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Controlling Bacterial Contamination During Fuel Ethanol Fermentation Using Thermochemically Depolymerized Lignin Bio-Oils

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Abstract

Lactic acid bacteria (LAB) contamination during fuel ethanol fermentation can lead to significant economic loses. To circumvent this, fuel ethanol plants add antibiotics prophylactically, but their overuse has resulted in the emergence of antibiotic-resistant LAB strains. Lignin is a sustainable biopolymer that can be found as a waste product from lignocellulosic biorefineries. Technical lignins and their smaller phenolic subunits have been shown to exhibit broad-spectrum antimicrobial properties, but there is a lack of demonstrations of lignin derivatives with highly selective properties in the literature. Here, corn stover lignin from a biorefinery was oxidatively depolymerized using an environmentally benign organic oxidant, peracetic acid, into a bio-oil that has selective antimicrobial properties against LAB and not yeasts. The resulting bio-oil demonstrated up to 90% inhibition of commercially sampled LAB (including antibiotic-resistant strains) at 4 mg/ml with no inhibition against an industrial yeast strain. These antimicrobial properties of the bio-oil are attributed to larger unidentified lignin oligomers, compared to monolignols, that have a membrane damaging mode of action. Using the bio-oil (4 mg/ml) during simultaneous saccharification and fermentation (SSF) of raw corn starch showed no inhibition of enzymatic activity, and in LAB contaminated fermentations the bio-oil treatments showed an 8% increase in ethanol yields at higher bacterial contamination ratios (l:100 yeast to LAB, CFU/ml). This study illustrates the efficacy of using lignin bio-oil as an antibiotic replacement during fuel ethanol fermentation and demonstrates the highly selective antimicrobial properties of lignin oligomers, which creates a viable lignin valorization strategy for biorefineries.

Keywords: Lignin, Bio-oil, Antimicrobial, Fermentation, Peracetic Acid, Lactic Acid Bacteria, Fuel Ethanol

Introduction

The U.S. alone produces over 16 billion gallons of fuel ethanol each year. Because fuel ethanol is primarily produced from the fermentation of corn starch, anything that limits yeast fermentation and subsequent ethanol yields will cause significant economic losses (Energy Information Administration). Since fuel ethanol fermentations are not conducted under completely aseptic conditions, chronic and acute bacterial contaminations can occur [1, 2]. Lactic acid bacteria (LAB) are considered to be the most problematic due to their production of by-products such lactic acids, polysaccharides and gummy biofilms that reduce yeast viability [3, 4]. Furthermore, LAB proliferation in fermentation reactors consumes essential micronutrients and sugar required for optimal yeast growth and ethanol production [2]. Therefore, these bacterial contaminations reduce ethanol yields and can result in "stuck" fermentations that cause costly shutdowns of facilities for cleaning [5]. While fermentation facilities attempt to prevent contamination through extensive sanitation practices, there are so many reservoirs of bacterial contamination that one of the most effective contamination preventatives is the use of antibiotics in the fermentation media [3, 5].

The most common antimicrobial agents used to treat LAB infection are virginiamycin, erythromycin, and penicillin [6]. These antibiotics are added prophylactically, even if no LAB contamination is detected, and they can often be used in combination during fermentations. However, due to the overuse of antibiotics, there is an increased incidence of antibiotic-resistant LAB strains isolated from dry-grind ethanol plants [7]. These antibiotics have also been shown to persist in downstream coproducts like distillers' grains [7, 8], which is becoming a major concern for consumers of distiller's grains fed livestock. Therefore, efforts in the development of new antimicrobial agents with good environmental biodegradability and high selectivity against LAB are needed to circumvent these issues.

Lignin is one of the most abundant naturally occurring sources of phenolic polymers on earth and is currently considered a major waste product in the paper and pulp industries and industrial lignocellulosic biorefineries [9]. Since lignin is a polyphenolic complex, much research has shown that its phenolic subunits can confer antimicrobial properties [10, 11]. Additionally, the antioxidant capacity and antimicrobial properties of lignin of various raw material sources, including epoxy-lignin and low-Mw lignins are also well documented [12-15]. Lignin's antimicrobial properties are dictated by the source of the lignin, its extraction methods, and chemical structure (i.e. monomers, oligomers and functional groups) [10, 16]. Nonetheless, it is believed that lignin phenolics can increase the ion permeability of cell membranes in microorganisms through ionophoric activity, causing cell lysis [17, 18]. Since ionophores are highly selective against Gram-positive bacteria compared to eukaryotes or Gram-negatives that have outer membranes (which confer insensitivity to ionophores), lignin phenolics with improved selective antimicrobial properties would be suitable for selectively inhibiting LAB in fermentation systems. Additionally, while a variety of technical lignins (i.e. Kraft lignin and organosolv lignin) have had notable antimicrobial properties, smaller depolymerized lignin oligomers and phenolic monomers are noted for increased antimicrobial activity [19]. Developing a lignin depolymerization method that produces compounds of high selectivity toward LAB but not yeast, will lead to an exciting alternative to traditional antibiotics while simultaneously valorizing lignin waste streams.

Some of the most popularly studied lignin depolymerization methods are pyrolysis, acid/base/metal catalyzed hydrolysis, hydrogenolysis and oxidation [20-22]. Pyrolysis and hydrolysis are characterized by increased condensation and repolymerization reactions that significantly reduce bio-oil yields [23, 24].While catalytic transfer hydrogenolysis provides increased bio-oil yields and more stable compounds it has energy intensive reactions that occur at high pressure and

temperatures ranging from 200-300°C [21, 25, 26]. On the other hand, oxidative procedures utilizing oxygen, hydrogen peroxide, or peroxyacids can be performed at significantly lower reaction temperatures (24-100°C), while still producing relatively high monomer yields [27]. More recent literature has focused on peracetic acid as an oxidizer due to its ability to cleave C-C and ether bonds, its higher monomer selectivity, high oil yields (18-22% w/w), and the fact that it is considered an environmentally benign oxidant [27, 28]. To that end, peracetic acid represents a viable lignin depolymerization strategy that could be low cost due to mild reaction conditions while maintaining high product yields.

Therefore, the main objective of this study was to use peracetic acid to depolymerize lignin and examine the resulting bio-oils' antimicrobial activity for use in a fuel ethanol fermentation environment. Specifically, the goals are to 1) depolymerize alkali-treated corn stover lignin from an ethanol biorefinery into a low molecular weight bio-oil by utilizing mild oxidative procedures with peracetic acid, 2) test the antimicrobial properties of the lignin bio-oil on yeast and LAB, 3) examine the effects of the lignin bio-oil on enzyme function for both α-amylase and glucoamylase, and 4) determine the efficacy of using the lignin bio-oil as an antibiotic to reduce contamination by LAB during the simultaneous saccharification and fermentation (SSF) of corn starch. We describe herein a successful demonstration of using a depolymerized lignin product as an alternative to traditional antibiotics for combating the deleterious effects of LAB on fuel ethanol production, while also creating a viable lignin valorization strategy.

Experimental

Lignin Purification

Corn stover was pretreated at the National Renewable Energy Laboratory (NREL) using 70 kg NaOH/ ton of corn stover with loading ratio of 1:12 solid: liquid at 92°C for 2 h. After pretreatment the lignin reside went under disk refining (200 kwh/ODMT, 36 inch disk, Sprout Waldon) at the Andritz pilot plant (Springfield, OH) and enzymatic hydrolysis (48 mg CTec2 and 12 mg HTec2 per gram of cellulose for 36 hour) [29]. The final lignin residue (alkaline enzymatic lignin - AEL) was collected after hydrolysis and further purified to remove remaining carbohydrates with a precipitation method [30]. The resulting lignin was then freeze-dried using FreeZone 6-liter console freeze dry system (Labconco, Kansas City, MO), at -50°C under 0.1-0.2 mBar vacuum for 72 hrs.

Structural carbohydrates and lignin composition of the resulting purified AEL samples were determined by compositional analysis according to an NREL laboratory analytical procedure [31]. The sugar concentration was determined by HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, US) equipped with a refractive index detector and using a Bio-Rad Aminex HPX-87H column and guard assembly.

Oxidative Depolymerization of Lignin

Oxidative depolymerization was carried out by following the procedures in an earlier study [28]. The purified AEL was treated with peracetic acid (PAA, Sigma-Aldrich, 32 wt. % in dilute acetic acid) at a PAA dosage of 0.8g PAA/g lignin, with acetic acid used to dilute the reaction mixture to 5% solid loading. The reaction occurred at 60 °C for 1 h while being hand mixed every 10 min. Once the reaction was completed, the reaction mixture was centrifuged at 4000 rpm to

remove unreacted solids and the supernatant was mixed with water at a 1:4 ratio to create an aqueous phase prior to liquid-liquid extraction. The lignin depolymerization compounds were extracted from the aqueous phase using ethyl acetate at a 1:4 ratio for three times. The ethyl acetate fractions were combined and dried under vacuum at 60 °C for 24 h to obtain the extracted lignin depolymerization compounds (namely bio-oil thereafter) that were then dissolved in ethanol and centrifuged to remove any undissolved solids. Bio-oil yield was determined by weighing the total bio-oil content before being dissolved in ethanol and dividing by the starting lignin weight.

Bio-Oil Characterization

The weight-average molecular weight (M_w) and the number-average molecular weight (M_n) of the purified AEL and PAA derived lignin bio-oils were determined using gel permeation chromatography (GPC) [32]. An Ultimate3000 HPLC system equipped with an Ultraviolet (UV) detector and Mixed-D PLgel column (Polymer Laboratories, Amherst, MA) were utilized. The mobile phase used was tetrahydrofuran (THF) at a flow rate of 0.5 ml min^{-1}, at 50°C. Elution profiles were monitored using UV (280 nm) and calibrated using low molecular weight polystyrene standards (Product No. 48937, Sigma-Aldrich). Polydispersity Index (PDI) was calculated utilizing methods previously described[32]. Furthermore, the bio-oil was further analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using previously described methods [33].

GC/MS was performed on the bio-oil to quantify monomer yields. The bio-oil was derivatized by first dissolving it in 0.5ml of pyridine then adding 0.5 ml of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and incubating at 50°C for 30 min. The GC was equipped with a 2-way splitter which direct the gas stream into both MS and flame ionization

detector (FID) after separated by a capillary GC column (Ultra Alloy-5, Frontier Lab, Fukushima, Japan). Monomers were identified and quantified by GC/MS using an Agilent 7890B GC coupled 5977B MS with an HP-5ms (60 m × 0.32 mm) capillary column utilizing previous methods [34]. Peak identification was conducted through the NIST MS spectra library matching. The FID detector was used for compound quantification. Calibration curves were created using five different concentrations of hydroquinone, guaiacol, syringic acid, vanillin, and 4-propylphenol (Sigma Aldrich, St. Louis, MO, USA) as standards. Based on previous work [34-36], these representative compounds were used as standards where each response factor was used according to the origin or number of carbons in the identified phenolic monomers.

NMR was performed on the purified lignin and the bio-oil. The samples were prepared by dissolving 100 mg of lignin/bio-oil in either DMSO-d_6/pyridine-d_5 (4:1) or DMSO-d_6 under mild heat and sonication. NMR spectra were acquired on a 500 MHz JEOL ECZR (Peabody, MA, USA) NMR spectrometer equipped with a 5-mm Royal Probe. Spectra processing and HSQC experimentation followed a previously published protocol [34]. The final spectra are displayed in absolute value mode and color coded (Adobe Illustrator) using literature reference standards [37].

Additionally, the total amount of phenolic compounds present in the bio-oil was estimated *via* the microtiter-plated *Folin–Ciocalteu* assay [38]. In short, reactions took place in 96-well microtiter plates where each well contained 150 uL of water, 10 uL of *Folin–Ciocalteu* (F-C) reagent, and 2 μL of the proper dilution of test compound. The wells were mixed for 5 min and 30 uL of a 20% aqueous sodium carbonate solution was added to each well. The contents of the well-plate were then incubated at 45 °C for 30 min in a dry bath. The absorbance of the aliquots at 765 nm after the reaction with F-C reagent was measured against a blank using deionized water. Total

phenolics were quantified by correlating absorbances to standard curves generated from phenol standards at different concentrations.

Microbial Cultivation

Lactobacillus fermentum (0315-1) was provided by Dr. Chris Skory (Renewable Product Technology Research Unit, USDA-Agricultural Research Service, Peoria, IL). The other lactic acid producing bacteria used were directly sampled from commercial ethanol refineries and were provided by Dr. Patrick Heist from Ferm-Solutions™ (Danville, KY) and consisted of: *Pediococcus pentosaceus, Enterococcus faecalis, Bacillus amyloliquefaciens*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus*. The yeast strain used in this study was a commercially available high-performance fuel ethanol yeast strain (*Saccharomyces cerevisiae*) from Ferm-Solutions™ called Fermpro S ®. Each microbe was grown on the liquid media recommended by the Agricultural Research Service Culture Collection (NRRL) with all LAB using De Man, Rogosa and Sharpe (M.R.S) broth (Oxoid, CM0359) and *S. cerevisiae* using yeast extract-peptone-dextrose (YPD) media (Fisher BioReagents™, BP2469). Prior to preparing frozen cultures, all LAB were incubated overnight at 37°C and a shaking speed of 180 rpm. Frozen cultures were prepared by mixing freshly washed cells with 50% sterilized glycerol in cryovial and frozen at -80°C. The yeast strain was provided as an active dried product and prior to experiments the dried product was suspended in YPD and allowed to grow overnight at 32°C and shaking speed of 180 rpm in a flask.

Antimicrobial Assay

To test for the bio-oil for antimicrobial properties, each microbe was cultivated in 96-well plates and the OD_{600} was monitored for 32 h with a microplate reader (Molecular Devices,

SpectraMax® M2). Time points were taken every 10 minutes during incubation at the growth temperatures described above. All wells were brought to an OD_{600} of 0.2 by mixing with seed cultures prior to growth, and the bio-oil was tested at 0.5, 1.0, 1.5, 2.0, 2.5, 3, and 4 mg/ml concentrations. Since the bio-oil was dissolved in ethanol prior to inoculation, all treated wells had a final ethanol concentration of 5% (v/v) to ensure homogeneity across treatment levels. Subsequently, two controls were used, one having the 5% ethanol concentration with bacterial cells, and one containing only microbes and media. All treatments and controls were performed in quadruplicates. The degree of inhibition was quantified by comparing the maximum OD_{600} during the exponential phase of growth with the minimum OD_{600} at the beginning of growth. The difference in these two values was calculated for each replicate and then averaged. The percent decrease in growth (degree of inhibition) was calculated for each treatment compared to the ethanol control, the following formula in Eq. 1 was utilized:

$$\text{Degree of Inhibition } (\%) = \left(1 - \frac{\text{Avg } (\text{Max } OD_{600} - \text{Min } OD_{600}) \text{of Growth with Oil}}{\text{Avg } (\text{Max } OD_{600} - \text{Min } OD_{600}) \text{ of Ethanol Control}}\right) * 100 \quad (1)$$

Cell membrane integrity

A cell membrane integrity assay was performed to elucidate the mode of action of the bio-oil against *L. fermentum* (0315-1). Bacterial staining was performed using the LIVE/DEAD Bac Light Bacterial Viability Kit L7012 (Invitrogen, CA), according to manufacturer's direction, on bacterial cells incubated with or without bio-oil (4 mg/ml) for 5 hr at 37°C in a 96 well plate (clear flat-bottom and black sides, Greiner Bio-One™) This kit used SYTO9 (green) and propidium iodide (red) nuclear stains to assess cell viability and membrane damage. SYTO9 is a fluorescent dye that can penetrate cell membranes freely and once bound to nucleic acids will fluoresce green,

while propidium iodide (PI) is a red fluorescent dye that can only bind to nucleic acids in cells with damaged membranes. Since PI has a higher affinity for nucleic acids compared to SYTO9, damaged cells will fluoresce red instead of green [39]. Green fluorescence was measured at Excitation/Emission (Ex/Em) wavelengths of 485 nm/530 nm while red fluorescence was measured at 485 nm/630 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Enzyme Inhibition Assays

To examine the effects of the lignin bio-oil on enzyme function during enzymatic saccharification, both α-amylase and glucoamylase were screened for activity while in the presence of the bio-oil at the highest concentration of 4 mg/ml. The dinitrosalicylic acid (DNS) method was used to screen α-amylase activity [40], which is described below. Prior to hydrolysis reaction, the α-amylase was suspended in phosphate buffer with and without the bio-oil, at a concentration of 4 mg/ml, and allowed to interact for 30 min at ambient temperature. During the DNS assay, hydrolysis reactions took place in 2 ml Eppendorf tubes, where 0.5% (w/v) of corn starch in phosphate buffer (20 mM Sodium Phosphate with 6.7 mM Sodium Chloride, pH 6.9) was reacted with ~1 unit of α-amylase for 10 min at 60°C. Additional bio-oil was added to the reaction mixture to ensure a constant concentration of 4 mg/ml. After the reaction DNS color reagent (5.3 M potassium sodium tartrate and 96 mM 3,5-Dinitrosalicylic acid solution) was added to the tubes and boiled for 15 min. The samples were immediately placed in an ice bath until they reached room temperature and then diluted with 9 ml of DI water prior to spectrophotometry. The absorbance at 540 nm was measured for the samples via spectrophotometry in 96 well plates. Enzymatic activity of control and bio-oil treated samples were determined and compared by using

amount of sugar released after hydrolysis, which was calculated by converting absorbance to maltose concentration using a standard curve of maltose.

Glucoamylase inhibition was quantified by measuring glucose content after hydrolysis using HPLC, as described below. Prior to hydrolysis reaction, the glucoamylase was suspended in acetate buffer (pH 5.6) with or without the bio-oil, at concentration of 4 mg/ml, and allowed to interact for 30 min at ambient temperature. For hydrolysis, the glucoamylase with or without bio-oil was added to a 10 mg/ml maltose solution (in acetate buffer) and allowed to react for 30 min at 60°C. Additional bio-oil was added to the mixture prior to reaction to ensure a constant concentration of 4 mg/ml. Afterwards, the reaction mixture was boiled for 15 min prior to glucose measurement. The glucose concentration released after hydrolysis was determined by HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, US) equipped with a refractive index detector and using a Bio-Rad Aminex HPX-87H column and guard assembly. Enzyme activity was determined by the amount of sugar released after hydrolysis, which was calculated by HPLC. The differences in sugar released was then used to determine any effects on enzyme function when comparing the controls and bio-oil treated samples.

Ethanol Fermentation

To test the antimicrobial properties of the PAA bio-oil in a fermentation system contaminated with LAB, the bio-oil was used at 4 mg/ml concentration in a yeast monoculture and a yeast/ *L. fermentum* (0315-1) co-culture. Only *L. fermentum* (0315-1) was tested in these model "stuck" fermentation experiments as it is the predominant strain causing contamination in the fuel ethanol industry [5, 41]. This strain is also known to be virginiamycin-resistant with a MIC value of 16 μg/ml compared to ≤2 μg/ml for susceptible strains [42]. This is important as our study aims to

utilize lignin bio-oil as an alternative antibiotic. Based on previous surveys of bacterial contaminants in fuel ethanol facilities that found bacterial loads can reach 10^8 CFU/ml [4], we challenged our model fermentations with yeast to lactobacillus ratios of 1:1, 1:10, and 1:100, which resulted in initial bacterial loads of 10^6, 10^7, and 10^8, respectively. Stock cultures of yeast and *L. fermentum* were prepared as previously described above. After 24 h incubation the microbial cells were pelletized via centrifugation and resuspended in phosphate buffered saline. One OD_{600} of yeast corresponds to 6×10^7 CFU/mL and one OD_{600} of *L. fermentum* is 1×10^8 CFU/mL. Yeast was inoculated at starting concentration of 10^6 CFU/ml and *L. fermentum* was inoculated at either 10^6 , 10^7 , or 10^8 CFU/ml.

Simultaneous saccharification and fermentation (SSF) was performed on raw corn starch (17% w/w) in sterile yeast extract-peptone (YP) medium (10 g of yeast extract and 20 g of peptone per liter of water). First, 1 ml α-amylase (Sigma-Aldrich, A8220) was added to 1 L of starch solution and the solution brought to 85 °C and held for 15 min. After enzymatic liquefaction, the starch solution was autoclaved at 121 °C for 15 min. The mixture was cooled to 85 °C, an additional 4 ml of α-amylase was added, and then the mixture was placed in a water bath at 85 °C for 1 h with intermittent stirring. The mixture was then cooled to 32 °C and glucoamylase was added to yield a concentration of 0.05% (v/v) glucoamylase (Sigma-Aldrich, A7095) immediately prior to inoculation and fermentation.

SSF was performed for 72 h at 32 °C in 50 ml serum bottles capped with a rubber septum that had a 20-gage needle inserted for gas release. Starch solution (30 ml) was added to the serum bottles and inoculated with 0.15 ml yeast solution and depending on the treatment 0.5ml of *L. fermentum* and/or 0.15 ml of bio-oil dissolved in ethanol. Treatments without *L. fermentum* had 0.5 ml of PBS added to serve as negative control. SSF treatments were performed in duplicate and

samples were withdrawn at 0, 12, 24, 36, 48, 60, and 72 h time points. The samples were analyzed using the same HPLC methods described above to monitor ethanol, lactic acid, acetic acid, and glucose concentrations. Additionally, lactobacillus density was enumerated at each time point using colony counting on MRS agar media supplemented with cycloheximide (10 μg/ml) to selectively inhibit the growth of yeast. For the bio-oi treatments, the fermentation solution had a 3.75 g/L starting concentration of ethanol, thus an additional control was used with the same starting concentration of ethanol for comparisons. The exogenous ethanol concentration was subtracted from the final ethanol concentration to arrive at the ethanol produced by the SSF.

Results and Discussion

Lignin depolymerization and molecular weight distributions

The ethyl acetate extracted bio-oil, which is the fraction used for all further experimentation, was found to be 36.1 ± 0.4 wt% of the starting lignin, whereas the remaining water soluble and undissolved solids were found to be 23.6 ± 0.9 and 40.3 ± 1.4 wt%, respectively (**Table 1**). While previous reports using similar reaction and extraction conditions with diluted acid corn stover lignin and kraft lignin found bio-oil yields of 58 and 16-45 wt% of starting lignin [28, 43, 44], respectively, the difference in lignin origin and purity can greatly affect depolymerization bio-oil yields. For example, some studies did not fully characterize their lignin source and without further purification there are likely large amounts of carbohydrates still present, which would inflate the conversion [28]. The purity of the AEL used in this study (after utilizing precipitation methods for purification) was found to be 95.11 ± 0.18% with 3.62 ± 0.16% glucan and 1.27± 0.03% xylan, so the comparison of yields may not be accurate. Furthermore, Ma et al. [28] also found that during their depolymerization reactions the starting lignin was completely dissolved. This was

not true during the reactions seen here and could indicate that the AEL is more resistant to oxidative depolymerization than the other lignin sources.

Table 1: Mass balance of lignin depolymerization into bio-oil as a percentage of starting lignin weight.

Fraction	Average Wt%
Ethyl Acetate Extracted Bio-oil	36.1 ± 0.4
Remaining Water Soluble	23.6 ± 0.9
Solids	40.3 ± 1.4

The weight-average (Mw) and number-average (Mn) molecular weight, as well as the polydispersity index (PDI) of unreacted AEL were compared with the PAA depolymerized bio-oil, as shown in **Table 2**. After treatment with PAA the weight average molecular weight (Mw) of AEL was reduced from approximately 4095 to 2277 Da (g/mol) in the bio-oil. The PDI was also reduced in the bio-oil, showing a narrower and more uniform distribution of smaller of molecular weight products compared to untreated AEL. This is also evident from the GPC chromatograms where MWD curves of the bio-oil shifted to the right (i.e., lower MW) compared to that of the untreated AEL (**Figure 1**). Previous studies depolymerizing kraft lignin with PAA at varying concentrations of PAA and temperature found that untreated kraft lignin was reduced from 2813 Da to bio-oils with Mw ranging from ~750-1500 Da [43, 44]. Even though their data also show an approximate two-fold reduction in Mw after depolymerization with PAA, the bio-oil created here is more similar in Mw to that of unreacted kraft lignin than the previously reported bio-oils. Thus, the GPC results provide a general trend on the size distribution of compounds in the PAA derived bio-oil and indicates that some depolymerization did occur.

Table 2: The molecular weight distribution of raw corn Stover lignin and PAA bio-oil.

Sample	Mw (g/mol)	Mn (g/mol)	Polydispersity index (PDI)
Corn Stover Lignin (AEL)	4095	1112	3.6
PAA Bio-oil	2277	785	2.9

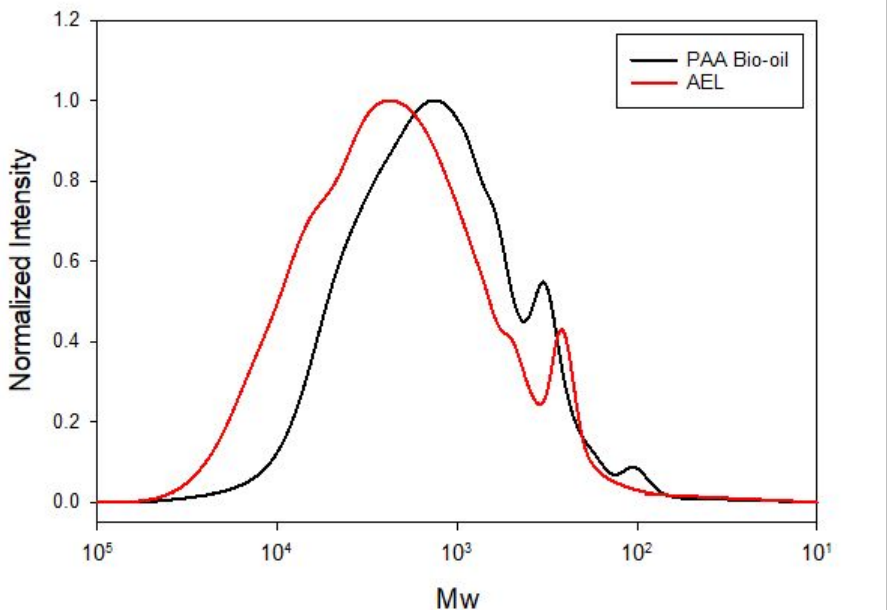

Figure 1: GPC chromatogram spectra of purified AEL and PAA derived bio-oil.

MALDI-TOF experiments were performed on the bio-oil to provide a more detailed examination of the lignin compounds in the dimer to trimer mass ranges. The MALDI spectra of the bio-oil is provide in Supplemental Figure S2. The spectra shows very low distributions of compounds in the monomer dimer (300-450 m/z) or trimer ranges (500 m/z) [33]. There is instead a much larger distribution of compounds in the 700-800 m/z range, which indicates the presence of large molecular weight oligomers above tetramer sizes. Therefore, the MALDI-TOF and GPC results corroborate each other in that this bio-oil is primarily composed of lignin oligomers. In order to identify and characterize the specific compounds formed after oxidative depolymerization, GC/MS analysis, the *Folin–Ciocalteu* assay, and HSQC NMR were performed.

Bio-oil compositional/structural characterization

The mechanisms of PAA oxidation on lignin has already be thoroughly evaluated in a previous study [28]. There work suggested that PAA depolymerizes lignin into monolignols by cleaving C-C and ether bonds in lignins polymeric structure, then individual phenolics can undergo multiple schemes to form primarily hydroxylated and acidic phenolic structures. For example, lignin subunits with phenylpropane side chains can undergo electrophilic HO^+ attack, replacing the alkyl side chain with a hydroxyl group. Additionally, hydroxylated-ether-linked phenylpropane units can be cleaved, which then undergo sequential pinacol rearrangement, Baeyer-Villiger oxidation, and hydrolysis to generate carboxylic acid side chains. In our GC-MS results, we did find the bio-oil was comprised of hydroxylated (i.e. hydroquinone and 2,6-Dimethoxyhydroquinone) and acidic (i.e. p-coumaric acid) phenolic monomers, but they only accounted for 1.77 wt% of the bio-oil (**Table 3**). This suggests that our reactions were not completely depolymerizing lignin into monomeric subunits, which is also supported by the large molecular weight ranges found in our GPC and MALDI-TOF results. Therefore, we believe our oxidative reactions predominantly cleaved inter-lignin ether bonds to produce oligomeric lignin structures, with limited monolignol production and subsequent side-chain replacement.

In terms of the depolymerization reactants acetic acid and peracetic acid, GC/MS analysis found that the bio-oil was comprised of 2.2% acetic acid, while no residual peracetic acid was found using peracetic acid test strips (MilliporeSigma™, MQuant™) that can detect 5-50 ppm of peracetic acid. The concentration of the bio-oil used for the test strips was around 500 mg/ml, so the amount of peracetic acid was below the detection limit of 5 ppm at this bio-oil concentration. This would mean that the bio-oil could contain less than 0.00001% peracetic acid, if peracetic acid is present below the detection limit. Neither Ma et al [28] nor Park et al [43] found any residual peracetic

acid in the resulting lignin depolymerization products, which makes sense as peracetic acid will quickly degrade to hydrogen peroxide and acetic acid during the dilution step with water and subsequent drying. This confirms that the oxidizers used during the depolymerization reaction were removed and should not a play a role in the antimicrobial properties of the bio-oil.

Table 3: GC/MS identifiable monomers in lignin bio-oil, with yields represented as mg/ml and wt% of total oil weight.

Compound	Yield (mg/ml)	Yield (wt %)
Hydroquinone	0.69	0.83
p-Coumaric acid	0.30	0.36
2,6-Dimethoxyhydroquinone	0.09	0.11
Syringic acid	0.09	0.11
Phloroglucinol	0.08	0.10
4-Hydroxybenzaldehyde	0.07	0.09
4-Hydroxyacetophenone	0.06	0.07
Ferulic acid	0.05	0.06
3-Ethylphenol	0.01	0.02
2-Hydroxybenzyl alcohol	0.01	0.01
Totals	**1.46**	**1.77**

While the monomeric phenolic yields seen here are very low, the *Folin–Ciocalteu* assay revealed that the bio-oil had a total phenolic content of 22.84±0.30% in terms of gallic acid equivalents. This is higher than the total monomer phenolic content found in the GC/MS results, but the *Folin–Ciocalteu* assay does not only measure monomeric phenolics, and thus larger oligomers can also be represented in this value [45]. These results indicate that the degradation compounds from AEL were primarily present as larger oligomers. Despite significant degradation occurring as evidenced by GPC results, PAA oxidation was limited in the production of monomers. This is also supported by the MALDI-TOF spectra that showed relatively low ion abundance in the dimer to trimer ranges. Other works also found a total phenolic yield of 22% using the *Folin–*

Ciocalteu assay, but they represented this value as total monomer yields and utilized total ion chromatogram (TIC) peak area instead of flame ionization detector (FID) for individual monomer quantification [28]. On the other hand, Park et al. [43] used FID for monomer quantification and found less than 0.08% of lignin monomers after treating kraft lignin with PAA, which is similar to the results of this study. Nonetheless, the data clearly illustrate that the bio-oil created here is comprised primarily of large molecular weight oligomers that are unidentifiable in GC/MS analysis.

^{1}H-^{13}C-HSQC NMR was also performed on the starting lignin and PAA depolymerized bio-oil (**Figure 2**). Notably, the AEL lignin showed only β-O-4 linkages in the linkage region. Additionally, the starting lignin had many conjugate esters as evident from the presence of pCA (p-coumaric acid ester) and FA (ferulic acid ester) in the HSQC. After treating the lignin with PAA, much of the conjugate esters and G-type structures remained relatively unchanged. This is not surprising given that acid catalyzed hydrolysis of esters is kinetically slower than the analogous base catalyzed reaction, and the potential for rearrangement of these esters on the lignin polymer under the current reaction conditions. Our GC/MS results support the low amounts of bond cleavage seen in the bio-oil and further indicate the lack of depolymerization into monomeric fragments occurring after PAA treatment. Moreover, HSQC of the PAA lignin revealed the complete loss of S-type structures from the bio-oil. We contend that this may be because the S-type lignin may have remained in the condensed solids after the oxidation reaction or is due to the increased lability of the S-lignin under acidic oxidizing conditions from the electron donation of the methoxy groups to the β-O-4 C_{α}-OH, however more studies are still needed to confirm these hypotheses [28].

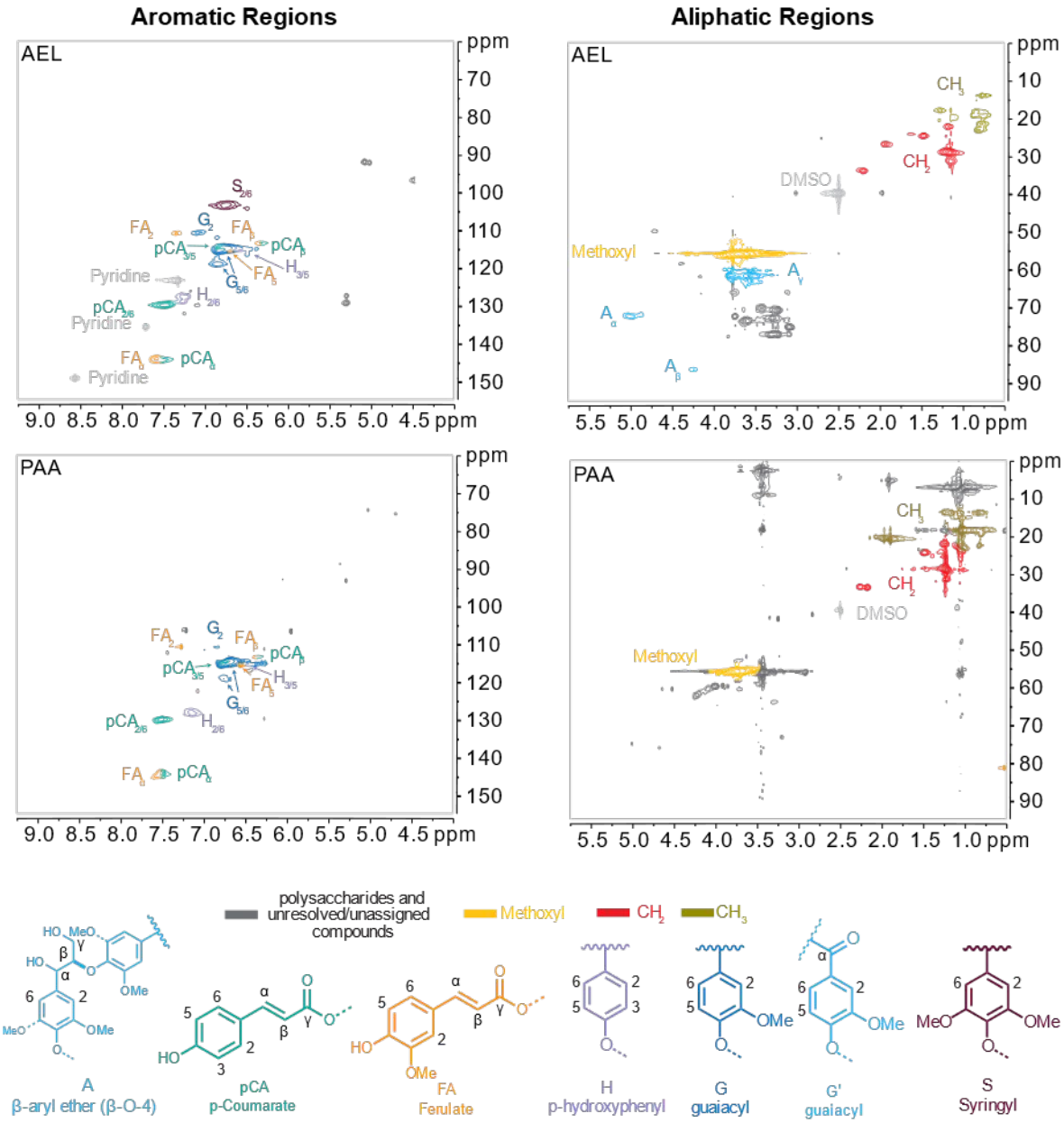

Figure 2: ^{1}H-^{13}C HSQC NMR of AEL and raw bio-oil derived from PAA oxidation. The structures of lignin compositional units and side-chain linkages were coded with colors corresponding to the cross peaks in the spectra.

Antimicrobial Assay

The PAA derived lignin bio-oil was tested against several commercially-relevant LAB strains and a commercially available high-performance fuel ethanol yeast strain (Fermpro S ®) for

antimicrobial properties by measuring growth differences utilizing spectrophotometry. Since the bio-oil is hydrophobic and becomes tar-like after drying, ethanol was used as a solvent. Consequently, an ethanol control was used in all further analyses to ensure ethanol's effects on microbial growth were accounted for. The results in **Table 4** illustrate that the bio-oil as no growth effects against yeast at any of the concentrations tested, but that the LAB showed significant growth reduction at all tested concentrations. The bio-oil was more inhibitory to both *L. fermentum* strains tested, which experienced a growth reduction of greater than 60% at bio-oil concentrations ranging from 1-2.5 mg/ml and then over 80% at 3 mg/ml. This is important as the *L. fermentum* (0315-1) strain used here is found to be one of the most prolific strains causing stuck fermentation in the fuel ethanol industry and is virginiamycin resistant [5, 41]. Therefore, the bio-oil was effective at reducing LAB growth while showing no effects on yeast growth. This suggests a selective mode of action that targets Gram-positive bacteria compared to eukaryotic yeast cells.

Table 4: Percent inhibition of PAA bio-oil at varying concentrations. Letters indicate differences at 95% confidence across all bio-oil concentrations for each organism, where values are mean±SE (n=3), using one-way ANOVAs with Tukey's pairwise comparisons or a T-test.

	Percent Inhibition						
	PAA Bio-Oil Concentration (mg/ml)						
Organism	0.5	1	1.5	2	2.5	3	4
S. cerevisiae (Fermpro S ®)	-6.88±1.56 ^{a}	4.96±2.03 ^{a}	1.73±1.44 ^{a}	-6.93±1.16 ^{a}	1.01±6.31 ^{a}	-6.83±1.78 ^{a}	-7.19±2.28 ^{a}
L. Fermentum (0315-1)	23.43±1.89 ^{a}	43.58±1.11 ^{b}	58.72±3.98 ^{bc}	61.06±4.02 ^{c}	59.74±5.06 ^{bc}	66.82±3.66 ^{c}	87.45±2.03 ^{d}
L. Fermentum	12.24±1.50 ^{a}	75.17±1.02 ^{bc}	69.57±4.46 ^{b}	74.97±0.76 ^{bc}	72.20±2.85 ^{bc}	91.25±0.63 ^{d}	83.96±3.61 ^{dc}
P. pentosaceus	N/A	N/A	N/A	74.99±5.07 ^{a}	N/A	N/A	75.18±0.00 ^{a}
E. faecalis	N/A	N/A	N/A	51.71±15.47 ^{a}	N/A	N/A	79.42±10.35 ^{a}
B. amyloliquefaciens	N/A	N/A	N/A	28.57±13.93 ^{a}	N/A	N/A	65.55±0.10 ^{a}
A. pasteurianus	N/A	N/A	N/A	42.48±1.86 ^{a}	N/A	N/A	56.40±12.41 ^{a}

These highly selective antimicrobial properties highlight the true novelty of this bio-oil and show it is not just another broad-spectrum antimicrobial lignin product. For example, in our previous publication we used the same AEL feedstock with a reductive depolymerization method to produce a bio-oil with broad spectrum activity [34]. This bio-oil was produced from catalytic transfer hydrogenolysis (CTH) depolymerization procedures and was composed of >30 wt% of phenolic monomers that were primarily in the form of alkylated phenolics (e.g. syringyl propane, 4-ethyl-phenol, 4-propylguaiacol, etc.). Antimicrobial activity of this bio-oil was screened against Gram-positive (*Bacillus subtilis*, *Lactobacillus amylovorus,* and *Staphylococcus epidermidis)*, Gram-negative (*Escherichia coli*) bacteria and yeast (*Saccharomyces cerevisiae*) and results showed inhibition of all tested organisms at concentrations less than 3 mg/mL. The alkylated phenolic monomers were suggested to be the main drivers of the CTH derived bio-oils broad-spectrum antimicrobial activity. On the other hand, Dong et al. [46] found that raw extracted lignin from dilute acid pretreated corn stover displayed more selective antimicrobial activities against Gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) and yeast (*Candida lipolytica*), but not Gram-negative bacteria (*Escherichia coli* O157:H7 and *Salmonella* Enteritidis) or bacteriophage MS2. Since Dong et al. used raw corn stover lignin, the antimicrobial properties are attributed to the larger technical lignin's structure. Interestingly, the larger molecular weight structures found in our PAA derived bio-oil and the technical corn stover lignins have more selective antimicrobial properties than the CTH bio-oil with higher monomer yields. This implies that larger molecular weight corn stover lignin structures can offer more selective antimicrobial products compared to lignin monomers, which could change some of the goals of typical depolymerization strategies. Taken together, this present study and the two aforementioned studies

illustrate that biorefinery corn stover lignin can have broad spectrum or selective antimicrobial properties depending on the processing strategy.

As stated previously, the bio-oil contained 2.2% acetic acid and less than 0.00001% peracetic acid. At the highest bio-oil concentration of 4 mg/ml this would represent a maximum of 0.088 mg/ml of acetic acid and 0.0000004 mg/ml of peracetic acid (based on the detection limit of the PAA test strips). Therefore, we tested these maximum concentrations of acetic and peracetic acid for antimicrobial properties against *L. Fermentum* (0315-1) and yeast (Fermpro). **Supplemental Table S1** shows that peracetic acid and acetic acid had no significant reductions in growth for either organism when tested individually and together compared to the control. In fact, there appeared to be a net positive increase in growth of the yeast with the addition of both acetic and peracetic acid. Thus, we are confident that the depolymerization reactants have no contributions to the observed antimicrobial properties of the bio-oil.

In order to infer the mode of action of the PAA derived lignin bio-oil, *L. Fermentum* (0315-1) was grown in the presence of the bio-oil and assessed for potential membrane damage by staining with SYTO9 and propidium iodide (PI) nuclear dyes. SYTO9 is a green fluorescent dye, and PI is a red fluorescence dye that both bind to nuclear material in the cell. However, while SYTO9 can penetrate cells freely, PI can only penetrate damaged membranes and due to its higher affinity for nucleic acids it can displace the weaker bound SYTO9 dye causing the damaged cell to show a strong red fluorescence instead of green [39]. Therefore, cells that fluoresce green (SYTO9) represent live cells without membrane damage and cells that fluoresce red (PI) can be considered membrane damaged or not metabolizing. **Figure 3** shows the ratio of SYTO9/PI fluorescence, representing the ratio of live cells to membrane damaged/dead cells, after *L. Fermentum* (0315-1) was incubated with the highest tested concentration of bio-oil (4 mg/ml) for 5 h at 37ºC.

The data show a significant decrease ($p<0.05$) in SYTO9/PI ratio when comparing the controls with cells in the presence of the bio-oil, where the ratios decreased from ~4.3 and 3.95 (control and ethanol control, respectively) to 0.7 when treated with the bio-oil. By significantly increasing the proportion of cells that fluoresce red when treated with bio-oil, it is assumed that these treated cells are PI-permeable membranes primarily due to damaged membranes and/or the inability to metabolize[39]. Lignin derivatives have been shown to directly cause cell membrane damage or have ionophoric activity that ultimately results in cell lysis and death [10, 47]. However, the mode of action may still be molecular in nature, which could affect protein synthesis or influence expression of genes, also resulting in cellular stasis or death [48]. Additionally, these data coupled with the percent inhibition data suggests that the bio-oil displays more bactericidal activity than bacteriostatic activity [49], due to the cell membrane damage or even death compared to just inhibiting cellular growth. In summary, the bio-oil is effective at selectively inhibiting a variety of LAB due to cell death without inhibiting yeast, which supports the use of this bio-oil as an alternative to control bacterial contamination in fuel ethanol fermentation.

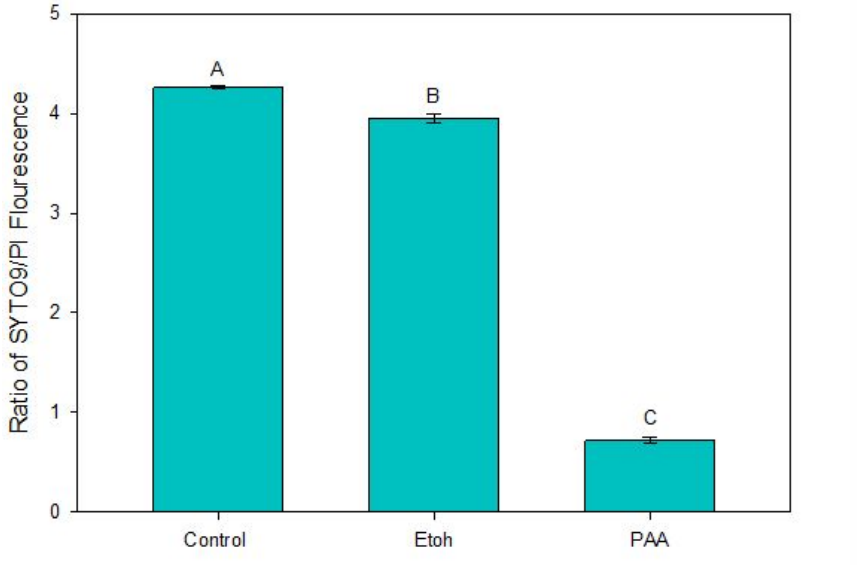

Figure 3: SYTO9/PI fluorescence ratios of *L. Fermentum* (0315-1) treated with or without bio-oil at a concentration of 4 mg/ml after incubating for 5 hr at 37 °C. These ratios indicate the ratio of live/dead or undamaged/membrane-damaged cells. In the **Figure**, Etoh is the control with ethanol added and PAA is the treatment with the bio-oil (4 mg/ml). Letters on the bars indicate differences at 95% confidence where values are mean±SE (n=3), using students T-tests.

Model Fermentations

Prior to conducting ethanol fermentation experiments, both α-amylase and glucoamylase were screened for inhibition when in the presence of the bio-oil at the highest concentration tested of 4 mg/ml. **Figure 4** shows that α-amylase had a statistically significant increase in activity, as measured by an increase in the amount of maltose released from hydrolysis of corn starch, while glucoamylase had no significant difference in the amount glucose released from hydrolysis of maltose when in the presence of bio-oil compared to the control. Thus, enzymatic saccharification during corn ethanol fermentation will not be impacted by the bio-oil and may be benefited by the slight increase in α-amylase activity.

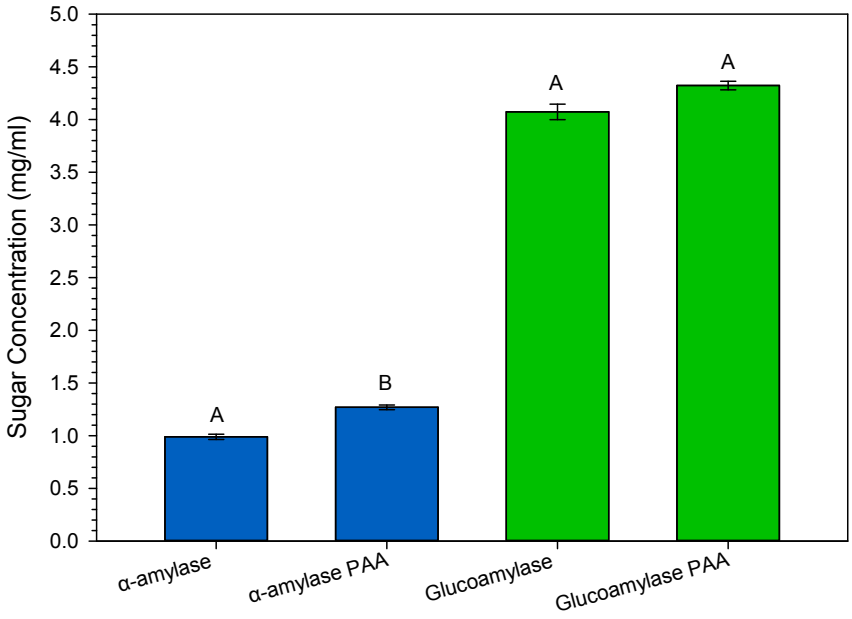

Figure 4: Sugar concentrations after enzymatic hydrolysis with or without the presence of PAA bio-oil at a concentration of 4 mg/ml. α-amylase bars (blue) indicate the amount of maltose released after 10 min of starch hydrolysis, while the glucoamylase bars (green) indicate amount of glucose released after 30 min of maltose hydrolysis. For the bio-oil treatments, the enzymes were pre-incubated for 30 min in the presence of bio-oil, and the same concentration of bio-oil was maintained during hydrolysis reactions. Letters on the bars indicate statistical differences at 95% confidence where values are mean±SE (n=3), using students t-test.

We tested our bio-oil as an antibiotic during the SSF of corn starch that was challenged with a previously reported antibiotic-resistant bacterial strain that causes "stuck" fermentation in fuel ethanol facilities (*L. Fermentum,* 0315-1) [5, 42]. **Figures 5** and **6** show the differences in ethanol production, glucose consumption, and lactic/acetic acid production for the uncontaminated and contaminated SSFs, respectively. The uncontaminated fermentations showed no significant difference in ethanol production, glucose consumption, or acetic acid production after 72 h of fermentation for the control, ethanol control, and bio-oil treatment. Available glucose was mostly consumed after 36 h (**Figure 5**), which was also marked by no significant increase in ethanol production. At the 72 h mark, there was also a large amount of starch solids left over in the fermentation broth and the ethanol production was only 76% of the theoretical yield (96.5 g/L compared to 75 g/L). Despite this, the data clearly indicate that the addition of the bio-oil had no significant effect on corn starch fermentation and yeast metabolism for the uncontaminated controls, supporting our previous results. .

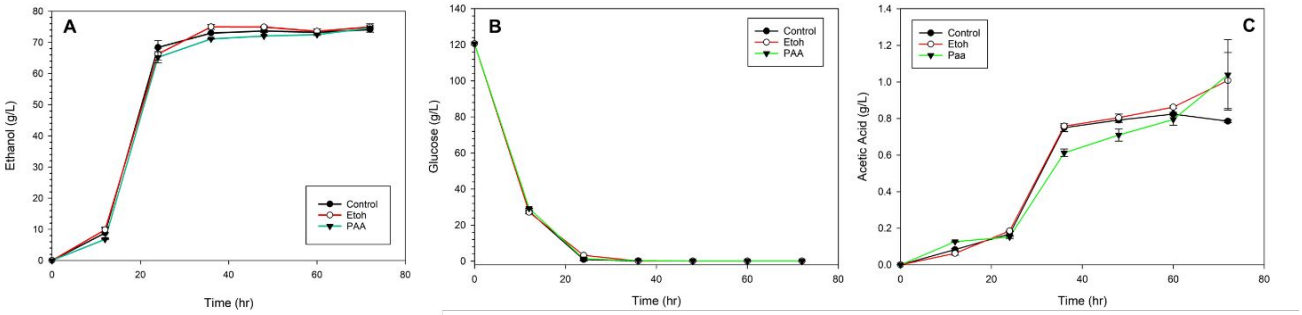

Figure 5: Ethanol (A), glucose (B), and acetic acid (C) concentrations during fermentation without contamination over time. In each graph Etoh is the control with ethanol added and PAA is the treatment with the bio-oil.

Figure 6 shows the fermentation products for the contaminated samples at yeast to LAB ratios of 1:1 (A,D,G), 1:10 (B,E,H), and 1:100 (C,F,I). After 72 h of fermentation, the 1:100 yeast to LAB ratio showed the greatest reduction in ethanol at 17% compared to the uncontaminated controls (**Figure 6 C**). The 1:1 ratio had no significant reduction in ethanol, and the 1:10 ratio had an 11% reduction in ethanol production. Conversely, Rich et al. [5] used yeast to LAB ratio of 1:6 and found an ethanol reduction of 23%, while Bischoff et al. [42] found a 17% reduction in ethanol at a ratio of 1:10, where both studies used the same lactobacillus strain applied here. Thus, the effect of lactobacilli contamination observed on our controls in the present study is less pronounced than that reported previously. These differences may be attributed to our use of corn starch instead of corn mash and/or the difference in our yeast strain, which could be more vigorous, causing the lactobacillus to be a less potent antagonist. Moreover, the bio-oil treatment did not significantly improve the ethanol yields for either the 1:1 or 1:10 yeast to LAB ratios (**Figure 6 A** and **B**), but it did significantly improve ethanol yields by 8% for the 1:100 ratio (**Figure 6 C**). This increase was even more pronounced at the 24 h time point, where the bio-oil treatment had 212% increase in ethanol produced compared to both the contaminated control and ethanol control (**Figure 6 C**). Similarly, the 1:1 and 1:10 contamination ratios also saw an increase in ethanol

production at the 24 h time point, but this did not impact total ethanol production. Therefore, as the amount of bacterial contamination increased, at earlier time points the bio-oil treatment did have a beneficial effect on ethanol production compared to untreated contaminated samples.

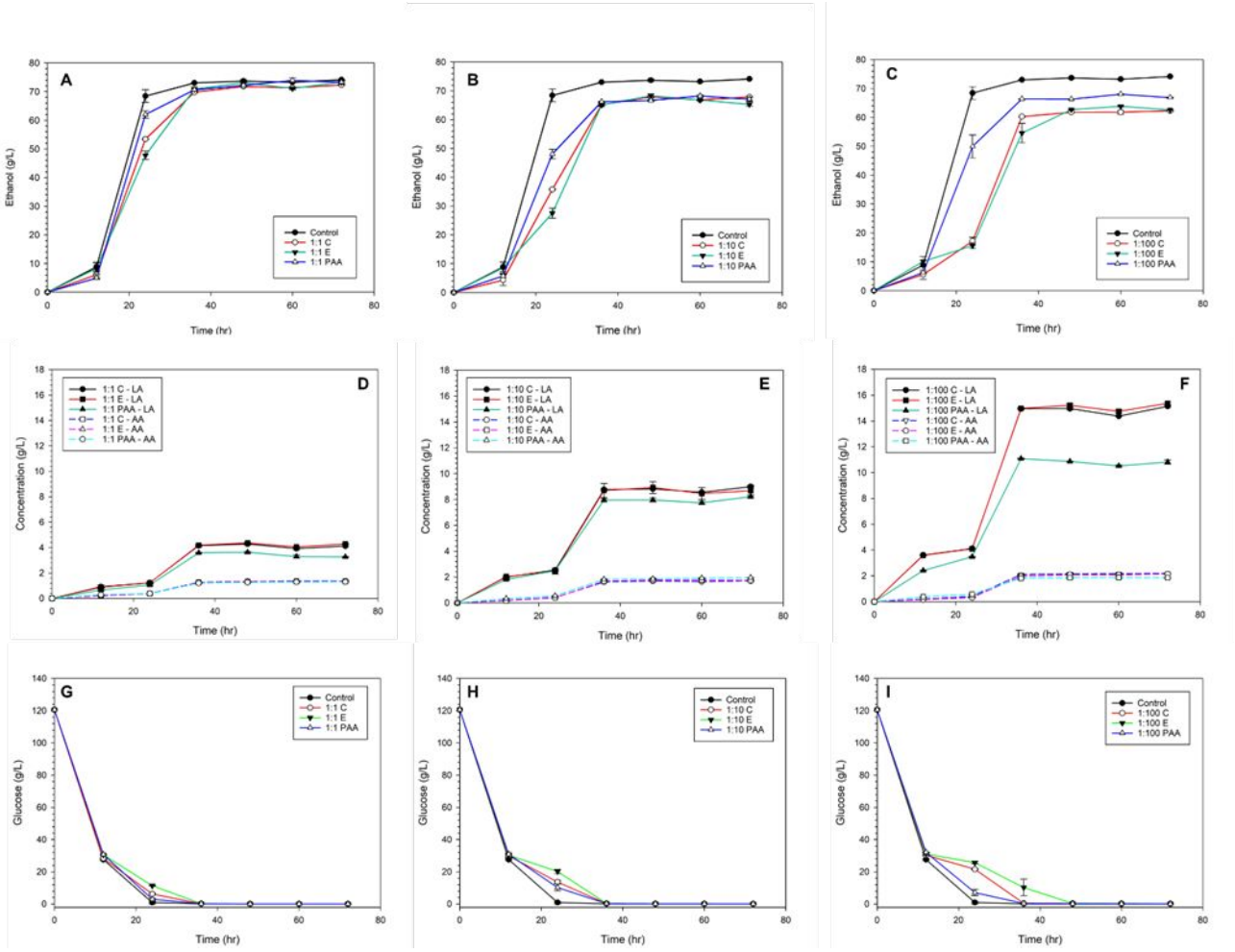

Figure 6: Ethanol (A-C), lactic/acetic acid (D-F), and glucose (G-I) concentrations during fermentation contaminated with *L. Fermentum* (0315-1) overtime for 72 hrs. The inoculation rates for the LAB were at yeast:LAB ratios of 1:1 (A,D,G), 1:10 (B,E,H), and 1:100 (C,F,I). For D-F the solid lines indicate lactic acid (LA) and the dotted lines indicate acetic acid (AA). The uncontaminated control from **Figure 4** is provided for comparisons of the ethanol and glucose concentrations and is labeled "Control". In each graph, C (i.e. 1:100 C) represents the control, E is control with ethanol added, and PAA is the treatment with the bio-oil (4 mg/ml).

While the lower bacterial contamination ratios of 1:1 and 1:10 did not show ethanol production improvements from the bio-oil treatment, there was a 10% reduction in lactic acid production, indicating that the bio-oil treatment had an effect on the lactobacillus growth/metabolism (**Figure 6 D and E**). This was even more pronounced in the 1:100 contamination ratios where lactic acid was reduced by 33% when the co-culture was treated with bio-oil (**Figure 6 F**). Figure 7 presents the lactobacillus population (CFU/ml) over the 72 h fermentation period for the 1:100 ratio. The lactobacillus population drastically decreased over time for the bio-oil treatment. When compared to the control and ethanol control there was an almost 100-fold reduction in the lactobacillus population. However, there was still a viable lactobacillus population at 3.6×10^6 CFU/ml after 72 h of fermentation. The presence of a viable lactobacillus population is consistent with our initial antimicrobial experiments showed a 90% reduction in growth (i.e. 10% of the population was still viable). at 4 mg/ml of bio-oil added to the fermentation broth. Despite the fact that the bio-oil did not completely inhibit LAB growth during SSF, the improvement in ethanol production based on the contamination controls clearly illustrates the effectiveness of lignin bio-oil used as an antibiotic replacement to control antibiotic-resistant LAB strains.

It is also noted that during the fermentation experiments with the bio-oil treatments, the bio-oil was seen mostly in a separate phase when added to the fermentation broth. Over time as the ethanol concentration increased, more of the bio-oil went into solution (as noted by a color change); thus, future work needs to improve the bio-oil's initial aqueous solubility in order to increase its efficacy as an alternative antibiotic. A formulation method that allows better introduction of concentrate bio-oil into the fermentation system should be examined and improved.

Furthermore, since traditional antibiotics such as virginiamycin are used at concentrations less than 2 ppm [42], the use of the bio-oil here at a maximum of 4 mg/ml is not directly comparable.

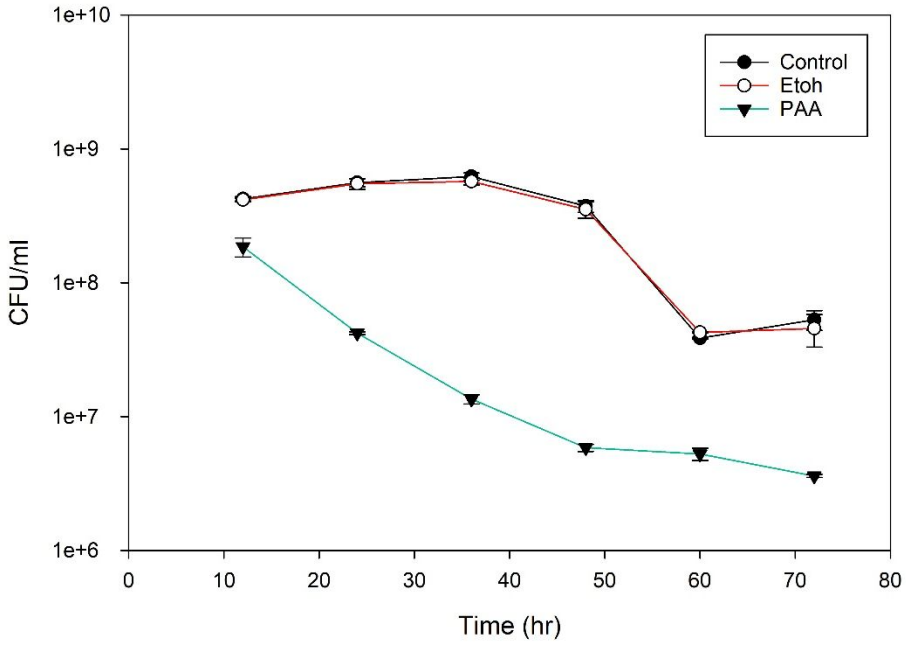

Figure 7: *L. Fermentum* (0315-1) colony forming units (CFU) per ml of fermentation broth during contaminated fermentation at a yeast:LAB ratio of 1:100 over 72 hrs. EtOH is the ethanol-added control and PAA is the treatment with the bio-oil (4 mg/ml) added to the fermentation broth.

Conclusions

In the present study, we have demonstrated that depolymerization of AEL by PAA oxidation produces mostly unidentified lignin oligomers with selective antimicrobial properties. Even though the resulting bio-oil contained less than 1.77 wt% of identifiable monomeric phenolic compounds, it demonstrated no inhibition against yeast and up to 90% inhibition of commercially relevant LAB at 4 mg/ml. The highly selective antimicrobial properties of the bio-oil are attributed to an ionophoric or membrane damaging mode of action that results in cell death, based on fluorescent staining. Using the bio-oil (4 mg/ml) as an alternative to antibiotic treatment during

SSF of raw corn starch showed an increase in ethanol production at high bacterial contamination levels when compared to the contaminated control. This indicates that the bio-oil freed up substate to be consumed by the yeast instead of LAB, by inhibiting LAB growth. At the highest contamination ratio of 1:100 yeast to lactobacillus (CFU/ml), the bio-oil treated samples had an ethanol yield increase of 8% compared to the contamination control. While the bio-oil did not completely inhibit lactobacillus growth, which still resulted in net losses of ethanol production (9%) compared to the uncontaminated control, the ability of the bio-oil to improve ethanol yields clearly show its efficacy as an alternative antibiotic. Future work should include increasing the bio-oils' solubility during fermentation, which would increase its antimicrobial action, resulting in beneficial effects on ethanol production. Therefore, the results obtained from this study offer a new application in lignin valorization and a better understanding of lignin-based bio-oil's selective antimicrobial properties and potential as a sustainable antibiotic alternative.

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