EXPLORING BIOCOMPATABILITY OF LACCASE IN IONIC LIQUID FOR SELECTIVE LIGNIN DEGRADATION

RESEARCH PROPOSAL

A proposal submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Engineering
at the University of Kentucky

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Abstract

Lignin makes up 20-30% of the plant biomass and is one of the most abundant aromatic polymers in nature. Converting lignin into high-value chemicals would add revenues for a biorefinery, thus improving the economic viability of biofuel production. Ionic liquids (ILs) have received increasing interest because of their ability to fractionate and pretreat lignocellulosic biomass. Given the unique properties of aqueous ILs that provide for lignin solubility and biocompatibility of enzymes and microbes, there is a great opportunity to develop new strategies for lignin extraction and depolymerization *via* biocatalysis in aqueous ILs. The stability and activity of commercial fungal and plant laccases and a recombinant bacterial laccase were evaluated in different concentration of ILs. Michaelis-Menten equations and Lineweaver-Burk curves were used to determine the inhibitory effects. Results suggest that certain ILs were fully compatible with laccases; while some appeared as non-competitive inhibitors. To elucidate the effect of IL on enzyme structure, folding of the enzyme was assessed by circular dichroism spectroscopy. Furthermore, lignin model compounds and native plant-derived lignin were tested with laccases in aqueous ILs and the correlation between solvent properties of aqueous IL and lignin deploymerization products was established. Results suggest that tuning the solvent properties of IL helps to channel specific products during the biocatalytic oxidation of lignin. Taken together, we have demonstrated an instructive concept of an aqueous phase lignin depolymerization process using laccase/IL pairs. This study provides insights into the oxidative degradation pathway of lignin in the presence of IL and the selectivity of biocatalyst towards product formation.

Chapter One: Introduction

Lignin makes up ~30% of the plant biomass and is one of the most abundant aromatic polymers in nature. The overall lignin structure consists of three highly crosslinked monolignols (H, G and S). These monolignols, as lignin depolymerization products, are valuable precursors to a variety of commodity and specialty chemicals with wide applications in fuels, food additives, antimicrobials and in the synthesis of other high-value materials. There are tremendous opportunities for the development of lignin based alternatives to petroleum-based products. Several thermochemical lignin depolymerization methods have been explored, including high temperature pyrolysis, hydrogenolysis and catalytic oxidation. Efforts have been focused on improving the catalyst performance in thermochemical processes; however, the efficiency and selectivity of the catalyst as developed on lignin model compounds often get compromised when applied to plant derived lignin due to its structural complexity. An emerging technology that has potential to improve catalyst efficiency and selectivity in thermochemical processes is the use of ionic liquids (ILs). ILs are salts that exist as liquids at ambient temperatures. They can have unique catalytic and solvent properties depending on the cation/anion combination (Rogers & Seddon, 2003). A few ILs have been shown to be highly effective at solubilizing and depolymerizing lignin via specific bond cleavage (Shi *et al*., 2014). ILs could prove useful for both catalytic performance and lignin solubility of an aqueous system.

Laccases, a group of metal-containing oxidative enzymes secreted by bacteria and fungi, are responsible for the degradation of lignin in nature. However, application of this lignolytic enzyme in an industrial lignin valorization process is hindered due to the very low lignin solubility in the aqueous environment compatible with enzyme activity. Additionally, most of the known effective lignin solvents such as DMSO and acetone are detrimental to enzyme activity in aqueous solutions and often require high temperature for complete lignin dissolution. Thus, a better solvent system with both high lignin solubility and enzyme compatibility is essential for developing a better biological lignin depolymerization process. There is an opportunity to develop a new strategy for lignin extraction and depolymerization *via* biocatalysis in aqueous ILs.

1.1 Screening Enzymatic Activity in Ionic Liquid

Tavares *et al.* (2008) sought to quantify the activity and stability of laccase in three different ILs. They used a colorimetric assay with ABTS to determine the activity of laccase in different concentrations of ILs. They also used the same ABTS assay to generate kinetic curves for determination of kinetic parameters of laccase. Finally, the effect of the pH of the solution on the laccase activity was also determined by using phosphate buffer at different concentrations. They found that at higher concentrations of ILs, as high as 50% v/v, the enzyme activity was severally inhibited when compared to the control of no IL. They also found that, depending on the IL present, the optimum pH ranged from 7-9. This provides evidence that ILs affect the stability and structure of laccase differently. Dominguez *et al.* (2011) expanded upon this work by focusing their efforts on different lengths of methylimidazolium cations. It has typically been thought that the longer the length of the cation, the more severe the inhibition of the enzyme will be. The different length cations were C2, C4 and C6 methylimidazolium cations. IL containing C4 methylimidiazolium was found to have preserved the activity of laccase best after 20 days, even better than the solution without any IL. Using C4 methylimidiazolium, it was found that the higher the concentration of IL, the lower the activity.

Galai *et al.* (2015) screened 56 different ILs on the activity of laccase. They selected 26 water-immiscible and 30 water-miscible ILs for testing. A colorimetric assay was used to determine activity at 25 ^{o}C and pH of 7.0. They also used several techniques, such as circular dichroism (CD) spectroscopy, to determine any changes in the secondary structure in the laccase. A wide range of effects were displayed by the ILs on the laccase, but perhaps the most critical is the effect of [Ch][H_2PO_4] on activity. The laccase incubated in this IL showed a 451% increase in activity; additionally, CD spectroscopy showed that the secondary structure of laccase was affected by [Ch][H_2PO_4]. Traditionally, ILs have only been shown to inhibit the activity of enzymes. The determination of the change in secondary structure is key as well, shedding further light on the mechanism through which ILs effect enzymatic activity.

Fernandez-Fernandez *et al.* (2014) improved enzymatic activity in IL by using an immobilized laccase from *Myceliophthora thermophile*, rather than focusing on the type of IL. Laccase was immobilized on agarose beads and activity was screened in [Emim][$EtSO_4$], a common IL. A colorimetric assay using ABTS was used to screen for activity of the immobilized laccase in IL. The immobilized laccase displayed high levels of activity in concentrations of ILs as high as 75% v/v, even after 7 days of incubation. Laccase typically displays high levels of inhibition in ILs at concentrations as low as 250 mM. The immobilized laccase in this study, however, is much more tolerant to the presence of IL.

1.2 Characterization of Enzymes from Different Sources

Varmana *et al.* (2016) shed light on the metabolic pathways of the soil bacterium *Sphingobium* sp. SYK-6 using a variety of analytic metabolic tools. ^{13}C metabolic flux analysis (^{13}C-MFA), ^{13}C-fingerprinting and RNA sequence analysis were used. A wide variety of carbon substrates, from vanillin to xylose, were labeled at different carbons to carry out the ^{13}C experiments. Use of these tools highlighted the effect of the lignin-degradation pathway on the NAD+/NADH ratio within the cell. Understanding how lignin degradation affects the metabolic pathways of this organism will provide for important insight that can be applied to other lignin-degrading organisms. Sahadevan *et al.* (2016) focused on a soil microbe as well, however they chose to characterize a series of lignin-degrading enzymes from a soil fungus rather than *Sphingobium* sp. SYK-6. The fungus was grown in media containing lignin, and the enzymes were extracted from the supernatant. They were then characterized and used to degrade lignin, the products of which were analyzed using GC-MS. All enzymes exhibited some secondary structural changes when exposed to lignin. Lignin peroxidase broke down lignin into a product profile different from that of both manganese peroxidase and laccase. Varmana *et al.* (2016) and Sahadevan *et al.* (2016) have offered ample evidence that looking into the soil for lignin degradation mechanisms could prove to be useful.

An *et al.* (2015) sought to characterize a form of laccase from the brown rot fungus *Postia placenta.* A variety of tests were performed on the laccase in order to determine many of its characteristics. SDS-Page was used to determine its molecular weight; colorimetric

assays were used to determine the pH and temperature range for optimal activity; and kinetic coefficients for different substrates were obtained by generating kinetic curves. It was found that the optimal temperature for activity for this form of laccase is about 60 ^{o}C, while the optimal pH varies from 3.5 to 5.0, depending on the substrate present. This form of laccase also prefers higher concentration of ABTS than DMP, due to the K_m of ABTS being double that of DMP. Laccase operates at an ideal temperature of 40 ^{o}C, so the discovery of a thermostable laccase could be used to examine how enzyme structure affects function. This study also highlights the importance of substrate selection when performing colorimetric assays.

Kiiskinen *et al.* (2004) expressed laccase from *Melanocarpus albomyces* in a recombinant strain of *Trichoderma reesei*. They characterized the laccase expressed by the system. *M. albomyces* laccase plasmid was transformed into and expressed by *T. ressei*. The expressed laccase was purified using gel chromatography and characterized using mass spectrometry, an optimal pH assay, a thermal stability assay and an enzyme activity assay. The recombinant laccase was compared to the native laccase in all studies performed. Overall, many of the characteristics of the recombinant laccase were similar to the native laccase. Showing the recombinant form of the laccase has characteristics similar to the native form indicates that recombinant expression may provide for a more efficient means of obtaining laccase.

1.3 Characterizing Impact of Ionic Liquids on Enzymes

Weingartner *et al.* (2012) discuss how a wide range of ILs alter the structure and stability of proteins. They also discuss different principles for ILs' effects on protein folding and stability. The cation and anion selected for the IL play a vital role in protein stability. The large number of cations and anions available to use in ILs also allows for careful selection and adjustment of IL based on the situation present. ILs provide a means for creating biocompatible solvents, but the type of IL that should be used will differ on a case-by-case basis. Lai *et al.* (2011) carried out an experiment to determine the effects of anions and cations present in ILs on enzymes. Two enzymes, lipase from *Penicillium expansum* and mushroom tyrosinase, were incubated with 14 different ILs. The activities of the enzymes in IL were determined using assays specific to each enzyme, pNPP for lipase and L-

DOPA for tyrosinase. It was found that [$NHMe_3$][$MeSO_3$] had the greatest impact on activity and half-life for lipase, while none of the ILs tested increased the half-life or activity of the tyrosinase when compared to the control. Using two enzymes in this study showed that not all ILs interact with enzymes in the same way. This lends further evidence that cation and anion choice needs to be considered on a case-by-case basis. Nordwald and Kaar (2013) further determined a relationship between enzyme stabilization and charge modification by ILs. Charge modification was first determined using chymotrypsin as a model enzyme, with [Emim][$EtSO_4$] and [Bmim][Cl] being the two ILs used for testing. Lipase and papain were then used to determine if the charge modification detected on chymotrypsin was enzyme specific or could be applied to other enzymes. It was found that the lower the amine:acid residue ratio of the modified enzymes, the higher the activity and longer the half-life of the enzyme.

1.4 Characterization of Enzyme Mediator Systems

Harwardt *et al.* (2014) determined how different concentrations of ILs affect the activity of laccase in a laccase-mediator system. The ILs [Emim][$EtSO_4$] and [Emim][Ac] were used at concentrations of 5, 15 and 30% v/v. A colorimetric assay was used to determine the kinetic parameters K_m and V_{max} of laccase in the IL. They also tested the effect of ILs on a system with laccase, mediator and lignin model compound (veratryl alcohol). The IL [Emim][Ac] was found to exhibit greater inhibitory characteristics than [Emim][$EtSO_4$], greatly reducing the V_{max} and increasing the K_m. [Emim][Ac], however, helped to retain activity of laccase over 12 days much better than [Emim][$EtSO_4$] and a solution without any IL. [Emim][Ac] was also found to improve the conversion of veratryl alcohol at low concentrations, but at higher concentrations it displayed inhibitory effects on the laccase. The different effects of ILs over different lengths of time is interesting, further supporting the idea that IL choice differs on a case-by-case basis. Rencoret *et al.* (2016) demonstrated the efficacy of a laccase mediator system in a practical process. They improved glucose yields from saccharification of wheat straw. They pretreated wheat straw with a form of laccase obtained from *Pycnoporus cinnabarinus* and a mediator, followed by peroxide treatment. They found that pretreating with the laccase greatly improved glucose yields from the wheat straw, as a result of the lignin removal. The yields were further improved when using the mediator. Pretreating biomass with a lignin

degrading enzyme with the addition of a mediator to improve glucose yields is a novel approach. There are a large number of mediators that can be used with laccase, so this study lays the groundwork for the implementation of other mediators.

Rehmann *et al.* (2014) improved laccase activity and lifetime in a laccase-mediator system. Water-immiscible ILs were selected as part of a two phase system. The water phase contained the laccase and the mediator. The IL phase would extract the oxidized mediator from the water phase, preventing the laccase from being inhibited by the toxic oxidized mediator. They found that $[C_6mim][AOT]$ greatly improved laccase activity in the presence of mediators; $[N_{1888}][Sac]$ also improved laccase activity, albeit it to a lesser extent. Laccase-mediator systems have previously been designed as one phase systems. Implementing a method that can selectively remove inhibitors from a laccase-mediator system is an important step towards designing more robust and efficient biocompatible laccase mediator systems. The vast number of ILs could also provide for even more efficient mediator-removal systems. Chen *et al.* (2014) analyzed the formation and characteristics of an IL formed in nature. Possible methods of the IL formation are examined in the paper, as are different properties of the IL. When the tawny crazy ant *Nylanderia fulva* is exposed to the venom of the fire ant *Solenopsis invicta*, it secretes a compound of formic acid which neutralizes the venom of *S. invicta*, forming a substance with characteristics representative of an IL. If other ILs are observed being formed in nature, a new area of biocompatible ILs could potentially be opened for research.

The end goal of the proposed work is to develop an innovative and sustainable biocatalysis process for effective extraction and selective deploymerization of lignin to high-value chemicals. This project will explore two key hypotheses that: 1) a relatively mild bioprocess will facilitate the selective deconstruction of lignin and retain the functionality of the monolignols, thus helping to channel the desirable aromatic products; and 2) by tuning the solvent properties (basicity, acidity and polarity) of aqueous ILs, we could improve lignin solubility, enzyme compatibility and product selectivity. The specific objectives are listed below.

Chapter Two: Objectives

Firstly, this study would like to determine the enzymatic activity and stability of laccase from *Trametes versicolor* in different ILs; this will act as a model system to determine the most biocompatible IL. The ILs [C_2C_1mim][OAc], [Ch][Lys] and [DEA][HSO_4] have been selected for screening because they represent neutral, basic and acidic pHs respectively. Once the ILs have been screened, different forms of laccase (plant and bacterial) will be screened with the previously used ILs; this will demonstrate the source of laccase has an effect on activity and stability in IL. Finally, this study will use model compounds and lignin to determine the products of the laccase-IL system. The ability to tailor systems for selectively decomposing lignin holds great potential.

The creation of lignin derived value-added chemicals will have significant impact on U.S. industries through the development of new feedstocks and new synthesis routes. The proposed study will hopefully deliver a novel lignin valorization process taking advantage of the effectiveness of aqueous ILs on solubilizing and depolymerizing lignin and the selectivity of enzymes as a biocatalyst.

Chapter Three: Methods & Materials

3.1 Ionic Liquid Screening

ILs will be synthesized using methods developed by George *et al.* (2015). The laccase activity in synthesized ILs will be determined using an ABTS assay by tracking the radical formation at a wavelength of 540 nm. Laccase will be loaded into the system at specific activities of 0.00, 0.01, 0.02 and 0.05 U/mL. Ionic liquids will be loaded into the system at concentrations of 0, 50, 100, 150, 200 and 500 mM. Michaelis-Menten kinetic curves will be used to determine the inhibition effects of the selected ILs on laccase activity using a specific activity of 0.05 U/mL, IL loadings of 0, 100 and 300 mM and ABTS concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.5 mM. Curves will be fit to the Michaelis-Menten formula to determine changes in V_{max} and K_m. Linearization of Michaelis-Menten curves will be performed using the Lineweaver-Burke method to determine method of inhibition. QCM-D and Nano-ITC will be used to determine binding affinity and thermodynamics, respectively, of laccase in aqueous ILs.

3.2 Effect of Source on Laccase Activity and Stability

The fungal laccase from *T. versicolor* and plant laccase from *Rhus vernicifera* will be purchased from Sigma-Aldrich (St. Louis, MO). The plasmid for *Thermus thermophilus* was obtained from California and expressed using recombinant *E. coli* strain BL21. An AKTA Start protein purification system from GE Healthcare Life Sciences was purchased to assist with purifying the expressed protein. The activity and stability of purified laccase in IL solutions will be further determined after incubation in IL using the ABTS method as described above. Circular Dichroism (CD) Spectroscopy will be used to determine any changes in the secondary structure of the recovered laccase.

3.3 Product Profile Screening

With selected IL and laccase, a system will be set up using lignin model compounds and Kraft lignin as substrates. The major lignin depolymerization products will be characterized by HPLC using prior methods developed by Deng *et al.* (2015). Reaction with either laccase or IL will be tested as a control. ABTS will be used as a mediator to

test the effect of a laccase-mediator system on product formation. These results will be used to select laccase/IL pairs for the best lignin conversion and product selectivity.

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Appendix 1: Budget

1. Direct Costs	Year 1	Year 2	Total
A. Salaries and Wages			
(1) Research Assistantship	$10,666.66	$10,666.66	$21,333.32
(2) Advisor	$10,562.51	$10,879.39	$21,441.90
(3) Post-doctoral Research Associate	$7,500.00	$7,500.00	$15,000.00
Total Salaries and Wages	**$28,729.17**	**$29,046.05**	**$57,775.22**
B. Fringe Benefits			
(1) Research Assistantship	$472.00	$472.00	$944.00
(2) Advisor	$3,627.53	$3,694.87	$7,322.40
(3) Post-doctoral Research Associate	$1,406.75	$1,406.75	$2,813.50
Total Fringe Benefits	**$5,506.28**	**$5,573.62**	**$11,079.90**
C. Travel			
(1) Biofuels Symposium	$2,292.00	$2,292.00	$4,584.00
Total Travel	**$3,950.58**	**$3,950.58**	**$7,901.16**
D. Materials and Supplies			
(1) Chemical reagents	$5,000.00	$5,000.00	$10,000.00
(2) Others	$1,000.00	$1,000.00	$2,000.00
Total Materials and Supplies	**$6,000.00**	**$6,000.00**	**$12,000.00**
E. Equipment			
(1) Air Flow Equipment	$500.00	$500.00	$1,000.00
(2) Protein Purification Equipment	$7,668.00	$0.00	$7,668.00
Total Equipment	**$8,168.00**	**$500.00**	**$8,668.00**
F. Other Direct Costs			
(1) Publication costs	$1,000.00	$1,000.00	$2,000.00
(2) Tuition and fees	$11,726.00	$12,500.00	$24,226.00
(3) Other	$2,500.00	$2,500.00	$5,000.00
Total Other Direct Costs	**$15,226.00**	**$16,000.00**	**$31,226.00**
G. Modified Total Direct Costs	**$46,027.50**	**$46,411.70**	**$92,439.20**
2. Indirect Costs	**$19,074.41**	**$19,228.10**	**$38,302.51**
3. Total Costs	**$65,101.90**	**$65,639.80**	**$130,742.71**

1. Direct Costs
    A. Salaries and Wages
        (1) Based on current departmental stipend of $10,333.33 per year for graduate student in M.S. program.
        (2) Based on current salary of $84,500.04 per year and contribution per year. Amount increased by 3% per year.
        (3) Post doc salary not listed, so estimated contribution at $7,500 per year. Amount held constant for following year.
    B. Fringe Benefits
        (1) Based on current fringe benefit rate of 8.85% of income for graduate students plus $2,500 of insurance coverage received per year for employee.
        (2) Based on current fringe benefit rate of 21.25% of contributed income for faculty plus estimated $11,064 per year of insurance coverage for employee + family.
        (3) Post doc salary not listed, so estimated based on current fringe benefit rate of 8.85% of contributed income for post doc plus estimated $5,940 per year of insurance coverage for employee.
    C. Travel
        (1) Attendance at 2017 Biofuels Conference in San Francisco, CA. Based on meeting registration cost of $385, round trip plane ticket currently priced at $543, 4 days lodging and per diem at $341 per day.
    D. Materials and Supplies
        (1) Estimated cost of total chemical reagents needed is $5,000 per year. Likely to get a more accurate figure as the project proposal progresses.
        (2) Other materials estimated to cost about $1,000 per year. Figure likely to get more accurate as proposal progresses.
    E. Equipment

(1) Air flow equipment needed to provide consistent air flow to samples being tested. Pieces to be finalized and ordered, but estimated total cost of tubing, needles, flow meter and scaffolding to be $500 with the possibility of building another rig in the second year.

(2) ÄKTA start Protein purification system purchased from GE to be used for purifying protein samples. Purchased at a cost of $7,668.

F. Other Direct Costs

(1) Publishing 10 page manuscript in *Transaction of ASABE* at $100 per page each year. Number likely to change with additional journal publications.

(2) Tuition and fees paid by the graduate school. For 2016-2017 school year $11,726, for 2017-2018 school year estimated to be $12,500.

(3) Health insurance provided by the school at $2,500 per year for graduate students.

G. Modified Total Direct Costs

(1) Total Direct Costs less tuition and fees and equipment costs.

2. Indirect Costs

A. Calculated as 40% of Modified Total Direct Costs per University of Kentucky Office of Sponsored Projects Administration

3. Total Costs

A. Modified Total Direct Costs plus Indirect Costs

Appendix 2: Research Plan

Appendix 2: Milestones

1. May 29: Optimal laccase and ionic liquid loading determined
2. June 5: Protein purification system set up complete
3. June 10: Biocompatible ionic liquid determined
4. September 4: Bacterial laccase expressed and purified
5. October 18: Model compound and lignin product analysis completed
6. November 1: Laccase mechanism determined, project completed