the presence of several
112 alkylimidazolium chloride ([C_nC_1Im][Cl]) ILs; [C_nC_1Im][Cl] ILs have been shown to severely
113 inhibit the activity of a mesophilic laccase at low concentrations [22; 59]. However, we showed
114 in a previous study that the most thermophilic laccase identified to date, produced by the
115 hyperthermophilic bacterium *Thermus thermophilus*, is highly sensitive to low concentrations
116 (<10% v/v) of [C_2C_1Im][OAc], suggesting that enzyme thermophilicity alone does not guarantee
117 high activity in ILs [21].

118 In this study we sought to determine the biocompatibility of aqueous [C_2C_1Im][OAc] with
119 *MtL*. To do this we first screened the activity of *MtL* in different concentrations of [C_2C_1Im][OAc]
120 up to 50% in water. We also measured the effect of low concentrations of [C_2C_1Im][OAc] on the

thermostability of *MtL*. Following the initial biocompatibility and stability screenings, we made several surface charge modifications with the aim of improving *MtL* activity and stability in $[C_2C_1Im][OAc]$. We also used molecular docking simulations to understand how $[C_2C_1Im][OAc]$ docks to the surface of *MtL* and the 4 charge variants we produced. The results of this study expand our understanding of laccase-IL interactions and the difficulties faced when trying to improve enzyme activity in ILs.

2. Methods and Materials

2.1. Materials

Laccase from *M. thermophila* expressed in *Aspergillus oryzae* (Batch # OMN07029) was kindly provided by Novozymes (Bagsværd, Denmark). The 1-Step™ ABTS Substrate Solution was purchased from ThermoFisher Scientific (Waltham, MA). All other reagents, including the IL $[C_2C_1Im][OAc]$, were purchased from MilliporeSigma (St. Louis, MO).

2.2 Surface Charge Modifications

MtL was purified by sequentially spin filtering with 100 kDa and 30 kDa MWCO filters at 8°C prior to surface charge modifications. Surface charges were modified according to previously reported protocols [60; 61] and the induced surface charge modifications were summarized in Table 1. Cationized *MtL* was produced by modification with 0.5 M ethylenediamine hydrochloride in 200 mM MES buffer (pH 4.5) containing a 20:1 molar ratio of the crosslinking reagent *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC)-to-acid sites for 2.5 h at room temperature; neutralized *MtL* was similarly produced using diethylamine in place of ethylenediamine hydrochloride. Succinylated *MtL* was produced by addition of 4 aliquots of succinic anhydride to the enzyme in 1 M sodium carbonate buffer (pH 8.5) over 3 h at room temperature. Acetylated

143 *MtL* was produced by addition of 3 aliquots of acetic anhydride to the enzyme in 100 mM sodium
144 phosphate buffer (pH 7.0) over 1.5 h at room temperature. The final molar ratio of acetic or
145 succinic anhydride to enzyme containing primary amines was approximately 30:1. Excess reagent
146 was removed with 30 kDa MWCO spin filters at 4°C and the enzymes were stored in 100 mM
147 sodium phosphate buffer (pH 7.0) prior to experiments. Protein content was measured using the
148 Bradford assay.

149 *2.3. Biocompatibility Screening*

150 The biocompatibility of $[C_2C_1Im][OAc]$ in aqueous solution (approximately 1-50% w/v),
151 was screened with all *MtL* variants. To reduce the effect of pH, the IL solution was adjusted to
152 pH 4.5 using 1 M hydrochloric acid prior to testing. Activity was screened with 50 mM citrate/100
153 mM phosphate buffer (pH 4.5), IL (0, 1, 2, 3, 4, or 10% w/v) and 50 µL ABTS solution in clear,
154 flat bottom, 96-well Costar assay plates (Corning Inc., Kennebunk, ME). Absorbance readings
155 were taken every 15 seconds for 10 min at 40 ºC in a SpectraMax M2 plate reader (Molecular
156 Devices, Sunnyvale, CA). Plates were shaken for 3 seconds prior to each reading to ensure a
157 homogeneous well solution. Oxidation of ABTS in buffer and AIL without laccase were measured
158 as blanks. The activity of *MtL* in ILs relative to buffer was calculated using equation 1.

159 $$Relative\ activity = \frac{Initial\ Velocity\ in\ IL}{Initial\ Velocity\ in\ buffer} \quad (1)$$

160 *2.4. MtL Thermostability*

161 To measure the thermostability of *MtL*, all charge variants were incubated at 40°C in 0,
162 2.5, or 5% $[C_2C_1Im][OAc]$ and 50 mM citrate/100 mM phosphate buffer, pH 4.5. At each time
163 interval (0, 30, 60, 120, 180, and 240 min) aliquots were removed and placed on ice prior to
164 measuring residual enzyme activity. Activity was screened using the same method in *4.3.3.*

Biocompatibility Screening. The residual activity of *MtL* and the charge variants after incubation was calculated using equation 2.

$$Residual\ activity = \frac{Initial\ Velocity\ after\ incubation}{Initial\ Velocity\ after\ 0\ \text{min}\ incubation} \quad (2)$$

2.5. Docking Simulations

The 3D structures and PDB files of the ligands were prepared in YASARA Structure (YASARA Biosciences GmbH, Vienna, Austria) using the SMILES strings obtained from PubChem. The PDBQT files of the ligands and *MtL* (PDBID: 6F5K) were prepared with AutoDock (version 4.2.6, MGLTools, La Jolla, CA). Charges for the coppers were added by manually editing the *MtL* PDBQT file. AutoGrid parameters were as follows: space value of 0.375Å, (x, y, z) grid centered at (4.840, 28.290, -18.567), and grid size of 126 in all directions. AutoDock parameters were as follows: Lamarckian GA, 100 genetic algorithm runs, and 25,000,000 max eval size. Docking results were visualized using PyMol (Schrodinger LLC, New York, NY). To simulate the surface charge modifications, target surface residues were mutated in PyMol. These mutations are shown in Table 1.

3. Results

3.1. Biocompatibility Screening

To understand the biocompatibility of *MtL* with $[C_2C_1Im][OAc]$, we measured the activity of *MtL* and all of the charged variants in aqueous solutions (0 – 50% w/v) of $[C_2C_1Im][OAc]$ in water. None of the surface charge modifications significantly improved the activity of *MtL* in aqueous $[C_2C_1Im][OAc]$ relative to the unmodified *MtL* (Figure 1a). Minimal inhibition of *MtL* activity is observed in 1 – 10% $[C_2C_1Im][OAc]$, however severe inhibition of *MtL* activity is observed in 15 – 50% $[C_2C_1Im][OAc]$. Since the coppers are not coordinated by any modifiable

187 residues, it is unlikely that the inhibition is due to the loss of the coppers. The range of
188 $[C_2C_1Im][OAc]$ concentrations tested in this study are based on recent reports that reduced lignin
189 removal, lower fermentable sugar yields, and lower cellulose solubilization are observed when less
190 than 50% IL is present in aqueous solutions of $[C_2C_1Im][OAc]$ and water [62].

191 The cation and anion of the IL play a role in determining the biocompatibility of the IL
192 with enzyme activity. Previous studies have shown that as the alkyl chain length of the cation
193 increases, alkylimidazolium ILs become more inhibitory [52; 59]. The inhibitory effect of the
194 anion has been shown to follow the Hofmeister series [63; 64]. ILs with chaotropic anions (*e.g.*
195 NTf_2^- and Br^-) more strongly destabilize proteins when compared to kosmotropic anions (*e.g.* SO_4^{2-}
196 and PO_4^{3-}). For example, the activity of *T. versicolor* laccase is increased 451% in 10 mM choline
197 dihydrogen phosphate ($[Ch][H_2PO_4]$), an IL with a kosmotropic anion, but is not affected by the
198 same concentration of choline bis([trifluoromethyl]sulfonyl)imide ($[Ch][NTf_2]$), an IL with a
199 chaotropic anion [51]. Nordwald et al. previously showed that acetylation and succinylation
200 improves enzyme activity and stability in 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$)
201 by reducing the interaction of the chaotropic Cl^- anion with the enzyme surface. Additionally, low
202 enzyme activity in higher concentrations of $[C_2C_1Im][OAc]$ can be attributed to the buffering
203 capacity of $[C_2C_1Im][OAc]$. Optimal ABTS oxidation occurs at pH < 5 for most fungal and
204 baterial laccases, but $[C_2C_1Im][OAc]$ buffers at a pH well above this range thereby lowering *MtL*
205 activity in high concentrations of $[C_2C_1Im][OAc]$ [65].

206 *3.2. Stability in $[C_2C_1Im][OAc]$*

207 Although the surface charge modifications did not improve the activity of *MtL*, we sought
208 to understand if the modifications improved the stability of *MtL* in $[C_2C_1Im][OAc]$. All charge
209 variants of *MtL* were incubated in 50 mM citrate/100 mM phosphate buffer (pH 4.5) with 0%,

2.5%, and 5% [C_2C_1Im][OAc] at 40°C and the activity was measured at different time intervals up to 4 hours. The surface charge modifications, by following the established protocols successfully tested on enzymes like cellulase and lipase, did not improve the stability of *MtL* in low concentrations of [C_2C_1Im][OAc] (Figure 1b). Minimal activity loss was observed after 4 h in buffer for all charge variants. When incubated in 2.5% [C_2C_1Im][OAc] >50% initial activity is lost after 2 h, while >50% initial activity is lost after only 1 h in 5% [C_2C_1Im][OAc]. The IL concentrations tested are based on the results of the biocompatibility screening: 2.5 and 5% [C_2C_1Im][OAc] represent ranges of IL concentrations in which no or minimal activity loss is observed, respectively.

Previously, enzymes with surface charge modifications in ILs were shown to have a higher or lower half-life relative to the wild type in the IL. We have summarized some of the previous studies using various methods to improve enzyme activity in ILs (Table 2). Among those, surface modification method has been applied to lipase and cellulase, however few reported surface modifications on laccases. Both succinylation and acetylation improved the half-life of lipase and α - chymotrypsin in 40% [C_4C_1Im][Cl] and 55% [C_2C_1Im][$EtSO_4$], respectively, while acetylation improved the half-life of papain in 30% [C_4C_1Im][Cl]; however, neutralization and cationization reduced the half-life of lipase and α - chymotrypsin in IL, respectively [61]. Succinylated and acetylated enzymes are thought to be more stable in ILs due to 2 factors: their similarity to halophilic enzymes and the formation of salt bridges. Halophilic enzymes have a large number of acidic surface residues which attract water molecules, increasing protein hydration and preventing the enzyme from aggregating, while the salt bridges further increase enzyme stability in saline environments [66; 67; 68].

3.3. Docking Simulations

Due to the unknown concentration of ABTS in the 1-Step solution used in the biocompatibility screening, we were unable to use enzyme kinetics analyses to understand the inhibition of *MtL* in $[C_2C_1Im][OAc]$. Therefore, docking simulations were used to better understand the effect of the surface charge modifications on the major binding locations of $[C_2C_1Im][OAc]$ to the enzyme surface. The results of the docking simulations show that the modification chemistry affects the major docking locations of the IL to the surface of *MtL* (Figure 2). The major docking location of the IL to the surface of the unmodified *MtL* was limited to a region close to the T1 copper (Figure 2a). When *MtL* was acetylated or succinylated the IL docked close to the T1 copper in addition to a new $[OAc]^-$ binding location away from the T1 copper (Figure 2b). When *MtL* was cationized or neutralized $[C_2C_1Im]^+$ docked close to the T1 copper while $[OAc]^-$ docked further from the T1 copper but still close to the active site entrance (cationized) or away from the active site (cationized and neutralized) (Figure 2c). In order to check whether the grid box size affects the docking simulation results, we have run additional simulations focusing the grid on the T1 copper + active site, a much smaller volume compared to our original simulation (supplemental Figure S1). We found all of the main binding locations were close to the T1 copper or in the active site. This confirms the results obtained on a grid box covering the entire protein.

A closer inspection of the $[C_2C_1Im]^+$ docking location reveals the presence of several aromatic residues that would not be affected by any of the surface charge modifications. These aromatic residues would be able to stabilize $[C_2C_1Im]^+$ *via* $\pi - \pi$ or π – cation interactions in all charge variants. While these results are consistent with a previous report that $[C_2C_1Im]^+$ docks to *MtL* in the presence of aromatic residues, we did not observe $[C_2C_1Im]^+$ entering the active site

[69]. This study did not restrict the possible $[C_2C_1Im]^+$ binding locations, whereas the previous study focused the docking around the *MtL* active site which could affect the degree to which the cation entered into the active site. Arg456 and His457 stabilize $[OAc]^-$ close to the active site of unmodified, succinylated, and acetylated *MtL*. When docking to succinylated and acetylated *MtL*, $[OAc]^-$ is stabilized by Arg226 and Arg228 at the 2nd $[OAc]^-$ binding site. The major $[OAc]^-$ docking location close to the active site of cationized *MtL* contains 2 modified residues (Asp360 and Glu420), in addition to several lysines. The 2nd [OAc]- binding site to cationized and neutralized *MtL* contains several arginines, lysines, and modified residues.

The shift in $[OAc]^-$ binding is determined by the modification chemistry. Acetylation and succinylation reduce the positive surface charges that stabilize $[OAc]^-$, driving the anion to the arginine-rich binding pocket. Neutralization and cationization further increase the favorability of anion binding to an arginine-rich pocket containing modified glutamate and aspartate. The previous work on modifying surface charges suggest that acetylation and succinylation discourage the chaotropic anion (*e.g.* Cl^- or $[OAc]^-$) from interacting with the enzyme surface, thereby improving enzyme activity and stability in IL [60; 61]. Our docking results show that all surface charge modifications encourage $[OAc]^-$ binding away from the active site, however neither *MtL* activity nor stability in $[C_2C_1Im][OAc]$ are affected by the modifications. Laccase activity is dependent upon the presence of a T1 copper in the active site, a trinuclear copper cluster, and a Cys-His pathway between the T1 copper and the trinuclear cluster. It is speculated that the loss of *MtL* activity and stability in $[C_2C_1Im][OAc]$ is likely due to destabilization of the coppers and the Cys-His pathway by the IL *via* a mechanism that cannot be influenced by modifying the enzyme surface charges. Further investigation on the surface properties of modified enzyme and enzyme kinetics will help to validate the proposed mechanism.

4. Discussion

In this study we examined the biocompatibility of dilute $[C_2C_1Im][OAc]$ with the activity of the thermophilic fungal laccase from *Myceliophthora thermophila*. Results show severe inhibition of *MtL* activity in only 15% (w/v) $[C_2C_1Im][OAc]$, far below the IL concentration necessary for effective biomass pretreatment. In an effort to reduce this inhibition of *MtL* activity, we made several surface charge modifications to increase the acid or amine surface residues. Although previous studies have shown that surface charge modifications can increase enzyme activity and stability in aqueous ILs, the stability and activity of all *MtL* charge variants in $[C_2C_1Im][OAc]$ were unchanged compared to the unmodified enzyme. Docking simulations show that the IL docks close to the T1 copper on all charge variants, with some additional $[OAc]^-$ docking locations on the surface of modified enzymes. Further work might seek to 1) optimize surface charge modification method on laccase and investigate the surface properties of the modified enzyme, 2) better understand the mechanism by which the IL inhibits *MtL* activity through the use of enzyme kinetics and molecular dynamics (MD) simulations and 3) employ additional techniques (*e.g.* rational design or immobilization) to improve *MtL* activity in $[C_2C_1Im][OAc]$.

Methods used to assess the effects of ILs on enzymes can be divided into *in vitro* (*e.g.* activity assays, stability assays, and enzyme kinetics) and *in silico* (*e.g.* docking simulations and molecular dynamics [MD] simulations) techniques. *In vitro* techniques can be used to provide a broad overview of how the ILs are affecting the enzyme activity, stability, and substrate affinity. By measuring the activity of *TvL* in ILs, past studies have shown that $[C_2C_1Im][EtSO_4]$ is the most biocompatible alkylimidazolium IL, suggesting that this laccase-IL combination would be best suited for lignin deconstruction applications [52; 58]. However, these activity assays are done on

301 a short time scale which does not provide any information about the stability of the laccase in the
302 IL. Although the initial activity of *MtL* immobilized on glyoxyl-agarose beads is lower in 25%
303 $[C_2C_1Im][EtSO_4]$ than the free laccase initial activity, the immobilized laccase is far more stable
304 in 75% IL than the free laccase over a 7 day period [58]. Enzyme kinetics studies, performed by
305 measuring the initial velocity of enzymes in different substrate concentrations, can further indicate
306 if the loss in activity is due to the IL affecting the enzyme activity or substrate binding. Kinetic
307 studies of *MtL* in $[C_2C_1Im][Cl]$ showed that the IL is a competitive inhibitor that competes with
308 the substrate for binding in the active site [69]. $[C_2C_1Im][EtSO_4]$ was found to interact with *MtL*
309 as an uncompetitive inhibitor that does not affect substrate binding, but rather affects substrate
310 oxidation once it has bound [58]. Some ILs are also able to affect both substrate binding and
311 oxidation, for example $[C_2C_1Im][OAc]$ and [Ch][Lys], which were found to be mixed inhibitors
312 that decreased substrate affinity and activity of *TvL* [53]. In contrast, several studies had
313 demonstrated synergy between IL and biocatalyst and promotion of enzyme activity in IL. For
314 example, Galai et al. found that 13 ILs increased the activity of the *T. versicolor* laccase with the
315 most significant improvement (451%) seen in 10 mM choline dihydrogen phosphate
316 ($[Ch][H_2PO_4]$) [51]. The authors attribute a shift to the α-helix structure induced by $[Ch][H_2PO_4]$
317 could be responsible for the enhancement of the enzyme activity. Synergetic effect was also
318 observed between 1-butyl-2,3-dimethylimidazolium cetyl-PEG sulfate and lipase catalyzed
319 transesterification of secondary alcohols [70].

320 To further understand the laccase-IL interactions seen with kinetic studies, recent studies
321 have used docking simulations with ILs and static protein models. Docking simulations showed
322 that the diffusion of $[C_nC_1Im]^+$ cations (n = 2, 4, 6, 8, 10) into the active site of *MtL* is determined
323 by the alkyl chain length [69]. Similarly, $[C_2C_1Im][OAc]$ and [Ch][Lys] were found to dock close

to the T1 copper of *TvL* [53]. In both of these cases the docking simulations supported the previous kinetics results showing the ILs can affect substrate binding and oxidation. MD simulations can also be used to better capture the dynamic nature of enzyme-IL interactions. After *in vitro* experiments showed $[C_2C_1Im][OAc]$ is more biocompatible with thermophilic cellulases than a mesophilic cellulase, MD simulations revealed the IL effect is limited to local disturbances of thermophilic cellulases or secondary structure unfolding of a mesophilic cellulase [54; 71]. MD simulations also provided further insight into enzyme-IL interactions that increase the thermostability of a *Fusarium solani* serine protease cutinase in 1-butyl-3-methylimidazolium hexafluorophosphate ($[C_4C_1Im][PF_6]$) [72]. Further insight into the effect of the IL on enzymes can be gained by applying different methods to understand the binding free energy of the IL or substrate to the enzyme surface or active site [73].

In this study we applied a single enzyme engineering approach to try and improve *MtL* activity and stability in $[C_2C_1Im][OAc]$. The enzyme used in this study was not produced recombinantly, therefore we were unable to apply and biotechnological (*e.g.* directed evolution or rational design) methods to improve *MtL* activity and stability. Future studies with *MtL* in $[C_2C_1Im][OAc]$ might apply immobilization techniques similar to those previously used to improve stability in $[C_2C_1Im][EtSO4]$ [58]. Additionally, recombinant expression of *MtL* would enable future researchers to apply other enzyme engineering techniques. The L1 loop can be targeted for mutations with a directed evolution or computationally assisted strategy, similar to previous studies with *TvL* [74; 75]. Additionally, novel targets for mutations can be identified using a computationally assisted approach like that used to identify the Glu170 target in *B. subtilis* CotA [59]. The application of more robust methods for analyzing laccase-IL interactions

alongside targeted enzyme engineering strategies will facilitate the identification of laccase-IL combinations suited for use in future lignin valorization systems.

Author Contributions

JCS and JS conceptualized the work, designed the experiments, and analyzed the data. JCS conducted the experiments and drafted the manuscript. JS acquired funding, supervised research, and revised the manuscript.

Acknowledgements

The authors acknowledge the support from the National Science Foundation under Cooperative Agreement No. 1929122 and 1632854 and the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch-Multistate project under accession number 1018315. We thank Novozymes for providing laccase samples and Dr. David Rodgers for useful suggestions.

359 **List of Tables and Figures**

360 **Table 1.** Summary of mutations to used mimic surface charge modifications in PyMol.

361 **Table 2.** Previous studies using various methods to improve enzyme activity in ILs.

362 **Figure 1.** Heatmap showing **a)** the activity of *MtL* and the charge variants in $[C_2C_1Im][OAc]$
363 relative to *MtL* activity in buffer, and **b)** the residual activity of *MtL* charge variants after
364 incubation in 50 mM citrate/100 mM phosphate buffer and 2.5 or 5% $[C_2C_1Im][OAc]$ at pH 4.5
365 and 40 ^{o}C.

366 **Figure 2.** Major docking locations of $[C_2C_1Im][OAc]$ to the surface of unmodified (**a**),
367 succinylated/acetylated (**b**), and cationized/neutralized *MtL* (**c**). $[C_2C_1Im]^+$ is shown as magenta
368 spheres, $[OAc]^-$ is shown as red spheres, and coppers are shown as brown spheres.

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Table 1. Summary of mutations to used mimic surface charge modifications in PyMol

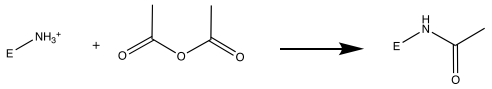
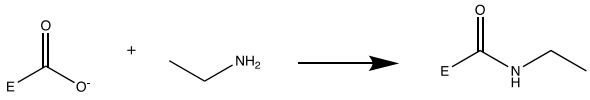
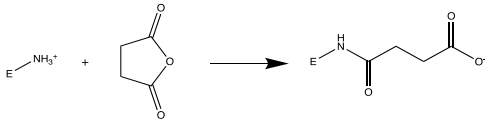
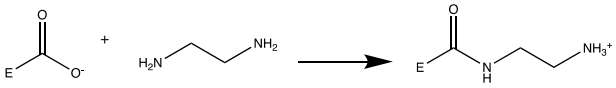
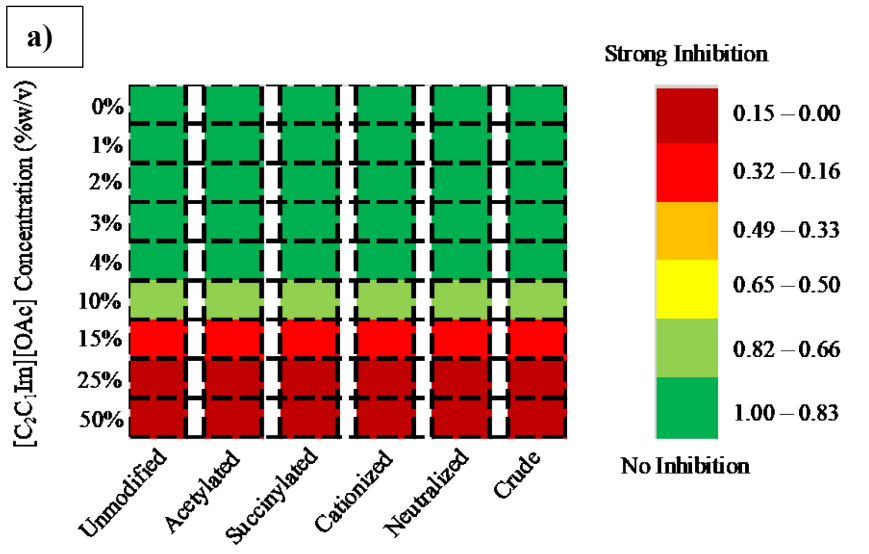
Charge Variant	Modification Chemistry	Target Residues	PyMol Mutations
Unmodified	N/A	N/A	N/A
Acetylated		Primary Amines (Lys)	Lys → Ala
Neutralized		Carboxylic Acid (Asp or Glu)	Asp → Ala Glu → Ala
Succinylated		Primary Amines (Lys)	Lys → Asp
Cationized		Carboxylic Acid (Asp or Glu)	Asp → Lys Glu → Lys

Table 2. Previous studies using various methods to improve enzyme activity in ILs.

Method	Enzyme	Outcome	Citation
Computationally assisted protein engineering	*T. versicolor* laccase	Triple and quadruple mutants with increased activity in $[C_2C_1Im][EtSO_4]$	[75]
Directed evolution	*T. versicolor* laccase	Double mutant with increased activity in $[C_2C_1Im][EtSO_4]$	[74]
Computationally assisted protein engineering	*B. subtilis* laccase	Single mutants with increased catalytic efficiency (k_{cat}/K_m) in 3 $[C_nC_1Im][Cl]$ ILs	[59]
Surface charge modification	Bovine pancreas α-chymotrypsin, *Carica papaya* papain, *Candida rugosa* lipase	Succinylation and acetylation improved activity and stability in $[C_4C_1Im][Cl]$ and $[C_2C_1Im][EtSO_4]$	[61]
Surface charge modification	*Trichoderma reesei* cellulase	Succinylation improved cellulose hydrolysis in $[C_4C_1Im][Cl]$	[60]
Immobilization	*M. thermophila* laccase	Immobilization on glyoxyl-agarose beads improved stability in $[C_2C_1Im][EtSO_4]$	[58]



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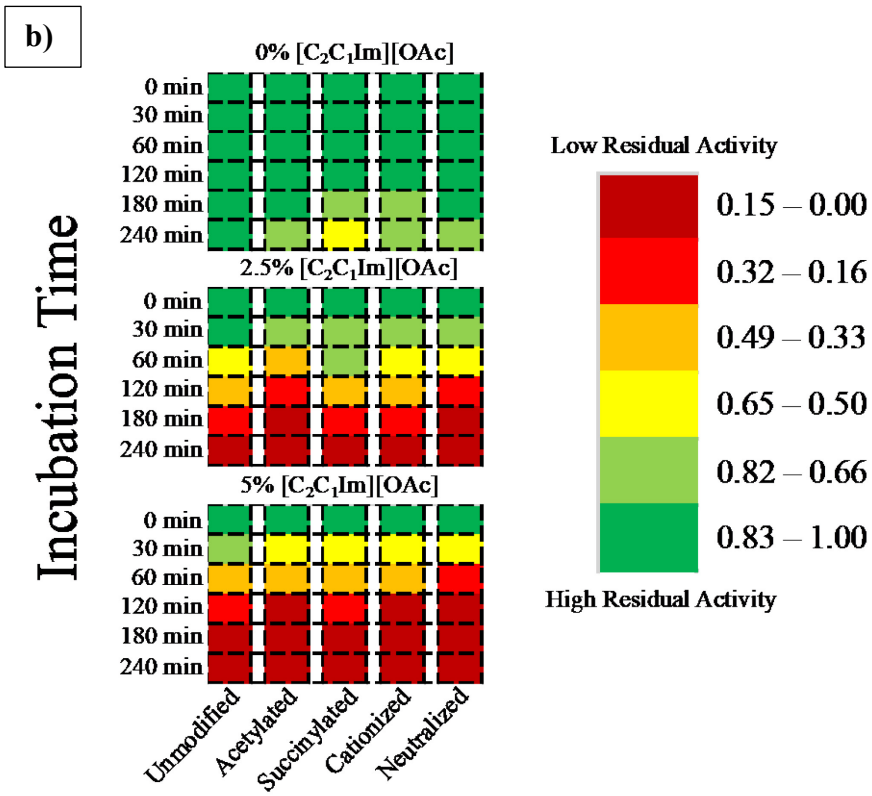

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377 **Figure 1.** Heatmaps showing **a)** the activity of *MtL* and the charge variants in $[C_2C_1Im][OAc]$
378 relative to *MtL* activity in buffer; and **b)** the residual activity of *MtL* charge variants after
379 incubation in 50 mM citrate/100 mM phosphate buffer and 2.5 or 5% $[C_2C_1Im][OAc]$ at pH 4.5
380 and 40ºC.

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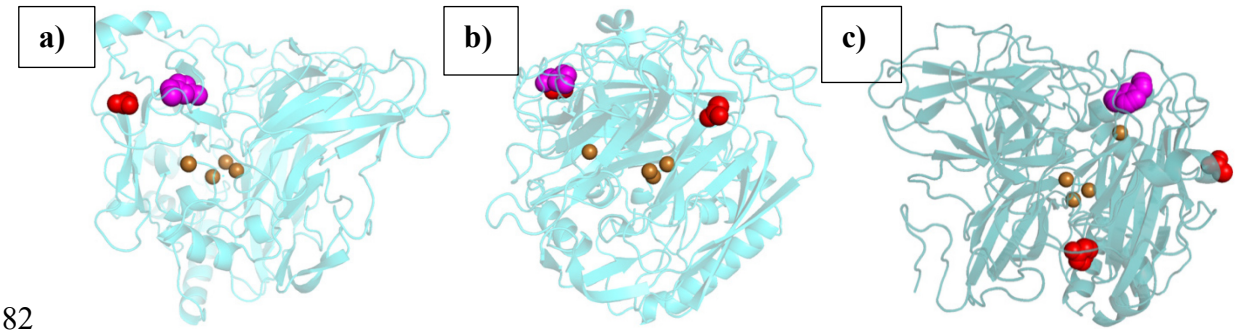

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383 **Figure 2**. Major docking locations of $[C_2C_1Im][OAc]$ to the surface of unmodified **(a)**,
384 succinylated/acetylated **(b)**, and cationized/neutralized MtL **(c)**. $[C_2C_1Im]^+$ is shown as magenta
385 spheres, $[OAc]^-$ is shown as red spheres, and coppers are shown as brown spheres.

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References

[1] J.-K. Weng, and C. Chapple, The origin and evolution of lignin biosynthesis. New Phytologist 187 (2010) 273-285.

[2] W.O.S. Doherty, P. Mousavioun, and C.M. Fellows, Value-adding to cellulosic ethanol: Lignin polymers. Industrial Crops and Products 33 (2011) 259-276.

[3] R. Vanholme, B. Demedts, K. Morreel, J. Ralph, and W. Boerjan, Lignin Biosynthesis and Structure. Plant Physiology 153 (2010) 895-905.

[4] M. Sette, R. Wechselberger, and C. Crestini, Elucidation of Lignin Structure by Quantitative 2D NMR. Chemistry – A European Journal 17 (2011) 9529-9535.

[5] W. Boerjan, J. Ralph, and M. Baucher, Lignin biosynthesis. Annual Review of Plant Biology 54 (2003) 33.

[6] J. Zeng, G.L. Helms, X. Gao, and S. Chen, Quantification of Wheat Straw Lignin Structure by Comprehensive NMR Analysis. Journal of Agricultural and Food Chemistry 61 (2013) 10848-10857.

[7] Suhas, P.J.M. Carrott, and M.M.L. Ribeiro Carrott, Lignin – from natural adsorbent to activated carbon: A review. Bioresource Technology 98 (2007) 2301-2312.

[8] N.A.o. Sciences, N.A.o. Engineering, and N.R. Council, Liquid Transportation Fuels from Coal and Biomass, 2009.

[9] D. Tilman, R. Socolow, J.A. Foley, J. Hill, E. Larson, L. Lynd, S. Pacala, J. Reilly, T. Searchinger, C. Somerville, and R. Williams, Beneficial Biofuels—The Food, Energy, and Environment Trilemma. Science 325 (2009) 270-271.

[10] A.J. Ragauskas, G.T. Beckham, M.J. Biddy, R. Chandra, F. Chen, M.F. Davis, B.H. Davison, R.A. Dixon, P. Gilna, M. Keller, P. Langan, A.K. Naskar, J.N. Saddler, T.J. Tschaplinski, G.A. Tuskan, and C.E. Wyman, Lignin Valorization: Improving Lignin Processing in the Biorefinery. Science 344 (2014).

[11] Y. Mottiar, R. Vanholme, W. Boerjan, J. Ralph, and S.D. Mansfield, Designer lignins: harnessing the plasticity of lignification. Current Opinion in Biotechnology 37 (2016) 190-200.

[12] M. Kleinert, and T. Barth, Towards a Lignincellulosic Biorefinery: Direct One-Step Conversion of Lignin to Hydrogen-Enriched Biofuel. Energy & Fuels 22 (2008) 1371-1379.

[13] L.M. Cotoruelo, M.D. Marqués, A. Leiva, J. Rodríguez-Mirasol, and T. Cordero, Adsorption of oxygen-containing aromatics used in petrochemical, pharmaceutical and food industries by means of lignin based active carbons. Adsorption 17 (2011) 539-550.

[14] D.D. Laskar, B. Yang, H. Wang, and J. Lee, Pathways for biomass-derived lignin to hydrocarbon fuels. Biofuels, Bioproducts and Biorefining 7 (2013) 602-626.

[15] P.J. De Wild, W.J.J. Huijgen, and R.J.A. Gosselink, Lignin pyrolysis for profitable lignocellulosic biorefineries. Biofuels, Bioproducts and Biorefining 8 (2014) 645-657.

[16] G.T. Beckham, C.W. Johnson, E.M. Karp, D. Salvachúa, and D.R. Vardon, Opportunities and challenges in biological lignin valorization. Current Opinion in Biotechnology 42 (2016) 40-53.

[17] Y. He, X. Li, H. Ben, X. Xue, and B. Yang, Lipid production from dilute alkali corn stover lignin by Rhodococcus strains. ACS Sustainable Chemistry & Engineering 5 (2017) 2302-2311.

[18] L. Das, S. Xu, and J. Shi, Catalytic Oxidation and Depolymerization of Lignin in Aqueous Ionic Liquid. Frontiers in Energy Research 5 (2017).

[19] L. Das, M. Li, J. Stevens, W. Li, Y. Pu, A.J. Ragauskas, and J. Shi, Characterization and Catalytic Transfer Hydrogenolysis of Deep Eutectic Solvent Extracted Sorghum Lignin to Phenolic Compounds. ACS Sustainable Chemistry & Engineering 6 (2018) 10408-10420.
[20] W. Li, S. Zhou, Y. Xue, Y.-J. Lee, R. Smith, and X. Bai, Understanding Low-Pressure Hydropyrolysis of Lignin Using Deuterated Sodium Formate. ACS Sustainable Chemistry & Engineering 5 (2017) 8939-8950.
[21] K. Miyazaki, A hyperthermophilic laccase from Thermus thermophilus HB27. Extremophiles 9 (2005) 415-425.
[22] M.-F. Hullo, I. Moszer, A. Danchin, and I. Martin-Verstraete, CotA of Bacillus subtilis Is a Copper-Dependent Laccase. Journal of Bacteriology 183 (2001) 5426-5430.
[23] H. An, T. Xiao, H. Fan, and D. Wei, Molecular characterization of a novel thermostable laccase PPLCC2 from the brown rot fungus Postia placenta MAD-698-R. Electronic Journal of Biotechnology 18 (2015) 451-458.
[24] A.M. Varmana, L. Heb, R. Follenfanta, W. Wua, S. Wemmera, S.A. Wrobela, Y.J. Tang, and S. Singh, Decoding how a soil bacterium extracts building blocks and metabolic energy from ligninolysis provides road map for lignin valorization. Proceedings of the National Academy of Science (2016) 10.
[25] T.D.H. Bugg, M. Ahmad, E.M. Hardiman, and R. Rahmanpour, Pathways for degradation of lignin in bacteria and fungi. Natural Product Reports 28 (2011) 1883-1896.
[26] M.E. Brown, and M.C.Y. Chang, Exploring bacterial lignin degradation. Current Opinion in Chemical Biology 19 (2014) 1-7.
[27] V.V. MOZHAEV, Y.L. KHMELNITSKY, M.V. SERGEEVA, A.B. BELOVA, N.L. KLYACHKO, A.V. LEVASHOV, and K. MARTINEK, Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures. European Journal of Biochemistry 184 (1989) 597-602.
[28] A.M. Klibanov, Improving enzymes by using them in organic solvents. Nature 409 (2001) 241-246.
[29] S. Park, and R.J. Kazlauskas, Biocatalysis in ionic liquids – advantages beyond green technology. Current Opinion in Biotechnology 14 (2003) 432-437.
[30] J.F. Brennecke, and E.J. Maginn, Ionic liquids: Innovative fluids for chemical processing. AIChE Journal 47 (2001) 2384-2389.
[31] K.E. Gutowski, G.A. Broker, H.D. Willauer, J.G. Huddleston, R.P. Swatloski, J.D. Holbrey, and R.D. Rogers, Controlling the Aqueous Miscibility of Ionic Liquids: Aqueous Biphasic Systems of Water-Miscible Ionic Liquids and Water-Structuring Salts for Recycle, Metathesis, and Separations. Journal of the American Chemical Society 125 (2003) 6632-6633.
[32] R.D. Rogers, and K.R. Seddon, Ionic Liquids--Solvents of the Future? Science 302 (2003) 792-793.
[33] M. Hajime, Y. Masahiro, T. Kazumi, N. Masakatsu, K. Yukiko, and M. Yoshinori, Highly Conductive Room Temperature Molten Salts Based on Small Trimethylalkylammonium Cations and Bis(trifluoromethylsulfonyl)imide. Chemistry Letters 29 (2000) 922-923.
[34] D.A. Fort, R.C. Remsing, R.P. Swatloski, P. Moyna, G. Moyna, and R.D. Rogers, Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-n-butyl-3-methylimidazolium chloride. Green Chemistry 9 (2007) 63-69.
[35] I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen, and D.S. Argyropoulos, Dissolution of Wood in Ionic Liquids. Journal of Agricultural and Food Chemistry 55 (2007)

9142-9148.
[36] P. Verdia, A. Brandt, J.P. Hallett, M.J. Ray, and T. Welton, Fractionation of lignocellulosic biomass with the ionic liquid 1-butylimidazolium hydrogen sulfate. Green Chemistry 16 (2014) 1617-1627.
[37] A.M. Socha, R. Parthasarathi, J. Shi, S. Pattathil, D. Whyte, M. Bergeron, A. George, K. Tran, V. Stavila, S. Venkatachalam, M.G. Hahn, B.A. Simmons, and S. Singh, Efficient biomass pretreatment using ionic liquids derived from lignin and hemicellulose. Proceedings of the National Academy of Sciences 111 (2014) E3587-E3595.
[38] A. George, A. Brandt, K. Tran, S.M.N.S. Zahari, D. Klein-Marcuschamer, N. Sun, N. Sathitsuksanoh, J. Shi, V. Stavila, and R. Parthasarathi, Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. Green Chemistry 17 (2015) 1728-1734.
[39] F.J.V. Gschwend, F. Malaret, S. Shinde, A. Brandt-Talbot, and J.P. Hallett, Rapid pretreatment of Miscanthus using the low-cost ionic liquid triethylammonium hydrogen sulfate at elevated temperatures. Green Chemistry 20 (2018) 3486-3498.
[40] E. Liu, L. Das, B. Zhao, M. Crocker, and J. Shi, Impact of Dilute Sulfuric Acid, Ammonium Hydroxide, and Ionic Liquid Pretreatments on the Fractionation and Characterization of Engineered Switchgrass. BioEnergy Research (2017).
[41] H. Yoshida, LXIII.-Chemistry of lacquer (Urushi). Part I. Communication from the Chemical Society of Tokio. Journal of the Chemical Society, Transactions 43 (1883) 472-486.
[42] K. Piontek, M. Antorini, and T. Choinowski, Crystal Structure of a Laccase from the FungusTrametes versicolor at 1.90-Å Resolution Containing a Full Complement of Coppers. Journal of Biological Chemistry 277 (2002) 37663-37669.
[43] J.K. Glenn, M.A. Morgan, M.B. Mayfield, M. Kuwahara, and M.H. Gold, An extracellular H2O2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete Phanerochaete chrysosporium. Biochemical and Biophysical Research Communications 114 (1983) 1077-1083.
[44] S.M. Jones, and E.I. Solomon, Electron transfer and reaction mechanism of laccases. Cellular and Molecular Life Sciences 72 (2015) 869-883.
[45] H. Claus, Laccases: structure, reactions, distribution. Micron 35 (2004) 93-96.
[46] J.A.F. Gamelas, A.P.M. Tavares, D.V. Evtuguin, and A.M.B. Xavier, Oxygen bleaching of kraft pulp with polyoxometalates and laccase applying a novel multi-stage process. Journal of Molecular Catalysis B: Enzymatic 33 (2005) 57-64.
[47] F. d'Acunzo, C. Galli, P. Gentili, and F. Sergi, Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. New Journal of Chemistry 30 (2006) 583-591.
[48] A. Rico, J. Rencoret, J.C. del Río, A.T. Martínez, and A. Gutiérrez, Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock. Biotechnology for Biofuels 7 (2014) 6.
[49] D.M. Mate, and M. Alcalde, Laccase engineering: From rational design to directed evolution. Biotechnology Advances 33 (2015) 25-40.
[50] D. Singh Arora, and R. Kumar Sharma, Ligninolytic Fungal Laccases and Their Biotechnological Applications. Applied Biochemistry and Biotechnology 160 (2010) 1760-1788.
[51] S. Galai, A. P. de los Rios, F.J. Hernandez-Fernandez, S. Haj Kacem, and F. Tomas-Alonso, Over-activity and stability of laccase using ionic liquids: screening and application in dye decolorization. RSC Advances 5 (2015) 16173-16189.

[52] A. Domínguez, O. Rodríguez, A.P.M. Tavares, E.A. Macedo, M. Asunción Longo, and M. Ángeles Sanromán, Studies of laccase from Trametes versicolor in aqueous solutions of several methylimidazolium ionic liquids. Bioresource Technology 102 (2011) 7494-7499.
[53] J.C. Stevens, L. Das, J.K. Mobley, S.O. Asare, B.C. Lynn, D.W. Rodgers, and J. Shi, Understanding Laccase–Ionic Liquid Interactions toward Biocatalytic Lignin Conversion in Aqueous Ionic Liquids. ACS Sustainable Chemistry & Engineering 7 (2019) 15928-15938.
[54] S. Datta, B. Holmes, J.I. Park, Z. Chen, D.C. Dibble, M. Hadi, H.W. Blanch, B.A. Simmons, and R. Sapra, Ionic liquid tolerant hyperthermophilic cellulases for biomass pretreatment and hydrolysis. Green Chemistry 12 (2010) 338-345.
[55] Y.-C. He, F. Liu, L. Gong, J.-H. Di, Y. Ding, C.-L. Ma, D.-P. Zhang, Z.-C. Tao, C. Wang, and B. Yang, Enzymatic in situ saccharification of chestnut shell with high ionic liquid-tolerant cellulases from Galactomyces sp. CCZU11-1 in a biocompatible ionic liquid-cellulase media. Bioresource technology 201 (2016) 133-139.
[56] Q. Li, J. Di, X. Liao, J. Ni, Q. Li, Y.-C. He, and C. Ma, Exploration of benign deep eutectic solvent–water systems for the highly efficient production of furfurylamine from sugarcane bagasse via chemoenzymatic cascade catalysis. Green Chemistry 23 (2021) 8154-8168.
[57] J. Ni, Q. Li, L. Gong, X.-L. Liao, Z.-J. Zhang, C. Ma, and Y. He, Highly Efficient Chemoenzymatic Cascade Catalysis of Biomass into Furfurylamine by a Heterogeneous Shrimp Shell-Based Chemocatalyst and an ω-Transaminase Biocatalyst in Deep Eutectic Solvent–Water. ACS Sustainable Chemistry & Engineering 9 (2021) 13084-13095.
[58] M. Fernández-Fernández, D. Moldes, A. Domínguez, M.Á. Sanromán, A.P.M. Tavares, O. Rodríguez, and E.A. Macedo, Stability and kinetic behavior of immobilized laccase from Myceliophthora thermophila in the presence of the ionic liquid 1-ethyl-3-methylimidazolium ethylsulfate. Biotechnology Progress 30 (2014) 790-796.
[59] B. Dabirmanesh, K. Khajeh, F. Ghazi, B. Ranjbar, and S.-M. Etezad, A semi-rational approach to obtain an ionic liquid tolerant bacterial laccase through π-type interactions. International Journal of Biological Macromolecules 79 (2015) 822-829.
[60] E.M. Nordwald, R. Brunecky, M.E. Himmel, G.T. Beckham, and J.L. Kaar, Charge engineering of cellulases improves ionic liquid tolerance and reduces lignin inhibition. Biotechnology and Bioengineering 111 (2014) 1541-1549.
[61] J.L.K. Erik M. Nordwald, Stabilization of Enzymes in Ionic Liquids Via Modification of Enzyme Charge. Biotechnology and Bioengineering 110 (2013) 9.
[62] J. Shi, K. Balamurugan, R. Parthasarathi, N. Sathitsuksanoh, S. Zhang, V. Stavila, V. Subramanian, B.A. Simmons, and S. Singh, Understanding the role of water during ionic liquid pretreatment of lignocellulose: co-solvent or anti-solvent? Green Chemistry 16 (2014) 3830-3840.
[63] R. Patel, M. Kumari, and A.B. Khan, Recent Advances in the Applications of Ionic Liquids in Protein Stability and Activity: A Review. Applied Biochemistry and Biotechnology 172 (2014) 3701-3720.
[64] H. Weingärtner, C. Cabrele, and C. Herrmann, How ionic liquids can help to stabilize native proteins. Physical Chemistry Chemical Physics 14 (2012) 415-426.
[65] V.M.a.S.S. Lele, Laccase: Properties and Applications. BioResources 4 (2009) 24.
[66] S. Uthandi, B. Saad, M.A. Humbard, and J.A. Maupin-Furlow, LccA, an Archaeal Laccase Secreted as a Highly Stable Glycoprotein into the Extracellular Medium by Haloferax volcanii. Applied and Environmental Microbiology 76 (2010) 733-743.

[67] A.H. Elcock, and J.A. McCammon, Electrostatic contributions to the stability of halophilic proteins11Edited by B. Honig. Journal of Molecular Biology 280 (1998) 731-748.
[68] O. Dym, M. Mevarech, and J.L. Sussman, Structural Features That Stabilize Halophilic Malate Dehydrogenase from an Archaebacterium. Science 267 (1995) 1344-1346.
[69] J. Sun, H. Liu, W. Yang, S. Chen, and S. Fu, Molecular Mechanisms Underlying Inhibitory Binding of Alkylimidazolium Ionic Liquids to Laccase. Molecules 22 (2017) 1353.
[70] K. Yoshiyama, Y. Abe, S. Hayase, T. Nokami, and T. Itoh, Synergetic Activation of Lipase by an Amino Acid with Alkyl–PEG Sulfate Ionic Liquid. Chemistry letters 42 (2013) 663-665.
[71] V. Jaeger, P. Burney, and J. Pfaendtner, Comparison of Three Ionic Liquid-Tolerant Cellulases by Molecular Dynamics. Biophysical Journal 108 (2015) 880-892.
[72] N.M. Micaêlo, and C.M. Soares, Protein Structure and Dynamics in Ionic Liquids. Insights from Molecular Dynamics Simulation Studies. The Journal of Physical Chemistry B 112 (2008) 2566-2572.
[73] V. Limongelli, Ligand binding free energy and kinetics calculation in 2020. WIREs Computational Molecular Science 10 (2020) e1455.
[74] H. Liu, L. Zhu, M. Bocola, N. Chen, A.C. Spiess, and U. Schwaneberg, Directed laccase evolution for improved ionic liquid resistance. Green Chemistry 15 (2013) 1348-1355.
[75] A.-M. Wallraf, H. Liu, L. Zhu, G. Khalfallah, C. Simons, H. Alibiglou, M.D. Davari, and U. Schwaneberg, A loop engineering strategy improves laccase lcc2 activity in ionic liquid and aqueous solution. Green Chemistry 20 (2018) 2801-2812.