ISOLATION AND CHARACTERIZATION OF THERMOSTABLE ALKALINE BACTERIA WITH LIGNINOLYTIC POTENTIAL

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ABSTRACT

Thermus thermophilus ST and S42 were isolated from high pH (9) and temperature (70°C) hot springs in the Alvord Desert, Oregon and the Heart Lake Geyser Basin in Yellowstone National Park, Wyoming, respectively. The two strains exhibited lignin degrading potential at pH 9 and 70°C, due to their ability to utilize the lignocellulose degradation products kraft lignin, ferulic acid, cinnamic acid, and p-coumaric acid for growth. Growth on the soluble fraction of alkaline pretreated lignocellulose sources, corn stover, corn cob and lodgepole pine was evaluated. The two isolates grew to higher cell yields due of the presence of kraft lignin, corn stover and lodge pole pine when supplemented with glucose. Dye decolorizing activity was confirmed with Remazol Brilliant Blue R (RBBR), an industrial dye and lignin analog compound. Laccase mediated 2,2' azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) activity was observed for both isolates when 0.05 mM $CuSO_4$ was supplemented to the growth medium. Ligninolytic bacteria capable of growth at pH 9 and 70°C are potentially useful with alkaline lignocellulose pretreatment methods to depolymerize and remove lignin prior to the production of second generation biofuel.

INTRODUCTION

Lignin is a cross-linked aromatic polymer composed of phenylpropanoidaryl-C3 units linked by ether or carbon-carbon bonds (Bugg 2011). It acts as a protective backbone to hold cellulose and hemicellulose fibers together in the secondary cell wall of plants (Bandounas 2011). Therefore, lignin is naturally resistant to chemical and microbial degradation, since it functions as a physical barrier (Huang 2010; Ahmad 2011). Lignin, along with cellulose and hemicellulose comprise lignocellulose, the biomass found in woody and fibrous plant materials (IEA 2008). It was estimated in 2008 that 10 to 50 billion dry tons of lignocellulose waste was produced annually, which translated to two thirds of the world's energy requirement (Kumar 2008). Lignocellulose material has been identified as a feedstock for second generation biofuel production, which utilizes biomass that does not compete with the food industry for resources (Galbe 2007). Three major processes are necessary to convert lignocellulose into biofuel, particularly bioethanol; pretreatment, hydrolysis and sugar fermentation (Margeot 2009) . Pretreatment is performed to remove lignin and make cellulose and hemicellulose more exposed to enzymatic attack during the hydrolysis step. Physical, chemical and biological pretreatment methods are currently employed in industry to remove lignin (Taherzadeh 2008). The pretreatment methods can be grouped by pH into acid, alkaline and near neutral (Galbe 2007). Chemical pretreatment under alkaline conditions has been called a promising technology because of low energy requirements, minimal recycling and sugar degradation (Cheng 2009). Enzymatic hydrolysis converts cellulose into glucose, and hemicellulose into pentose and hexose sugars via cellulose enzymes.

Finally, the glucose monomers are released during the hydrolysis step, and fermented to produce ethanol (Kumar 2008). The focus of this work was to isolate and characterize bacteria that have the potential to degrade lignin under high temperature and alkaline conditions. The isolates could be used to potentially decrease the costs associated with the pretreatment of lignocellulose material.

A recent report by the International Energy Agency (IEA, 2008) stated that second generation biofuel production is not financially feasible on a commercial level. Several technological improvements to the lignocellulose pretreatment process must be implemented including, feedstock pretreatment and enzyme discovery, to increase effieciency and reduce costs. Specifically, feedstock pretreatments are inefficient and expensive, therefore new combinations of physical, biological, and chemical pretreatments must be achieved before the technology can be commercialized. The work presented here in was performed to identify bacterial isolates with ligninolytic potential capable of growth under the alkaline conditions of pH 9, and thermostable at 70°C. These conditions are industrially relevant as alkaline methods are common for the pretreatment of lignocellulose feedstock in second generation biofuel production processes.

Two approaches were taken when evaluating the ligninolytic potential of bacteria isolated from high temperature and pH hot springs. The influence of lignocellulose degradation products, especially those associated with lignin, on the growth yield of the bacteria was evaluated. The compounds tested were kraft lignin, the aromatic monomers ferulic acid, p-coumaric acid and cinnamic acid, along with the soluble fraction of

alkaline pretreated lignocellulose material. Secondly, enzymatic activity assays were performed to identify the specific activity of known ligninolytic enzymes. There are two major classes of ligninolytic enzymes found in microorganisms, peroxidase and phenol oxidase (Perez 2002; Bugg 2011). Of these, the three enzyme types that are held responsible for lignin degradation are lignin peroxidase, manganese peroxidase and laccase. In particular, bacterial laccases are promising biocatalysts of oxidation reactions because they operate in an aqueous solvent at neutral to basic pH, are temperature stable, cofactor independent, produce water as the only by-product, and are nonspecific oxidizers (Reiss 2011). Laccase is a multi-copper blue phenoloxidoreductase that catalyzes the one electron oxidation of phenolic lignin, and non-phenolic lignin units in the presence of low molecular weight mediators (inorganic compounds like thiol, or phenol aromatic derivatives) which can lead to polymer cleavage (Perez et al. 2002; Li 2009). Due to its broad substrate specificity, laccase can act on a range of compounds including diphenols, polyphenols, substituted phenols, diamines, aromatic amines, benzethiols and other xenobiotic compounds (Singh 2007). This makes laccase a very useful biocatalyst and the discovery of ligninolytic enzyme systems that are stable under harsh conditions, like high temperature and pH, would be industrially relevant (Hildén 2009).

Lignin degradation has been extensively studied in Basidiomycetes, like the white rot fungi, *Phanerochaete chrysosporium* which produce extracellular laccase, an oxidative, unspecific lignin and manganese peroxidase (Perez 2002). Low molecular weight cofactors like manganese and copper were necessary for their ligninolytic activity

(Ahmad 2011; Bandounas 2011). A comparatively small amount of work has been done with bacterial ligninolytic enzymes which may be superior to fungal enzymes due to specificity, thermostability, mediator dependency, difficulties with fungal protein expression and the propensity for genetic manipulation (Ahmad 2011; Bandounas 2011; Bugg 2011). *Streptomyces viridosporus* T7A was one of the first lignin depolymerizing bacteria found to produce an extracellular lignin peroxidase with hydrogen peroxide as a mediator (Crawford 1983). Actinomycetes and bacteria have been shown to remove methoxyl groups and carbon-carbon linkages from the lignin molecule (Tuomela 2000). Several soil bacteria with lignin degrading activity from the genus Nocardia and Rhodococcus, species *P. putida* mt-2 and *Rhodococcus jostii* RHA 1 which were also found to be aromatic ring degraders (Ahmad 2011; Bugg 2011). The activity of *P. putida* mt-2 and *Rhodococcus jostii* RHA 1 was attributed to a mediator independent, extracellular lignin peroxidase (Bugg 2011). Three bacterial isolates, *Pandoraea norimbergensis* LD001, *Pseudomonas sp.* LD002 and *Bacillus sp.* LD003 were tested for their ability to grow on aromatic monomers, industrial dyes and kraft lignin (Bandounas 2011). They found that *P. norimbergensis* LD001 and *Pseudomonas sp.* LD002 exhibited best growth on kraft lignin, but limited growth on Remazol Brilliant Blue R (RBBR). *Bacillus sp.* LD003 did not grow significantly on kraft lignin but was capable of decolorizing several industrial dyes. The current study focused on discovering bacterial ligninolytic systems which were stable under thermoalkaliphilic conditions.

Several additional studies have discovered bacterial laccases that were either heat or temperature stable. Thermostable bacterial laccases were isolated from the soil

bacterium, *Azospirillium lipoferum* (Diamantidis 2000), *Thermus thermophilus* HB27 (Miyazaki 2005), *Aquifex aeolicus* (Fernandes 2007), *Bacillus subtilis* (Enguita 2003) and *Bacillus sphaericus* (Claus 1997). Lignin degrading activity was observed in a thermophilic microbial consortium in a compost pile at 50°C, composed of the fungus *Aspergillus fumigatus* and gram positive bacteria *Bacillus sp.*, *Clostridium thermocellum*, *Clostridium cellulyticum, Flavobacterium sp.* and ξ and ε subclass Proteobacteria (Huang 2010). Alkaline laccase activity was found in a cloned *Bacillus halodurans* (Ruijssenaars 2004), a mixed bacterial consortium isolated from dyeing wastewater (Yang 2011), and the soil isolate, gamma-proteobacterium JB (Singh 2007). The industrial processes in which ligninolytic enzymes would be used are under high temperature (above 60°C) and salt concentrations, extreme pH and with short reaction times (Hildén 2009).

In the present study, two bacterial strains, *Thermus thermophilus* ST and *Thermus thermophilus* S42 were isolated from hot spring samples, and shown to possess ligninolytic potential at pH 9 and 70°C. The strains were capable of utilizing lignocellulose degradation products for increased growth yield and tested positive for laccase mediated 2,2'azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) oxidation.

MATERIALS AND METHODS

Sample Collection, Growth Medium and Bacterial Isolation

Several bacterial isolates were grown from Mickey Hot Spring in the Alvord Desert of Oregon and the Heart Lake Geyser Basin in Yellowstone National Park, Wyoming. Liquid samples which contained plant biomass were collected from each of the hot spring locations. Mickey Hot Springs, with conditions at 80.9°C and pH 8.92, was sampled along with the spring outflow at 66.9°C and pH 8.52. Several 50 mL sterile vials of geothermal water containing plant material like pine needles and bark were collected from five hot springs in the Heart Lake Geyser Basin. The conditions of the features varied from pH 8.41- 9.31 and 50 – 89.8°C. A small aliquot of the environmental samples was inoculated into Castenholz salts growth medium (Ramaley 1970). Castenholz salts medium was prepared by adding per liter: Nitrilotriacetic acid (NTA), 0.2 g; $FeCl_3$, 0.0006 g; $CaSO_4 \cdot 2H_2O$, 0.12 g; $MgSO_4 \cdot H_2O$, 0.2 g; NaCl, 0.016 g; KNO_3, 0.21 g; $NaNO_3$, 1.4 g; and Na_2HPO_4, 0.22 g: Two stock trace element solutions were used, Solution A was composed of per liter: $MgCl_2$ 25 g, $CaCl_2 \cdot H2O$ 6.6 g, Boric Acid 0.58 g, $FeCl_3 \cdot 6H_2O$ 5 g, $Co(NO_3)_2$ 0.05 g, and $NiCl_2 \cdot 6H_2O$ 0.02 g. The second solution, Trace Elements B, had per liter: $MnSO_4 \cdot H_2O$ 2.0 g, $ZnSO_4 \cdot 7H_2O$ 0.5 g, $CuSO_4 \cdot 5H_2O$ 0.15 g and sodium molybdate$\cdot 5H_2O$ 0.025 g. The stock solutions were concentrated so 1 mL of solution was added per liter of medium. A 10 L carboy of twice concentrated (2x) Castenholz salts with trace elements was made for ease of media preparation. For each liter of growth medium, 500 mL of the 2x Castenholz salts with the trace element solutions was added to 500 mL of nanopure water and buffered with

sodium borate (2.94 mM) at pH 9. Kraft lignin (0.1 g/L) was added to the medium prior to sterilization at 121°C for 20 min. Filter sterilized stock solutions of the remaining nutrient sources were added post-sterilization.

The cultures were initially enriched on 1 g/L tryptone and yeast extract (TYE). Liquid cultures (25 mL) were incubated at 70°C and pH 9, aerobically in 100 mL screw cap bottles until turbidity was present to indicate growth. Samples from the bottles with turbidity were streaked onto Castenholz salt plates containing (3.5 g/L) a thermostable solidifying agent, Gelzan ™ (Gelrite®). To make 500 mL of the plates, 2x Castenholz Salts medium (250 mL) was placed in a 1 L bottle and sodium borate (1.12 g/L) was added, and the solution was titrated to pH 9. Sodium borate buffer in 250 mL of nanopure water was titrated to pH 9, and then Gelzan ™ (3.5 g) was added. Solutions were autoclaved for 25 min at 121°C. The solutions were allowed to cool to approximately 60°C, then 0.002 M $MgSO_4 \cdot 7H_2O$ and 0.5 M $CaCl_2 \cdot 2H_2O$ were added to the 2x Castenholz salts solution and then mixed with the Gelzan ™ solution. The plates were themostable, but prone to evaporation at 70°C. To prevent evaporation the plates were stored in a sealed portable anaerobic chamber in the incubator. After several subsequent transfers on solid plates, individual colonies were picked, and reintroduced into liquid medium.

Chemicals

The lignocellulose degradation compounds, or lignin analogs used in this study were prepared in nanopure water and autoclaved with the growth medium. A 50 mM stock solution of Remazol Brilliant Blue R (Acros Organics) was prepared for 1:1000

dilutions into the growth medium. Remazol Brilliant Blue R (RBBR) is a lignin analog compound whose concentration is linearly related to absorbance at 600 nm (Shin 1997). Kraft lignin (sodium lignin sulfonate, MP Biomedicals) a byproduct of the Kraft process (Chandra 2007) was used throughout the study. The Kraft process cleaves β-O-4 linkages, the most common bond found between the monolignol units in the lignin molecule. This depolymerizes lignin and allows enzymatic access to the free cellulose fibers (Keshwani 2009). There are many structural similarities between lignin and the partially depolymerized kraft lignin, including the distinctive phenylpropanoid aryl-C3 units linked by ether or carbon-carbon bonds (Bugg 2011). A concentration of 0.1 g/L was chosen to provide a lignin source without influencing the spectrophotometric readings for growth at 600 nm. The lignin associated aromatic monomers ferulic acid (MP Biomedical), trans-cinnamic acid (cinnamic acid, Acros organics) and p-coumaric acid (MP Biomedicals) were added to the growth medium at a concentration of 0.1 g/L. The particle size of the raw lignocellulose sources, corn stover (Ames, IA), corn cob (Benson, MN) and lodgepole pine (West Yellowstone, MT) were reduced to 2 mm by milling. The material was exposed to a ^{60}Co-gamma radiation source for 3 hours, receiving approximately a 3 kilogrey (kGy) dose. The lignocellulose sources were then alkaline pretreated with a modified version of slake lime ($Ca(OH)_2$) method developed for the treatment of corn stover (Kaar 2000). The pretreatment was performed in 1 L borosilicate glass bottles with lignocellulose (1g), lime (0.075 g) and nanopure water (5 mL), the mixture was incubated at 70°C for 3 days.

Remazol Brilliant Blue R Decolorization

The liquid cultures generated from single colonies were screened for dye decolorizing activity with the industrial dye, Remazol Brilliant Blue R (RBBR), a lignin analog compound (Shin 1997). Triplicate cultures were grown on 250 mL of Castenholz Salts medium in 1 L glass screw cap bottles, with kraft lignin (0.1 g/L), TYE (1 g/L), 50 uM RBBR, 0.5 mM copper sulfate ($CuSO_4$) and buffered at pH 9 with sodium borate (1.12 g/L). A 1 mL sample of the cultures was removed daily and centrifuged at 10,000 g for 5 min to remove the bacterial cells. The absorbance maximum for RBBR at 592 nm was correlated to concentration and monitored on a spectrophotometer (Teixeira 2010). Six bacterial isolates (MOA, ST, S42, Stwhite, FD, DOME) were capable of decolorizing RBBR, and exhibited growth in the presence of kraft lignin. The cultures, MOA, ST, Stwhite and FD were isolated from Mickey Hot Springs. Dome and S42 were isolated from different hot springs in the Heart Lake Geyser basin. Two bacterial isolates (ST and S42) were selected for the remaining experiments due to their robust growth on kraft lignin and decolorization of the industrial dye, RBBR. Supplemental information on industrial dyes including RBBR and their role as ligninolytic indicator compounds is provided in Appendix C.

16S rRNA Identification of Isolated Bacterial Strains

Genomic DNA was extracted from the isolates using two methods; one with a liquid nitrogen (LN) freeze/thaw cycle followed by a PowerMax® DNA Isolation kit (MOBIO;(Bowen De León 2012) and another that used a high pH extraction buffer (pH

8.6) specific for alkaline tolerant organisms (Moll et al. not yet published). All six isolates were extracted with the Bowen De Leon method, however only three (DOME, S42 and STwhite) provided enough DNA for amplification. DNA from the remaining three cultures, ST, MOA and FD, was extracted with a method designed to extract DNA from alkaline tolerant microorganisms. In this method, cultures were collected by centrifugation at 8000 rpm for 5 min, the supernatant was decanted and the pellet was resuspended in a 2 mL microcentrifuge tube. The cells were centrifuged again at 13000 rpm for 1 min, and the supernatant was discarded. The pellet was resuspended in 200 uL of extraction buffer (1M NaCl, 70 mM Tris, 30 mM EDTA buffered at pH 8.6) and centrifuged at 13000 rpm for 1 min. The supernatant was discarded and resuspended in 500 uL of extraction buffer. Glass beads were added to fill the 2 mL microcentrifuge tube along with 200 uL of chloroform, and then the tube was mixed on a vortex for 10 min. The samples were centrifuged at 13000 rpm for 15 min and the supernatant was transferred to 1.5 mL centrifuge tube. Sodium acetate (3 M) and isopropanol were added to a fraction of one tenth and three-fourths the sample's volume, respectively. The tube was inverted several times and incubated at -20°C overnight. The tube was centrifuged at 13000 rpm, and the supernatant decanted. The pellet was washed with 500 uL of 80% ethanol and centrifuged at 13000 rpm for 5 min. The supernatant was removed and the pellet was allowed to dry for 30 min. The DNA in the pellet was resuspended in 50 uL of nuclease free, diethylprocarbonate (DEPC) treated water (Ambion).

PCR mediated amplification of the 16S rRNA region was performed with the universal bacterial primers 8F, 907R and 1392R. The PCR product was sequenced at the

Idaho State University, Molecular Core Research Facility on their Applied Biosystems 3130XL Genetic Analyzer. The sequence alignment was performed using the Sequencer program (Gene Codes) and 1350±10bp contigs were generated for the isolated cultures. The contigs were submitted to Basic Local Alignment Search Tool (BLAST) analysis on the NCBI website (Altschul 1990) and the identity was indicated based on the percent similarity to other organisms.

Bacterial Growth on Lignin Associated Compounds

The ability of the isolates to have increased growth yields on several lignocellulose degradation products was tested. The two bacteria isolated from the enrichments, *T. thermophilus* ST and *T. thermophilus* S42, were routinely cultured on Castenholz salts medium with kraft lignin (0.1 g/L), and buffered at pH 9 with sodium borate (1.12 g/L). The strains were grown in 25 mL glass screw caps bottles in 15 mL of growth medium with the lignin associated compound (0.1 g/L) and supplemented with an additional nutrient source of TYE (0.025 g/L) or glucose (0.5 g/l). The lignocellulose degradation products kraft lignin, ferulic acid, cinnamic acid and p-coumaric acid were added to the growth medium in an experiment run in triplicate along with an uninoculated medium control. The water soluble fraction of the alkaline pretreated lignocellulose material (corn stove, corn cob and lodge pole pine) was added to the Castenholz salts medium. For these experiments, growth medium was composed of per liter: 500 mL 2x Castenholz salts medium, 250 mL of nanopure water and 250 mL of the soluble lignocellulose material, buffered at pH 9 with sodium borate (1.12 g/L). All experiments with lignin like compounds were performed in a shaking incubator (125 rpm) at 70°C.

Samples were collected every 12 h for growth analysis via protein concentration or optical density 600 nm. The growth measurements were performed on a spectrophotometric 96-well plate reader with sample volumes of 200 uL. It was necessary to remove 700 uL when protein concentration was measured. The protein concentration was assessed using the manufacturers' protocol for the Better Bradford Assay (Pierce)(Bradford 1976). After the bacterial cells were pelleted by centrifugation at 10,000 g for 5 min, the supernatant was decanted and the cells were lysed with the BugBuster® Protein Extraction reagent (Novagen). This method employs the Coomasie Reagent which has an absorbance maximum at 595 nm. The protein concentration was used to correlate the absorbance units recorded at 600 nm to a bovine serum albumin (BSA) protein standard actual value and was recorded at the initial, middle and final growth point. The complete data set with growth curves can be found in Appendix D.

Ligninolytic Enzyme Activity Assays

Activity assays for, laccase and lignin peroxidase, the enzymes which are usually responsible for ligninolytic activity in bacteria, laccase and lignin peroxidase, were performed on the isolates *T. thermophilus* ST and *T. thermophilus* S42. A protein extraction protocol was used to isolate the proteins by cellular location. The activity of intracellular proteins located in the cytoplasm and membrane of the two bacterial strains was evaluated. Two week old cultures grown on Castenholz salts medium with kraft lignin (0.1 g/L), glucose (0.5 g/L) and $CuSO_4$ (0.0, 0.05 and 0.5 mM) was subjected to a protein extraction protocol. Protein from the strains was extracted and separated into two cytoplasmic fractions (S1 and S2) and one membrane associated fraction (S3). The

cultures were centrifuged at 4500 rpm for 15 min, the growth medium was decanted and the cells were resuspended in 4 mL of 25 mM phosphate buffered saline (PBS, pH 7.3). This was repeated twice, but the volume of PBS was dropped to 1.5mL and then 1mL. Benzonase® Nuclease (5 uL), and a bacterial protease inhibitor cocktail (PIC; Sigma Aldrich) (20 uL) was added to the cells suspended in PBS. The centrifuge tubes containing the washed bacterial cells were submerged in liquid nitrogen (LN) until completely frozen. The samples were allowed to thaw at room temperature before being placed into the LN, this process was repeated for a total of three freeze/thaw cycles. After the cultures were allowed to thaw for a third time, they were decanted into 15 mL falcon tubes and sonicated for 1 min. The lysed cells were centrifuged at 15,000 g for 35 min in 2 mL centrifuge tubes. The supernatant collected contained the first cytoplasmic protein faction (S1) and was stored at -80°C. The pellet was resuspended in 500 uL of 0.5 M NaCl in 20 mM sodium acetate, and centrifuged for 25 min. The supernatant contained the second cytoplasmic protein fraction (S2) and was collected in a new 2 mL centrifuge tube then stored at -80°C. The pellet was resuspended in 200 uL of 0.4% triton X-100 in 25mM PBS, and centrifuged at 13,000 g for 25min. The supernatant was transferred to a new 2 mL centrifuge tube and stored at -80°C. This was the membrane associated protein fraction (S3). PIC (20 uL) and Benzonase® Nuclease (5 uL) were added to all three protein fraction samples (S1, S2 and S3). The protein separated by location into two cytoplasmic (S1 and S2) and one membrane associated fraction was subjected to activity assays to determine which enzymes were present.

The lignin peroxidase assay used 15 mM 2, 4- dicholorophenol as a substrate in the presence of 1.64 mM 4-aminoantipyrine and initiated by 40 mM hydrogen peroxide while buffered at pH 9 with 0.1 M sodium borate (Ishida 1987). The substrate oxidation was monitored at 510 nm on a spectrophotometer. Two assays were used to determine if laccase activity was present in the strains, one with 0.216 mM syringaldazine (SYR) (Ride 1980) and another with 500 uM 2,2' azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)(ABTS)(Bourbonnais 1990) as the substrate. Both assays were buffered with 0.1 M sodium borate at pH 9 and incubated at 70˚C. The increase in absorbance at 530 nm for 10 min was monitored for the SYR laccase assay. The ABTS oxidation product at 420 nm was recorded at time zero and after two minutes of incubation. All assays were performed at room temperature, and at 70°C to differentiate the activity of thermostable enzymes. The enzyme assays were modified for a spectrophotometric plate reader which accepted 96 well plates with sample volumes of 200 uL.

RESULTS AND DISCUSSION

Identification of Bacterial Strains with RBBR Decolorization Activity

Several bacterial cultures were isolated from liquid samples containing plant biomass collected near the edge of alkaline hot springs in the Heart Lake Geyser Basin, Yellowstone National Park, Wyoming and Mickey Hot Springs, Alvord Desert, Oregon. Aliquots of the liquid samples containing biomass was added to Castenholz Salts medium with kraft lignin and supplemented with tryptone/yeast extract (TYE) as an additional nutrient source. The isolates were inoculated into Castenholz Salts medium buffered at pH 9, with 50mM RBBR and TYE as a nutrient source. (Miyazaki 2005; Li 2009). Laccase is one of three enzymes usually responsible for ligninolytic activity (Perez 2002). The absorption at 592 nm linearly correlates to RBBR concentration. The percent change or decolorization was reported as the difference between the initial and final reading over the initial concentration. In Figure 1, the isolated cultures labeled MOA, Dome, S42, ST and MOA caused 39% to 61% RBBR decolorization after five days of incubation. The uninoculated control (U.C.) caused 8% RBBR decolorization. The possibility that RBBR was adhering to the cell surface as the removal method versus direct enzymatic activity was not eliminated. The RBBR data along with the lignin substrate experiments and ligninolytic enzyme assays were used to select two cultures ST and S42 for further study. The supplemental data is shown in Appendix D.

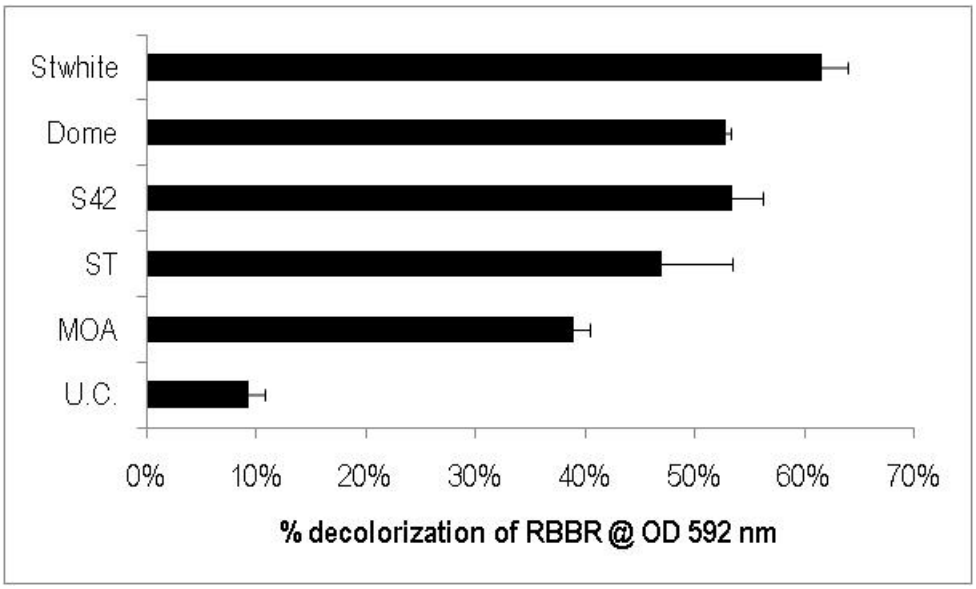

Figure 1. The change in concentration of RBBR, presented as a percentage of the initial concentration, caused by the presence of the isolated strains Stwhite, Dome, S42, ST and MOA. Triplicate data for the culture FD was not collected.

The ability of the isolates to decolorize the industrial dye, Remazol Brilliant Blue R (RBBR), a lignin analog compound was tested. RBBR can be decolorized by lignin degrading enzymes and has been found to induce lignin degrading activity (Singh 2011). Several studies have used industrial dyes when searching for bacteria with ligninolytic activity (Nigam 1996; Khehra 2005; Joshi 2008; Bandounas 2011). Lignin and RBBR are polycyclic aromatic compounds which are resistant to microbial degradation, however organisms with ligninolytic activity can decolorize RBBR (Nigam 1996;Shin 1997). Unlike lignin, RBBR linearly absorbs light at an absorbance maximum of 592 nm which correlates to concentration and can be monitored on a spectrophotometer (Machado 2005; Faraco 2009).

16S rRNA Identification of the Bacterial Isolates

Sequence information is provided in Appendix A. All six isolates were identified to be 99% similar to *Thermus thermophilus* HB8 (Williams 1995), however only two were given strain names *T. thermophilus* ST and *T. thermophilus* S42. There is a high degree of sequence similarity within the genus *Thermus* despite distinct physiological differences, especially in the case of *T. thermophilus* HB8 and AT62 (Fujino 2007). 16s rRNA sequence similarities range from 94-96 % for the genus and 99.4-100 % for strains of *T. thermophilus* (Costa 2006). All six cultures originally isolated from this project were phylogenetically identical to *T. thermophilus* HB8 and each other, even though they displayed different growth rates, yields and enzymatic activity. *T. thermophilus* HB8 was originally isolated from Yellowstone National Park, Wyoming, maintained on Castenholz Salts medium at pH 7.5 and incubated at 65°C (Williams 1995). *T. thermophilus* ST and *T. thermophilus* S42 were also thermophilic and grew at 70°C, but were isolated from alkaline conditions. There are no reports of *T. thermophilus* HB8 exhibiting laccase or ligninolytic activity, although a thermostable laccase was isolated from *T. thermophilus* HB27 (Miyazaki 2005).

The STRING database (string-dg.org; (Snel 2000)) was used to search for known or predicted protein interactions, by searching for protein name or sequence similarity among organisms whose genomes have been fully sequenced. The full genome of *T. thermophilus* HB8 was annotated in 2004 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?name=Thermus+thermophilus+HB8) (Maybe (Masui et al. unpublished 2004) although a reference was not

provided for the researchers responsible. The sequence data for the thermostable laccase identified from the closely related organism *T. thermophilus* HB27 (Miyazaki 2005), was submitted to the STRING database to search the genome of *T. thermophilus* HB8 for a similar sequence. The sequence is provided in Appendix A for TTC1370, the laccase found in HB27. This query provided no results for sequences in HB8 that match TTC1370 in HB27, the genes neighboring TTC1370 that were predicted to have similar functions were also not found in HB8. Genes coding for hypothetical proteins are present in this region and more analysis would have to be performed to determine if one of them has laccase like function (Morita 2011).

Degradation of Aromatic Monomers

The lignin degrading potential was evaluated for two bacterial strains isolated from hot springs which were capable of growth under high pH and temperature conditions. This means that the isolates will grow in the presence of or have increased growth yields due to lignin, its degradation products or associated compounds. Two strains of *T. thermophilus* ST and S42 were grown on the basal salts medium, Castenholz Salts, with lignocellulose degradation products. The monoaromatic degradation products ferulic acid, cinnamic acid and p-coumaric acid, along with kraft lignin were added to the growth medium. Tryptone/yeast extract (TYE) was supplemented to increase the growth yield and rate of the strains. The strains were grown on TYE alone and TYE plus one degradation product. An uninoculated medium control was run in parallel to determine the impact of abiotic changes to the medium. The experiment was performed in triplicate at 70˚C, in pH 9 buffered medium and rotated at 125 rpm. Supernatant samples were

collected every 12 hours and cell growth was monitored by OD (600 nm) on a spectrophotometer.

Figure 2 displays the cell growth, as the difference between the final and initial OD at 600 nm readings. This difference represents the growth observed on the compounds supplemented in the medium and removes an increase in OD caused by the inoculum. The two strains were grown in the presence of 0.1 g/L of kraft lignin and the same concentration of the monoaromatic compounds ferulic acid, cinnamic acid and p-coumaric acid. Tryptone/Yeast Extract (TYE) was added as a rich nutrient source to encourage the strains to grow. An uninoculated medium control was run in parallel with the experimental replicates, denoted by "U.C." for uninoculated control. Growth was visualized as the difference in OD between the compound and its uninoculated control. For example, growth on TYE would be the difference between the columns labeled "TYE" and "TYE (U.C.)". The impact of kraft lignin and the monoaromatics is visualized as the difference between their columns and the one labeled "TYE".

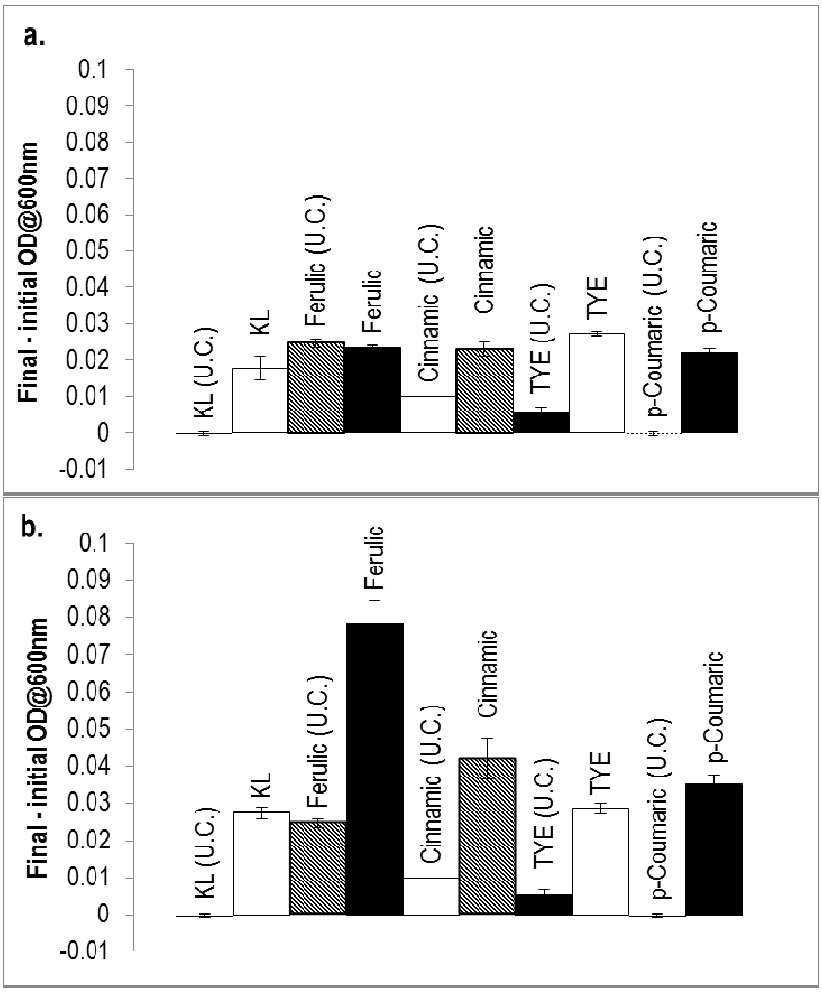

Figure 2. The strains *T. thermophilus* a) ST and b) S42 were grown in the presence of 0.1 g/L kraft lignin (KL), ferulic acid, p-coumaric acid or cinnnamic acid with 0.025 g/L tryptone/yeast extract (TYE) as a nutrient source. "U.C." represents the uninoculated medium control. The initial OD measurement at time zero was subtracted from the final OD reading at 96 h.

In Figure 2a, *T. thermophilus* ST grew to a higher OD than the uninoculated control (U.C.) for each of the compounds tested. The presence of the kraft lignin, ferulic acid, cinnamic acid or p-coumaric acid did not cause significantly higher growth than TYE which was supplemented along with the aromatic source. Bandouras et al. 2011 showed that a ligninolytic isolate *Bacillus sp.* LD003 exhibited limited growth off of kraft

lignin and several monoaromatic compounds. *Bacillus sp.* LD003 did show decolorization activity for several industrial dyes including RBBR when supplemented with yeast extract and $CuSO_4$. The suggested role of *Bacillus sp.* LD003 as a lignin degrader which must rely on external sources for nutrients could potentially be applied to *T. thermophilus* ST. Like *Bacillus sp.* LD003, *T. thermophilus* ST was capable of decolorizing RBBR, but did not grow to increased cell yields due to the presence of aromatic monomers or kraft lignin. Figure 2b shows that the strain *T. thermophilus* S42 grew to higher optical densities than the uninoculated medium in the presence of all compounds. *T. thermophilus* S42 grew to higher growth yields due to the addition of ferulic acid, cinnamic acid and p-coumaric acid as compared to TYE alone. The most significant increase in growth in S42 was caused by the presence of ferulic acid in the medium in contrast with the growth of ST on any of the compounds. A two sample t-test assuming equal variances analysis was used to confirm the significance of the differences between growth due to TYE and the aromatic compounds. The p-values of ferulic, cinnamic and p-coumaric acid were 0.006, 0.057 and 0.002 respectively, which are below an α=0.05. The differences in growth caused by the aromatic sources were significantly higher than the nutrient source TYE. The complete data set for the t-tests performed are in Appendix D. A distinction has been made between the ability to depolymerize and modify lignin, and the capacity to metabolize lignin degradation products (Bandounas 2011). Both should be evaluated to determine the lignin degrading potential of bacterial strains. *T. thermophilus* S42 has shown the potential to metabolize the aromatic monomers ferulic acid, cinnamic acid, and p-coumaric acid.

Bacterial Growth on Lignin Associated Compounds

The ligninolytic potential of the isolates *T. thermophilus* ST and *T. thermophilus* S42 was evaluated for growth on kraft lignin, ferulic acid and the soluble fraction of alkaline pretreated lignocellulose material (Kaar 2000). Glucose (0.5 g/L) was supplemented as an additional carbon source to guarantee growth in presence of the lignin associated compounds. Tryptone/yeast extract was removed as the nutrient source in order to have completely defined medium and glucose has been found to induce laccase activity. The growth on glucose alone was confirmed in several experiments and is shown in Figure 3, along with additional growth due to the presence of kraft lignin, ferulic acid and the soluble lignocellulose material. Cell free medium controls (U.C.) were run along with the experiment triplicates at pH 9 and 70˚C under agitation at 125 rpm.

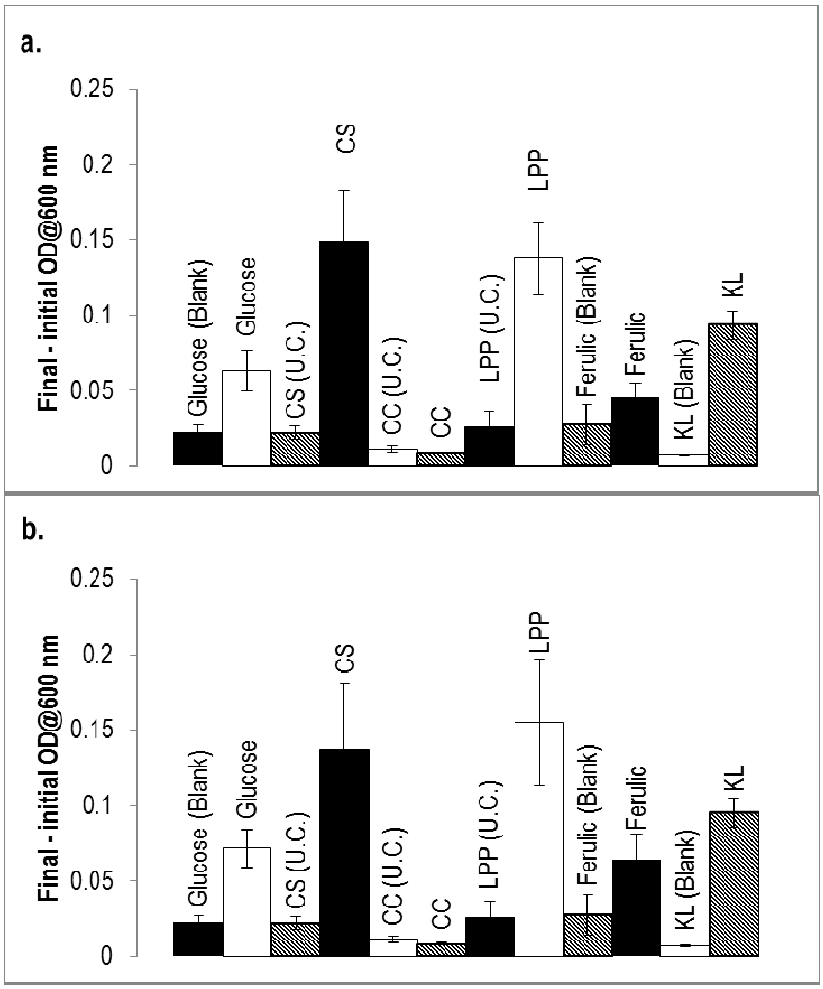

Figure 3. The impact of 0.1 g/L of kraft lignin, ferulic acid and the soluble fraction of alkaline pretreated lignocellulose with 0.5 g/L of glucose on the growth of *T. thermophilus* a) ST and b) S42. An uninoculated medium control (U.C.) was run in parallel with the bacterial replicates for each of the compounds.

In Figure 3 it is important to recognize the difference between growth due to glucose alone and growth caused by glucose combined with the lignin associated compound. Displayed in Figure 3a, *T. thermophilus* ST grew to a significantly higher yield due to the addition of kraft lignin and lodge pole pine in the medium. A two sample t-test assuming equal variances was performed to discriminate the difference between the growth on glucose and the lignin like compound. The p-values for kraft lignin and lodge

pole pine were 0.006 and 0.012 respectively. Although it is not explicitly known what compounds were released into the soluble lignocellulose fraction by the alkaline pretreatment, it is interesting that the *T. thermophilus* ST was capable of growth utilizing some compound(s) present. When processing lignocellulose as the feedstock for biofuel production it is important that compounds inhibitory to the enzymatic hydrolysis are removed during the pretreatment step (Kumar 2008). Dissolved lignin will diminish the efficiency of cellulase, xylanase and glucosidase used during the enzymatic hydrolysis of cellulose and hemicellulose (Taherzadeh 2008). Alkaline pretreatment methods usually solubilize major portions of the lignin and some of the hemicellulose present in the treated lignocellulose (Galbe 2007). If the isolate ST can use some of the released lignin associated compounds for growth it could potentially increase the overall efficiency of the pretreatment process. *T. thermophilus* ST utilized kraft lignin and lodge pole pine for increased growth when only a carbon source glucose, not the rich nutrient source TYE, was added to the medium. Shown in Figure 3b, *T. thermophilus* S42 grew on kraft lignin, corn stover and lodge pole pine in addition to glucose. The p values generated from the t-tests for kraft lignin, corn stover and lodge pole pine are 0.033, 0.067 and 0.041 respectively. There was a conflict between the one and two tail p-values for corn stover and lodge pole pine. The complete data is shown in Appendix D. *T. thermophilus* ST and *T. thermophilus* S42 together have ligninolytic potential due to their ability to grow to higher cell yields because of the presence of kraft lignin, corn stover and lodge pole pine when supplemented with glucose.

Ligninolytic Enzyme Activity Assays

The activity of three enzymes, lignin peroxidase, manganese peroxidase and laccase are usually responsible for ligninolytic activity in microorganisms (Perez 2002). Lignin peroxidase and laccase activity assays were performed on the two isolates, *T. thermophilus* ST and *T. thermophilus* S42, to determine if their ligninolytic activity was caused by these enzymes. The two isolates were grown on Castenholz Salts medium buffered at pH 9, with 0.5 g/L glucose as an additional carbon source and supplemented with Cu^{2+} atoms. The concentration of Cu^{2+} in the form of copper sulfate was varied from 0.0 mM to 0.5 mM to try to induce laccase activity without significantly inhibiting the growth of the bacteria.

The concentration of total protein (the sum of all three fractions) decreased with increased copper sulfate ($CuSO_4$) concentration, as shown in Figure 4. The protein concentrations at 0.0 mM $CuSO_4$ were 0.5 and 0.343 mg/L for *T. thermophilus* ST and S42, respectively. The concentration decreased to 0.051 mg/L for *T. thermophilus* ST and 0.144 mg/L for *T. thermophilus* S42 at 0.5 mM $CuSO_4$. The increased concentration of $CuSO_4$ significantly inhibited the growth of the bacteria.

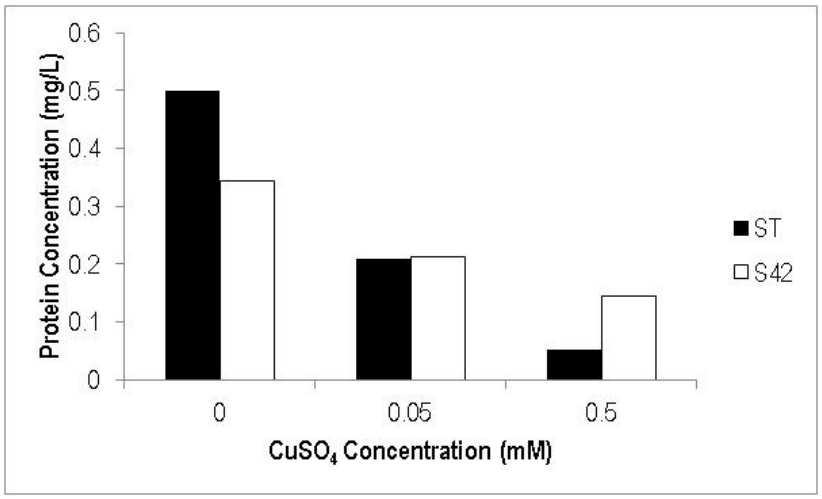

Figure 4. The change in total protein concentration with increased $CuSO_4$ concentration for the two isolates *T. thermophilus* ST and *T. thermophilus* S42.

The change in concentration caused by the increase in $CuSO_4$ to each fraction is displayed in Figure 5 for *T. thermophilus* ST and S42. When $CuSO_4$ was not added to the medium all fractions contained protein in both strains. The cytoplasmic fraction (S1) had slightly greater protein than S3, and S2 contained the least amount (S1>S3>S2). At 0.05 mM $CuSO_4$ the distribution of protein followed the same trend, S1>S3>S2, but the total protein concentration was decreased at least in half. At the highest $CuSO_4$ concentration tested, 0.5 mM $CuSO_4$, only a small amount of protein was removed with the cytoplasmic fractions (S1 and S2), the majority was extracted in the membrane associated fraction (S3). The increase in concentration from 0.0 to 0.5 mM $CuSO_4$ not only decreased the total protein concentration of the two isolates *T. thermophilus* ST and S42, but also shifted the distribution of protein in the extracted fractions.

Specific enzyme assays for laccase and lignin peroxidase were performed on the extracted fractions (S1, S2 and S3) of *T. thermophilus* ST and *T. thermophilus* S42. The

lignin peroxidase (LiP) assay was performed using 2, 4- dicholorophenol as the substrate in the presence of 4-aminoantipyrine mediated by hydrogen peroxide while buffered at pH 9 with 0.1 M sodium borate. Both isolates tested negative for LiP activity at 70˚C and pH 9. Two assays were used to determine if laccase activity was present in the strains, one with syringaldazine (SYR) and another with 2,2' azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) as the substrate. Both assays were buffered with 0.1 M sodium borate at pH 9 and incubated at 70˚C. The SYR laccase assay tested negative for an increase in oxidation products at 530 nm over 10 min. The results for laccase mediated ABTS oxidation are shown in Figure 5 for *T. thermophilus* ST and *T. thermophilus* S42. The reaction was carried out for two minutes at 70˚C and room temperature, while the increase in ABTS oxidation product was monitored on a spectrophotometer at 420 nm. The results shown in Figure 5 are the difference in activity at 70˚C and room temperature. When the room temperature reaction showed higher activity than the 70˚C reaction, a negative value resulted.

Intracellular (Salano 1997; Miyazaki 2005; Fernandes 2007; Bandounas 2011) and extracellular (Crawford 1983; Bugg 2011) ligninolytic enzymes have been isolated from bacteria. Preliminary data suggested that the enzymes produced by *T. thermophilus* ST and *T. thermophilus* S42 were intracellular. All bacterial laccases reported have been intracellular except for laccase from the spores of *B. subtilis* (Singh 2007). An enzyme is considered intracellular if it was extracted from the cell pellet instead of supernatant of the medium (Santhanam 2011). An extraction protocol was used to lyse the cells and separate proteins by cellular location. The cytoplasmic and membrane associated

proteins from the bacteria were isolated with the extraction protocol yielding three fractions. Two of the fractions contain cytoplasmic proteins (S1 and S2) and one contains membrane associated proteins

(S3).

There was little laccase activity from either strain when no $CuSO_4$ was added to the growth medium. Increasing the concentration to 0.05 mM and 0.5 mM $CuSO_4$ caused ABTS oxidation product to be present in the membrane associated fraction (S3) of *T. thermophilus* ST as shown in Figure 5c. From Figure 5d, laccase activity was induced in the cytoplasmic fractions (S1 and S2) of *T. thermophilus* S42 when 0.05 mM $CuSO_4$ was provided. When the growth medium was supplemented with 0.05 mM $CuSO_4$, laccase activity was induced in *T. thermophilus* ST and *T. thermophilus* S42. Miyazaki 2005 showed a hyperthermophilic laccase stable at 92°C was isolated from *T. thermophilus* HB27 and expressed as a recombinant enzyme in *Escherichia coli* (). The conditions used to characterize the native laccase were similar to the process used during the current study. Laccase mediated ABTS oxidation was tested to verify the activity of the *T. thermophilus* HB27 laccase which required 0.5 mM $CuSO_4$ to be supplemented into the growth medium to induce activity (Miyazaki 2005). *T. thermophilus* HB27 was grown on a medium similar to Castenholz salts supplemented with tryptone and yeast extract, buffered at pH 7 with NaOH. The laccase activity of *T. thermophilus* ST and S42 was found after growth on kraft lignin and glucose while buffered at pH 9, meaning the two strains found in this study did not require a rich nutrient source like TYE to produce laccase.

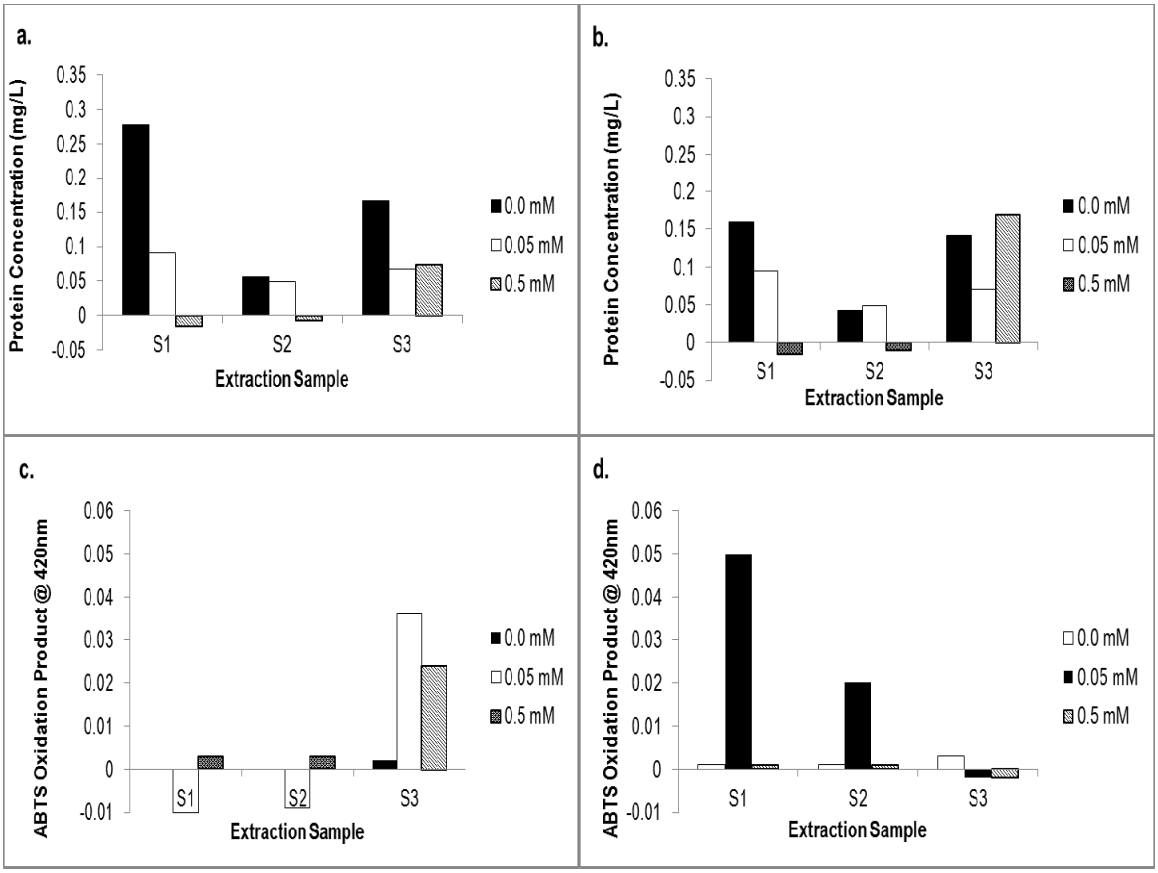

Figure 5. The protein concentration (mg/L) within the three extraction fractions of *T. thermophilus* a) ST and b) S42, along with the laccase activity reported as an increase in ABTS oxidation product at 420 nm, for *T. thermophilus* c) ST and d) S42. The impact of $CuSO_4$ (0.0, 0.05 and 0.5 mM) on the protein concentration and laccase activity was evaluated.

CONCLUSIONS

Two bacterial strains were isolated from hot springs in Yellowstone National Park, Wyoming and the Alvord Desert, Oregon, which were capable of growth at high temperature (70˚C) and pH (9). The two isolates, *T. thermophilus* ST and *T. thermophilus* S42 exhibited ligninolytic potential due to their ability to utilize lignin associated compounds for growth and tested positive for laccase mediated ABTS oxidation. *T. thermophilus* ST and *T. thermophilus* S42 have the ability to partially decolorize the industrial dye, RBBR, a lignin analog compound in five days when incubated at 70˚C and pH 9, with TYE and 0.5 mM $CuSO_4$ supplemented to the medium. *T. thermophilus* ST grew to higher cell yields due to the presence of kraft lignin and lodge pole pine when supplemented with glucose (0.5 g/L) at 70˚C and pH 9. The growth on kraft lignin and the lignocellulose sources suggest that *T. thermophilus* ST can depolymerize lignin, but is not capable of metabolizing the released aromatic monomer compounds tested in these experiments. *T. thermophilus* S42 was capable of growth on ferulic acid, cinnamic acid and p-coumaric acid when supplemented with TYE, as well as kraft lignin, corn stover, and lodgepole pine when glucose was added to the medium. *T. thermophilus* S42 utilized lignin and metabolized lignocellulose degradation products yielding increased cell densities. The ligninolytic bacterial strains isolated during this project were stable under the conditions of the alkaline pretreatment at 70°C and grew on a fraction of the lignin present. The removal of lignin would potentially increase the efficiency of the alkaline pretreatment method by removing a portion of lignin which was inhibitory to enzymatic hydrolysis. The presence of 0.05 mM $CuSO_4$ in the medium

caused a decrease in the total protein concentration of the isolates but it was found to induce laccase activity in both *T. thermophilus* ST and *T. thermophilus* S42 at 70˚C and pH 9. The two isolates have ligninolytic potential because they grew on lignocellulose degradation products, a lignin analog compound (RBBR), and tested positive for laccase activity using the ABTS assay. Thermostable alkaline bacteria with ligninolytic potential have an assortment of applications as sustainable biocatalysts in the bioremediation of recalcitrant industrial waste compounds and as a biological pretreatment for lignocellulose material prior to biofuel production.

FUTURE WORK

If sufficient time and funding were provided to continue this project, in general a high throughput method would be employed to find extremophilic bacteria with ligninolytic systems present. The approach that was taken to complete the work presented in this thesis are common to industrial or environmental microbiology. The overall process includes, taking source samples, enriching them in liquid growth medium, isolating some of the bacteria present in the sample and characterizing them for the activity of interest. This general process has been employed by several other researchers who successfully identified ligninolytic enzymes from industrial or environmental sources including pulp paper mill effluent sludge (Chandra 2007), waste water drained soil (Bains 2003), textile soil and sludge (Joshi 2008), and a Yellowstone hot spring (70°C, pH 7)(Williams 1995). A few places to improve the procedure used in this thesis would be to attempt to cultivate a diversity of isolates during the initial enrichment stage by culturing a larger initial volume of sample in the growth medium. As well as varying the pH of the initial cultures by a pH unit around the optimal pH (8-10), to allow organisms with different optima to flourish until a sustainable cell density was reached. The same procedure would be repeated with the incubation temperature. Enrichment in the presence of varied concentration of kraft lignin (0.1-0.6 g/L) could also show which isolates have a greater tolerance for kraft lignin. Chandra et al 2007 found that out of eight isolates screened. the three that were capable of growth above 0.3 g/L kraft lignin also had ligninolytic and de-colorization activity. After single cultures are isolated from the sample that display high tolerance for growing on kraft lignin and robust growth, they

will be grown in the presence of a matrix of compounds that have been found to either induce lignin depolymerization activity, are known lignin analog or degradation products (Bandounas 2011). This matrix would include aromatic acids, (4-hydroxybenzoic acid, syringic acid and vanillic acid) which have been found less likely to inhibit bacterial growth than the aldehyde or alcoholic forms of the aromatic monomers (Bandounas 2011). The industrial dye Remazol Brilliant Blue R, an anthraquinone dye, was tested in this experiment but dyes representing the other common classes, azo dyes (Acid Red 88, Acid Orange 7, Xylidine ponceau), phthalocyanine dyes (Remazol Turquoise Blue G 133) and quinone-imide dyes (Azure B, Methylene Blue, Toluidene Blue O) (Nigam 1996; Khehra 2005; Joshi 2008; Bandounas 2011). The addition of copper to stimulate laccase production was tested in the concentrations of 0.0 mM, 0.05 mM and 0.5 mM $CuSO_4$. The growth on different molecular weight fractions of kraft lignin, and the lignocellulose sources; corn stover, corn cob and lodgepole pine should be monitored by optical density (OD) 600 nm and protein concentration. The change to the absorption peaks between 200 to 300 nm will be observed especially in regions that are known to be associated with lignin, around 280 nm (Vermerris 2006). Preliminary data is shown in Appendix E for impact of growth on kraft lignin separated by molecular weight. This will help to quantify the direct impact of the bacterial isolates on lignin sources, although growth on the lignin polymer is not always an indicator of having ligninolytic activity. A distinction must be made between lignin depolymerizing activity and the ability to metabolize released lignin degradation products. Some bacteria may possess the ability to depolymerize lignin without being able to metabolize lignin degradation products

(Bandounas 2011). Therefore, it is important to use activity assays that test for the phenol oxidase and oxidase activity associated with lignin peroxidase and laccase as well as substrate experiments to quantify growth on lignin degradation products. High performance liquid chromatography (HPLC) will be used to quantify the change in concentration the bacterial isolates have on the lignin degradation products and on the low molecular weight fractions of kraft lignin (Chandra 2007; Bandounas 2011).

An approach that was not taken in this thesis that should be explored is the use of bioinformatics and proteomics to identify novel laccases and lignin peroxidases. In general this involves cloning the DNA from an environmental sample into recombinant *Escherichia coli.* Then searching the clones directly for the laccase gene, translating this to a protein sequence and using bioinformatics databases to model the structure and compare this laccase to others. This approach would begin with an environmental or industrial sampling trip; the bacterial DNA would be isolated and ligated into a vector to create recombinant plasmids. These would be transformed into an *E. coli* strain to generate clones that can be screened for containing conserved regions of laccase, particularly for copper binding sites within the laccase genes. Degenerate primers have been created, Cu1F and Cu4R, to amplify the region coding for copper binding sites I and IV in bacterial laccases (Hoegger 2006). After this round of amplification with laccase specific primers, inverse polymerase chain reaction (PCR) would be used to obtain full length laccase genes. These plasmids would be digested, ligated and used as PCR template DNA for amplification with new primers in order to identify the bacterial species the gene was extracted from. This method was used to identify several bacterial

strains, *B. halodurans*, *B. licheniformis*, *B. subtilis* (Enguita 2003), *E. coli*, gamma proteobacterium JB (Bains 2003), *T. thermophilus* HB27 (Miyazaki 2005) that contain laccase like genes (Fang 2010).

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APPENDICES

APPENDIX A

MOLECULAR DATA

Full Length 16S rRNA Sequence

The six cultures were 99% identical on the genomic level, so this contig is representative all six cultures. The lengths varied from 1340-1360bp.

GGGCCGCGGGGTTTTACTCCGTGGTCAGCGGCGGACGGGTGAGTAACGCGTG
GGTGACCTACCCGGAAGAGGGGGACAACCCGGGGAAACTCGGGCTAATCCC
CCATGTGGACCCGCCCCTTGGGGTGTGTCCAAAGGGCTTTGCCCGCTTCCGGA
TGGGCCCGCGTCCCATCAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGAC
GACGGGTAGCCGGTCTGAGAGGATGGCCGGCCACAGGGGCACTGAGACACG
GGCCCCACTCCTACGGGAGGCAGCAGTTAGGAATCTTCCGCAATGGGCGCAA
GCCTGACGGAGCGACGCCGCTTGGAGGAAGAAGCCCTTCGGGGTGTAAACTC
CTGAACCCGGGACGAAACCCCCGAGGAGGGGACTGACGGTACCGGGGTAAT
AGCGCCGGCCAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGCGCGAGC
GTTACCCGGATTCACTGGGCGTAAAGGGCGTGTAGGCGGCCTGGGGCGTCCC
ATGTGAAAGACCACGGCTCAACCGTGGGGGAGCGTGGGATACGCTCAGGCTA
GACGGTGGGAGAGGGTGGTGGAATTCCCGGAGTAGCGGTGAAATGCGCAGA
TACCGGGAGGAACGCCGATGGCGAAGGCAGCCACCTGGTCCACCCGTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACCGGATTAGATACCCGGGTAGTCC
ACGCCCTAAACGATGCGCGCTAGGTCTCTGGGTCTCCTGGGGGCCGAAGCTA
ACGCGTTAAGCGCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCAGGCCTTGACATGCTAGGGAACCCGGGTGAAAG
CCTGGGGTGCCCCGCGAGGGGAGCCCTAGCACAGGTGCTGCATGGCCGTCGT
CAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCG
CCGTTAGTTGCCAGCGGTTCGGCCGGGCACTCTAACGGGACTGCCCGCGAAA
GCGGGAGGAAGGAGGGGACGACGTCTGGTCAGCATGGCCCTTACGGCCTGG
GCGACACACGTGCTACAATGCCCACTACAAAGCGATGCCACCCGGCAACGGG
GAGCTAATCGCAAAAAGGTGGGCCCAGTTCGGATTGGGGTCTGCAACCCGAC
CCCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACGCCATGGGAGCGGGCTCTA
CCCGAAGTCGCCGGGASCCT

Laccase Sequence Data for TTC1370 found in *T. thermophilus* HB27

MLARRSFLQAAAGSLVLGLARAQGPSFPEPKVVRSQGGLLSLKLSATPTPLALAG
QRATLLTYGGSFPGPTLRVRPRDTVRLTLENRLPEPTNLHWHGLPISPKVDDPFLE
IPPGESWTYEFTVPKELAGTFWYHPHLHGRVAPQLFAGLLGALVVESSLDAIPEL
REAEEHLLVLKDLALQGGRPAPHTPMDWMNGKEGDLVLVNGALRPTLVAQKA
TLRLRLLNASNARYYRLALQDHPLYLIAADGGFLEEPLEVSELLLAPGERAEVLV
RLRKEGRFLLQALPYDRGAMGMMDMGGMAHAMPQGPSRPETLLYLIAPKNPK
PLPLPKALSPFPTLPAPVVTRRLVLTEDMMAARFFINGQVFDHRRVDLKGQAQT
VEVWEVENQGDMDHPFHLHVHPFQVLSVGGRPFPYRAWKDVVNLKAGEVARL
LVPLREKGRTVFHCHIVEHEDRGMMGVLEVG

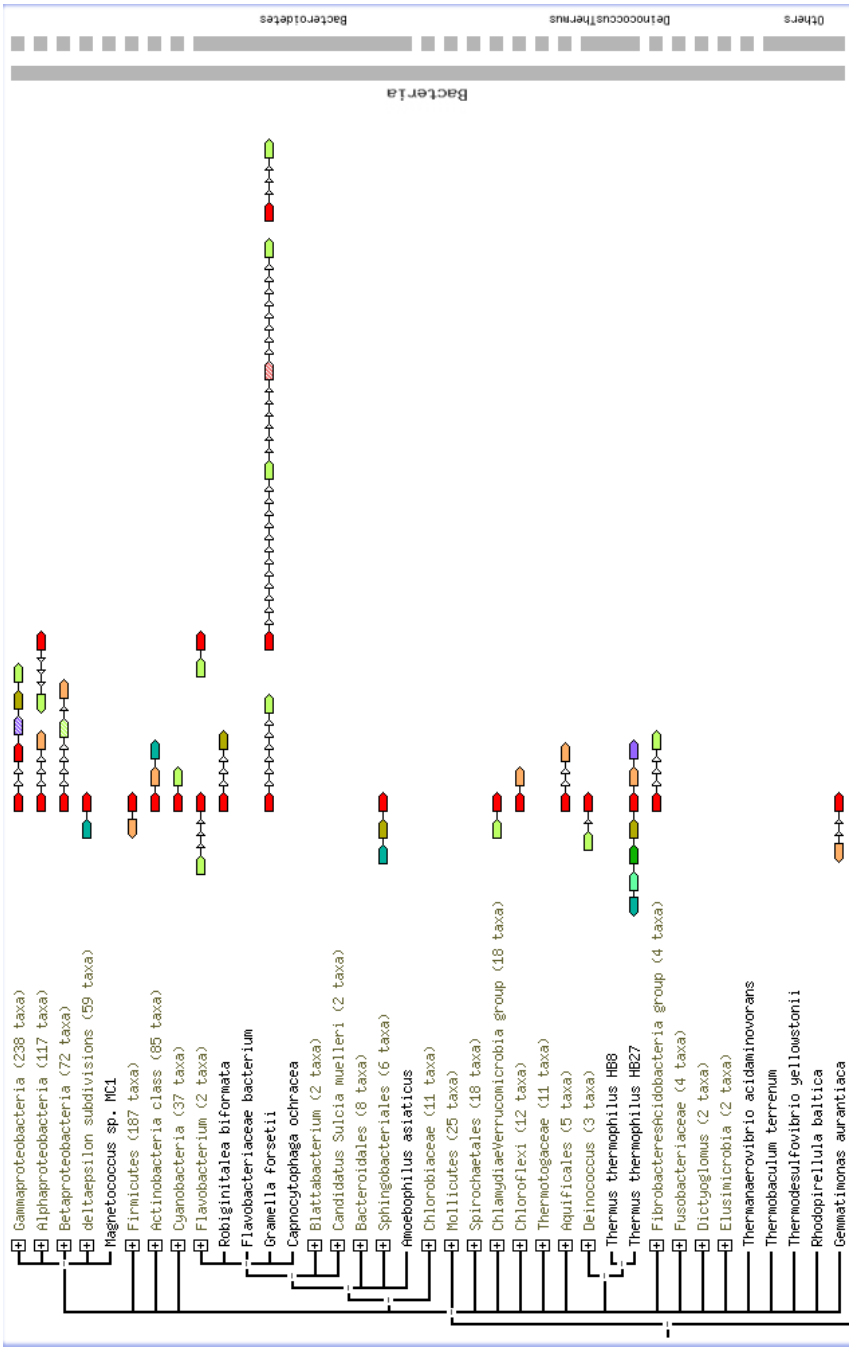

Figure A1. A neighborhood homology figure from the STRING database result for TTC1370 laccase isolated from HB27. This figure shows that HB8 does not contain the same laccase gene location.

APPENDIX B

CASTENHOLZ SALTS MEDIUM

Mix aseptically 5 parts double strength Castenholz Salts (see below) with one part 1% TYE (see below) and 4 parts distilled water. Final pH of complete medium should be 7.6.

Castenholz Salts, 2X:

Nitrilotriacetic acid.....0.2 g
Nitsch's Trace Elements (see below).....2.0 ml
$FeCl_3$ solution (0.03%).....2.0 ml
$CaSO_4 . 2H_2O$.....0.12 g
$MgSO_4 . H_2O$.....0.2 g
NaCl.....0.016 g
KNO_3.....0.21 g
$NaNO_3$.....1.4 g
Na_2HPO_4.....0.22 g
Agar (if needed).....30.0 g
Distilled water.....1.0 L
Adjust pH to 8.2.

Nitsch's Trace Elements:

H_2SO_4.....0.5 ml
$MnSO_4$.....2.2 g
$ZnSO_4 . 7H_2O$.....0.5 g
H_3BO_3.....0.5 g
CuSO . 5HO.....0.016 g
$Na_2MoO_4 . 2H_2O$.....0.025 g
$CoCl_2 . 6H_2O$.....0.046 g
Distilled water.....1.0 L

1% TYE:

Tryptone (BD 211705).....10.0 g
Yeast extract.....10.0 g
Distilled Water……1L

APPENDIX C

SUPPLEMENTAL INFORMATION ON LIGNOCELLULOSE, ITS DEGRADATION PRODUCTS AND LIGNIN ANALOG COMPOUNDS

Chemical Structure of Lignocellulose

Lignocellulose is made of cellulose, a long chain polysaccharide made up of D-glucose units; hemicellulose a heterogeneous, branched polymer of sugar derivatives and lignin a phenolic polymer. The cellulose fibers intact with hydrogen bonding and are immersed with hemicellulose to form a crystalline structure encased by lignin. Cellulose has an organized fibrous structure of D-glucose linked by B-(1, 4)-glycosidic bonds forming a repeating unit called cellobiose. Hemicellulose has short branched chains made up of various polysaccharides, primarily pentose and hexose sugars like xylans and mannans. The most common type of hemicellulose in hardwoods is xylan which is made up of linked D-xylose units and in softwoods glactoglucomannan which are β-(1-4) linked D-glucose and D-galactose (Kumar 2008; Bugg 2011). Lignin acts as a backbone to hold the cellulose and hemicellulose fibers together and provide support to the plant. It makes up 15-25% of the total biomass for grasses and hardwoods and 40% for softwoods (Cheng 2009). Resistance to chemical and microbial breakdown is a consequence of its natural function as a physical barrier to protect the carbohydrates, hemicellulose and cellulose (Huang 2010; Ahmad 2011). Lignin is a cross linked aromatic polymer of phenylpropane units, the monolignols, p-coumaryl, coniferyl and sinapyl alcohol, shown in Figure C1, which differ from one another by the number of methoxy (OCH_3) units (Perez 2002).

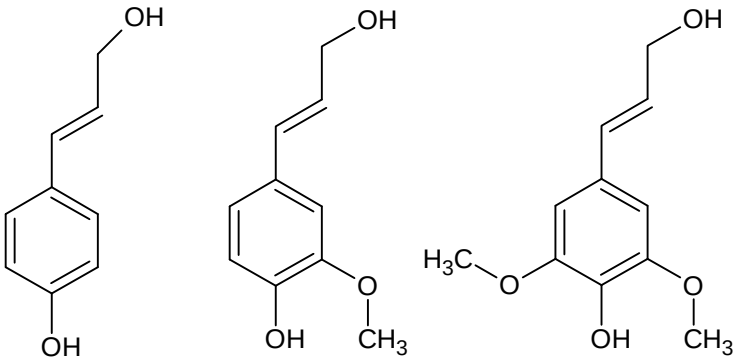

Figure C1. The monolignols are base units of the lignin molecule, p-coumaryl, coniferyl and sinapyl alcohol, they differ by the number of methoxyl units off the side of the aromatic ring.

The types of monolignols composing lignin vary by the source of the lignocellulose material; softwood lignin is 95% coniferyl alcohol, hardwood lignin has a mixture of coniferyl and sinapyl alcohol, and grasses have a mixture of all three monolignol units. The monolignols are bonded together by dehydration reactions to form ether or carbon-carbon linkages, although ether bonds are the most common in plants and animals (Keshwani 2009).

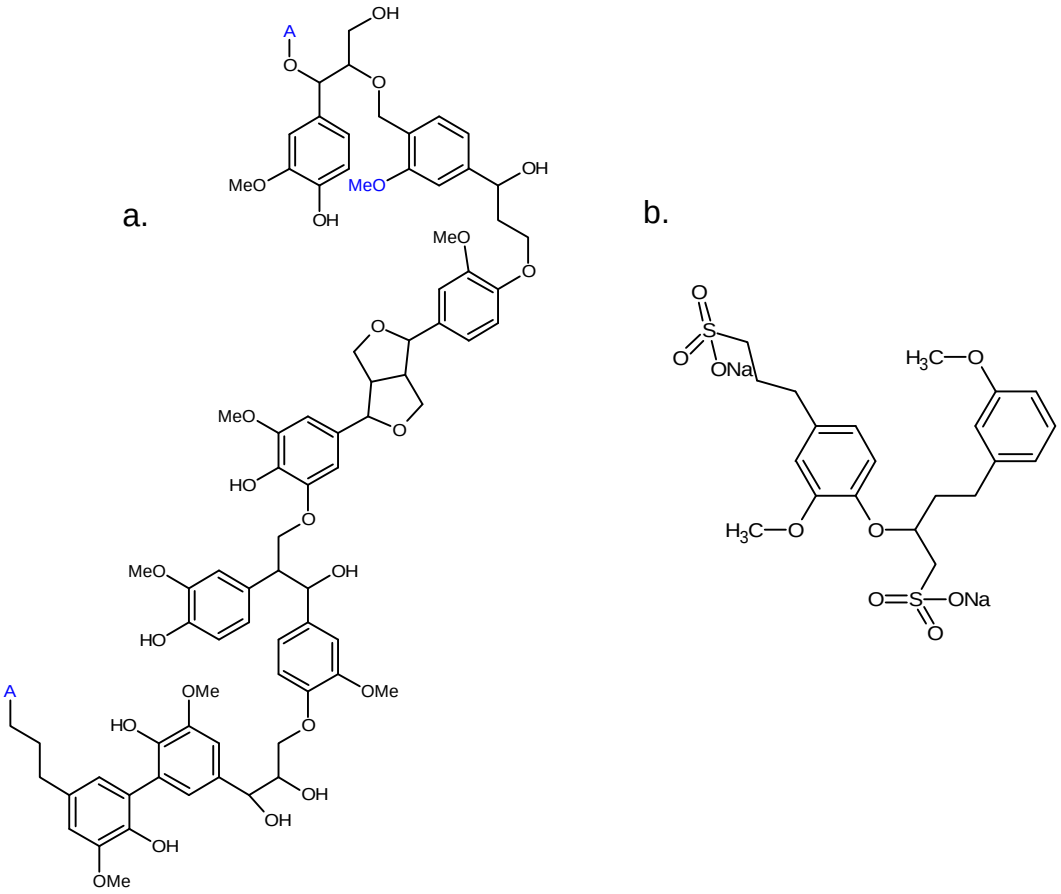

Figure C2. Kraft lignin a waste residue of the industrial kraft process which has undergone many chemical conversions from the natual lignin molecule. A typical repeating unit for a) natural lignin and b) kraft lignin.

Industrial Dyes as Ligninolytic Indicator Compounds

One application of lignin degrading bacterial enzymes is the degradation of industrial dyes, which have structural similarities with the lignin molecule. Industrial dyes can be decolorized by lignin degrading enzymes and have been found to induce lignin degrading activity as well as be decolorized by the same enzymes (Singh 2011). The ability to decolorize synthetic dyes has been attributed to the presence of lignin

degrading enzymes including laccase, lignin peroxidase and manganese peroxidase, which have also been shown to detoxify detoxify polyaromatic hydrocarbons, and other dyes (da Silva 2010). Therefore industrial dyes are often used when characterizing ligninolytic activity in bacterial isolates to monitor the change in absorption of the molecule.

During industrial textile processing up to 40% of the used dye can remain in the effluent stream affecting the toxicity, transparency and gas solubility of the water. The high discharge volume and composition causes the wastewater from the textile industry to be considered one of the most polluting among all industrial sectors (Faraco 2008). Azo, anthraquinone, and phthalocyanine derivatives represent the three classes of synthetic dyes of the greatest economic importance. Azo class dyes are the most common, representing 60 to 70% of dyestuff currently being manufactured. They are given this name due to the azo bond (–N=N–) found in their structure and are further classified into mono, diazo, and triazo dyes depending on the number of bonds (Ramirez 2009). Azo dyes have the widest range of shades from greenish yellow to black. Anthraquinone dyes are derived from anthraquinone, the oxidation product of anthracene, a tricyclic aromatic hydrocarbon separated from anthracene oil, a fraction of coal tar at temperatures above 270˚C (Britannica 2010). They create brilliant blue and green shades with increased light fastness, the ability to resists fading, caused by the stable fused ring structure at its base. This also causes the anthracene dyes to be resistant to degradation. (Venkataraman 1972 (Banat 1996). The last major class of dye is the phthalocyanine derivatives which are usually complexed with metal to create turquoise shades. Figure

C.3 displays the anthraquinone dye, RBBR. Phthalocyanine dyes have the distinctive cyclical structure which coordinates with metals in the center of the molecule to generate different dye colors (Marchis 2011).

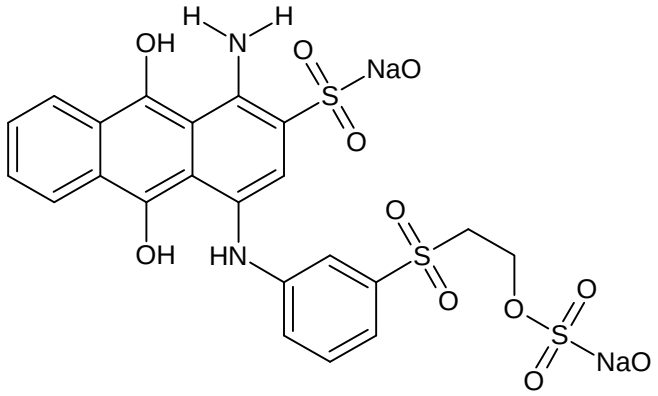

Figure C3. The RBBR structure is similar to lignin because they are both polycyclic aromatic compounds and therefore ligninolytic enzymes can decolorize anthracene and other synthetic dyes (Shin 1997).

The use of synthetic dyes in the textile industry is common because they are more cost effective, stable and easier to use than natural dyes, but some are toxic and recalcitrant organopollutants. Due to its highly aromatic structure, complex molecules and synthetic origin, synthetic dyes cannot be removed aerobically by municipal sewerage systems (Robinson 2001; da Silva 2010). Physical, chemical and biological methods have been employed to decolorize synthetic dyes. Physical and chemical methods generate large amounts of waste which need to be disposed; therefore biological treatments have been studied in recent years (Robinson 2001). Azo dyes are the most commonly used in industry so many studies have found microorganisms that are able near completely decolorize dyes of this class. Various azo dyes have been degraded by the fungi *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, and *Aspergillus oryzae*

(Palmieri 2005; Faraco 2008) (Teixeira 2010), the plant enzyme horseradish peroxidase (da Silva 2010), and bacterial consortia of *Alcaligenes faecalis*, *Commamonas acidovorans* (Nigam 1996), *Aeromonas caviae*, *Proteus mirabilis*, *Rhodococcus globerulus* (Joshi 2008) and *Bacillus cereus*, *Pseudomonas putida*, *Pseudomonas fluorescence* and *Stenotrophomonas acidaminiphila* (Khehra 2005). Anthraquinone based dyes are most resistant to degradation because of the fused ring structure (Banat 1996). The fungi, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Aspergillus oryzae* and *Polyporus sp. S133* (Palmieri 2005; Faraco 2008; Teixeira 2010; Hadibarata 2011), and bacterial consortia *Alcaligenes faecalis* and *Commamonas acidovorans* (Nigam 1996) can decolorize anthraquinone class dyes. Phthalocyanine dyes have been decolorized by the plant enzymes soybean peroxidase and horseradish peroxidase (Marchis 2011) and the bacterial consortia *Alcaligenes faecalis* and *Commamonas acidovorans* (Nigam 1996). The ability to decolorize synthetic dyes has been attributed to the presence of lignin degrading enzymes, laccase, lignin peroxidase and manganese peroxidase which have been proven to detoxify detoxify polyaromatic hydrocarbons, polychlorinated biphenyls and other dyes (da Silva 2010).

APPENDIX D

COMPLETE DATA SETS

Kraft lignin and Aromatic Monomer Growth Experiments

Table D1. The OD data as a function of time, the average and standard deviation values are reported for the culture MOA that was eliminated during the screening process and the uninoculated controls represented by "Blank".

0.025 g/l TYE & 0.1 g/l KL										
Time (h)	MOA	MOA	MOA	Average	Stdev	Blank	Blank	Blank	Average	Stdev
0	0.066	0.069	0.069	0.068	0.001732	0.054	0.054	0.054	0.054	0
6	0.061	0.063	0.065	0.063	0.002	0.054	0.054	0.054	0.054	0
18	0.061	0.061	0.062	0.061333	0.000577	0.054	0.054	0.055	0.054333	0.000577
30	0.085	0.071	0.072	0.076	0.00781	0.053	0.082	0.055	0.063333	0.016197
42	0.078	0.076	0.094	0.082667	0.009866	0.053	0.054	0.054	0.053667	0.000577
54	0.082	0.076	0.098	0.085333	0.011372					
0.025 g/l TYE & 0.1 g/l Ferulic Acid										
Time (h)	MOA	MOA	MOA	Average	Stdev	Blank	Blank	Averag	Stdev	
0	0.062	0.064	0.062	0.062667	0.001155	0.046	0.046	0.047	0.046333	
6	0.062	0.068	0.068	0.066	0.003464	0.056	0.057	0.056	0.056333	
18	0.065	0.066	0.069	0.066667	0.002082	0.062	0.059	0.068	0.063	
30	0.072	0.08	0.075	0.071	0.004041	0.066	0.066	0.072	0.068	
42	0.069	0.075	0.07	0.071333	0.003215	0.07	0.07	0.073	0.071	
54	0.078	0.085	0.082	0.081667	0.003512					
0.025 g/l TYE & 0.1 g/l Cinnamic Acid										
Time (h)	MOA	MOA	MOA	Average	Stdev	Blank	Blank	Blank	Average	
0	0.06	0.062	0.061	0.061	0.001	0.044	0.044	0.044	0.044	
6	0.061	0.064	0.061	0.062	0.001732	0.05	0.049	0.048	0.049	
18	0.07	0.059	0.066	0.065	0.005568	0.053	0.055	0.06	0.056	
30	0.071	0.082	0.083	0.0825	0.000707	0.05	0.05	0.049	0.049667	
42	0.068	0.066	0.068	0.067333	0.001155	0.054	0.054	0.054	0.054	
54	0.076	0.074	0.078	0.076	0.002					
66	0.062	0.063	0.067	0.064	0.002646					
0.025 g/l TYE										
Time (h)	MOA	MOA	MOA	Average	Stdev	TYE	Blank	Blank	Average	
0	0.061	0.061	0.061	0.061	0	0.046	0.044	0.045	0.001155	
6	0.055	0.059	0.064	0.059333	0.004509	0.048	0.047	0.048	0.000577	
18	0.055	0.055	0.061	0.057	0.003464	0.051	0.049	0.05	0.001155	
30	0.087	0.069	0.067	0.074333	0.011015	0.05	0.051	0.05	0.000577	
42	0.079	0.073	0.081	0.077667	0.004163	0.05	0.049	0.05	0.001414	
54	0.083	0.088	0.091	0.087333	0.004041					
0.025 g/l TYE & 0.1 g/l pCoumaric Acid										
Time (h)	MOA	MOA	MOA	Average	Stdev	Blank	Blank	Blank	Average	Stdev
0	0.059	0.061	0.059	0.059667	0.001155	0.054	0.054	0.054	0.054	0
6	0.058	0.056	0.06	0.058	0.002	0.054	0.054	0.054	0.054	0
18	0.062	0.061	0.062	0.061667	0.000577	0.054	0.054	0.055	0.054333	0.000577
30	0.079	0.08	0.094	0.084333	0.008386	0.053	0.082	0.055	0.063333	0.016197
42	0.071	0.07	0.088	0.076333	0.010116	0.053	0.054	0.054	0.053667	0.000577
54	0.073	0.073	0.094	0.08	0.012124					

Table D2. The OD data as a function of time, the average and standard deviation values are reported for the two isolates ST and S42, which were selected as having the highest ligninolytic potential.

0.025 g/l TYE & 0.1 g/l KL										
Time (h)	ST	ST	ST	Average	Stdev	S42	S42	S42	Average	Stdev
0	0.07	0.07	0.07	0.069667	0.000577	0.066	0.07	0.069	0.068	0.001732
6	0.07	0.07	0.07	0.070333	0.001528	0.1	0.1	0.107	0.103	0.003606
18	0.092	0.1	0.07	0.084333	0.015948	0.093	0.1	0.104	0.099333	0.005686
30	0.089	0.09	0.08	0.085667	0.005774	0.095	0.1	0.101	0.098667	0.003215
42	0.09	0.09	0.08	0.087333	0.003786	0.093	0.1	0.098	0.095333	0.002517
0.025 g/l TYE & 0.1 g/l Ferulic Acid										
Time (h)										
0	ST	ST	ST	Average	Stdev	S42	S42	S42	Average	Stdev
6	0.0006	0.07	0.06	0.064	0.064	0.001	0.06	0.064	0.062	0.062667
18	0.0006	0.08	0.08	0.081	0.081	0	0.11	0.112	0.107	0.108
30	0.0046	0.08	0.09	0.087	0.083667	0.0042	0.12	0.129	0.132	0.126333
42		0	0.09	0.083	0.082	0.0833	0	0.127	0.14	0.138
54		0.09	0.09	0.087	0.087333	0.0006	0.13	0.142	0.147	0.141
0.025 g/l TYE & 0.1 g/l Cinnamic Acid										
Time (h)	ST	ST	ST	Average	Stdev	S42	S42	S42	Average	Stdev
0	0.059	0.06	0.06	0.059667	0.000577	0.06	0.06	0.061	0.061	0.001
6	0.001	0.08	0.09	0.085	0.085	0.001	0.1	0.082	0.098	0.092333
18	0.0036	0.08	0.08	0.082	0.083	0.001	0.1	0.106	0.106	0.103333
30	0.0006	0.08	0.08	0.08	0.080667	0.0006	0.1	0.101	0.099	0.099333
42	0.08	0.08	0.09	0.082667	0.002517	0.096	0.11	0.108	0.103	0.006245
0.025 g/l TYE										
Time (h)	ST	ST	ST	Average	Stdev	S42	S42	S42	Average	Stdev
0	0.061	0.06	0.06	0.062333	0.001528	0.061	0.06	0.061	0.061	0
6	0.066	0.07	0.07	0.068333	0.003215	0.095	0.09	0.094	0.093667	0.001528
18	0.083	0.08	0.09	0.084333	0.003215	0.092	0.09	0.095	0.093333	0.001528
30	0.081	0.08	0.08	0.083	0.001732	0.093	0.09	0.093	0.093333	0.000577
42	0.088	0.09	0.09	0.0905	0.000707	0.088	0.09	0.09	0.089667	0.001528
0.025 g/l TYE & 0.1 g/l pCoumaric Acid										
Time (h)	ST	ST	ST	Average	Stdev	S42	S42	S42	Average	Stdev
0	0.061	0.06	0.06	0.060333	0.001155	0.059	0.06	0.059	0.059667	0.001155
6	0.08	0.08	0.08	0.079333	0.003055	0.106	0.1	0.101	0.101667	0.004041
18	0.082	0.08	0.08	0.081333	0.000577	0.095	0.1	0.099	0.098667	0.003512
30	0.081	0.08	0.08	0.082333	0.001155	0.098	0.1	0.101	0.100333	0.002082
42	0.082	0.08	0.08	0.082333	0.000577	0.091	0.1	0.096	0.094667	0.003215

Table D3. The OD data as a function of time, the average and standard deviation values are reported for the culture STw (STwhite) which was eliminated during the screening process and for the uninoculated control (Blank).

1.0 g/l glucose & 0.1 g/l KL										
Time (h)	blank	blank	blank	Average	Stdev	STw	STw	STw	Average	Stdev
0	0.067	0.068	0.07	0.06833	0.0015	0.068	0.069	0.07	0.069	0.001
6	0.062	0.066	0.066	0.06467	0.0023	0.067	0.07	0.07	0.069	0.001732
12	0.063	0.064	0.064	0.06367	0.0006	0.071	0.076	0.071	0.07267	0.002887
24	0.061	0.065	0.062	0.06267	0.0021	0.168	0.282	0.181	0.21033	0.062405
56		0.056	0.057	0.0565	0.0007	0.203	0.31	0.232	0.24833	0.055338
73	0.046	0.066	0.07	0.0565	0.0007	0.237	0.312	0.295	0.28133	0.039323
101	0.046	0.066	0.07	0.0565	0.0007	0.222	0.315	0.323	0.28667	0.056146
1.0 g/l glucose & 0.1 g/l Ferulic Acid										
Time (h)	blank	blank	blank	Average	Stdev	STw	STw	STw	Average	Stdev
0	0.079	0.08	0.082	0.08033	0.0015	0.076	0.079	0.077	0.07733	0.001528
6	6	0.078	0.076	0.076	0.0767	0.001	0.084	0.083	0.08	0.082333
12	12	0.076	0.078	0.075	0.0763	0.002	0.079	0.086	0.082	0.082333
24	24	0.073	0.076	0.076	0.075	0.002	0.079	0.083	0.082	0.081333
56		56	0.075	0.08	0.076	0.077	0.003	0.323	0.18	0.197
73	73	0.076	0.079	0.084	0.077	0.003	0.304	0.276	0.249	0.276333
101	0.08	0.084	0.077	0.00265	0.295	0.275	0.285	0.285	0.01	
1.0 g/l glucose & 0.1 g/l Cinnamic Acid										
Time (h)	blank	blank	blank	Average	Stdev	STw	STw	STw	Average	Stdev
0	0.077	0.08	0.081	0.07933	0.0021	0.076	0.076	0.077	0.07633	0.000577
6	6	0.081	0.082	0.085	0.0827	0.002	0.083	0.085	0.083	0.083667
12	12	0.072	0.075	0.083	0.0767	0.006	0.086	0.083	0.082	0.083667
24	24	0.069	0.074	0.068	0.0703	0.003	0.081	0.079	0.08	0.08
56	0.049	0.05	0.052	0.05033	0.0015	0.313	0.315	0.199	0.27567	0.066403
73	0.052	0.051	0.05	0.00153	0.312	0.311	0.285	0.30267	0.01531	
101	0.05	0.051	0.05	0.00153	0.186	0.319	0.276	0.26033	0.06787	
1.0 g/l glucose										
Time (h)	blank	blank	blank	Average	Stdev	STw	STw	STw	Average	Stdev
0	0.079	0.081	0.083	0.081	0.002	0.076	0.078	0.076	0.07667	0.001155
6	0.078	0.077	0.076	0.077	0.001	0.079	0.083	0.084	0.082	0.002646
12	0.075	0.073	0.07	0.07267	0.0025	0.083	0.083	0.081	0.08233	0.001155
24	0.066	0.066	0.068	0.06667	0.0012	0.083	0.085	0.089	0.08567	0.003055
56	0.051	0.049	0.048	0.04933	0.0015	0.282	0.265	0.259	0.26867	0.01193
73	0.046	0.046	0.047	0.04933	0.0015	0.263	0.289	0.293	0.28167	0.016289
101	0.047	0.046	0.047	0.04933	0.0015	0.253	0.296	0.294	0.281	0.024269
1.0 g/l glucose& 0.1 g/l pCoumaric Acid										
Time (h)	blank	blank	blank	Average	Stdev	STw	STw	STw	Average	Stdev
0	0.079	0.083	0.084	0.082	0.0026	0.079	0.078	0.078	0.07833	0.000577
6	0.075	0.079	0.081	0.07833	0.0031	0.082	0.089	0.09	0.087	0.004359
12	0.078	0.082	0.078	0.07933	0.0023	0.086	0.086	0.088	0.08667	0.001155
24	0.077	0.079	0.083	0.07967	0.0031	0.083	0.087	0.089	0.08633	0.003055
56	0.07	0.089	0.092	0.08367	0.0119	0.188	0.201	0.345		

Table D4. The OD data as a function of time, the average and standard deviation values are reported for the culture MOA.

1.0 g/l glucose & 0.1 g/l KL					
Time (h)	MOA	MOA	MOA	Average	Stdev
0	0.069	0.069	0.069	0.069	0
6	0.067	0.069	0.07	0.068667	0.001528
12	0.063	0.073	0.07	0.068667	0.005132
24	0.191	0.176	0.185	0.184	0.00755
56	0.2	0.316	0.201	0.239	0.066686
73	0.303	0.312	0.265	0.293333	0.024947
101	0.291	0.333	0.294	0.306	0.023431
1.0 g/l glucose & 0.1 g/l Ferulic Acid					
Time (h)	MOA	MOA	MOA	Average	Stdev
0	0.078	0.077	0.077	0.077333	0.000577
6	0.078	0.082	0.083	0.081	0.002646
12	0.086	0.086	0.083	0.085	0.001732
24		0.079	0.08	0.083	0.080667
56	0.184	0.188	0.18	0.184	0.004
1.0 g/l glucose & 0.1 g/l Cinnamic Acid					
Time (h)	MOA	MOA	MOA	Average	Stdev
0	0.077	0.076	0.076	0.076333	0.000577
6		0.082	0.084	0.087	0.084333
12		0.078	0.081	0.081	0.08
24		0.074	0.075	0.072	0.073667
56	0.171	0.182	0.15	0.167667	0.016258
73	0.237	0.181	0.209667	0.028024	
101	0.23	0.178	0.204	0.026	
1.0 g/l glucose					
Time (h)	MOA	MOA	MOA	Average	Stdev
0	0.079	0.076	0.076	0.077	0.001732
6	0.079	0.084	0.083	0.082	0.002646
12	0.084	0.084	0.08	0.082667	0.002309
24	0.071	0.087	0.077	0.078333	0.008083
56	0.258	0.268	0.213	0.246333	0.029297
73	0.245	0.28	0.236	0.253667	0.023245
101	0.245	0.263	0.225	0.244333	0.019009
1.0 g/l glucose& 0.1 g/l pCoumaric Acid					
Time (h)	MOA	MOA	MOA	Average	Stdev
0	0.11	0.079	0.079	0.089333	0.017898
6	0.081	0.083	0.089	0.084333	0.004163
12	0.086	0.098	0.084	0.089333	0.007572
24	0.094	0.088	0.089	0.090333	0.003215
56	0.2	0.123	0.088	0.137	0.057297
73	0.198	0.093	0.207667	0.119793	
101	0.239	0.168	0.244333	0.079135	

Table D5. The OD data as a function of time, the average and standard deviation values are reported for the cultures ST, S42 and the uninoculated control "Blank".

1/20/2012	**0.5g/L glucose**					1/26/2012	**0.5 g/L glucose**				
Time [h]	**ST**	**ST**	**ST**	Average	StDev	**Time [h]**	**ST**	**ST**	**ST**	Average	StDev
0	0.056	0.06	0.061	0.059	0.0026	0	0.048	0.052	0.053	0.051	0.002646
24	0.074	0.079	0.077	0.07667	0.0025	8	0.065	0.062	0.069	0.0653	0.003512
48	0.077	0.084	0.147	0.10267	0.0386	20	0.07	0.067	0.07	0.069	0.001732
72	0.123	0.087	0.136	0.11533	0.0254	32	0.098	0.071	0.079	0.0827	0.013868
96	0.112	0.135	0.126	0.12433	0.0116	44	0.158	0.1	0.119	0.1257	0.029569
120	0.101	0.115	0.095	0.10367	0.0103	56	0.151	0.13	0.129	0.1367	0.012423
144	0.094	0.117	0.107	0.106	0.0115	70	0.144	0.121	0.125	0.13	0.012288
						94	0.136	0.113	0.116	0.1217	0.012503
1/20/2012	**0.5g/L glucose**					1/26/2012	**0.5 g/L glucose**				
Time [h]	**S42**	**S42**	**S42**	Average	StDev	**Time [h]**	**S42**	**S42**	**S42**	Average	StDev
0	0.054	0.058	0.058	0.05667	0.0023	0	0.052	0.053	0.052	0.0523	0.000577
24	0.07	0.069	0.064	0.06767	0.0032	8	0.066	0.071	0.068	0.0683	0.002517
48	0.132	0.141	0.073	0.11533	0.0369	20	0.065	0.072	0.072	0.0697	0.004041
72	0.126	0.143	0.132	0.13367	0.0086	32	0.07	0.074	0.068	0.0707	0.003055
96	0.112	0.143	0.126	0.127	0.0155	44	0.116	0.082	0.074	0.0907	0.022301
120	0.119	0.136	0.12	0.125	0.0095	56	0.129	0.079	0.077	0.095	0.029462
144	0.102	0.15	0.116	0.12267	0.0247	70	0.123	0.132	0.076	0.1103	0.030072
						94	0.118	0.129	0.134	0.127	0.008185
1/20/2012	**0.5 g/L glucose**					1/26/2012	**0.5 g/L glucose**				
Time [h]	**Blank**	**Blank**	**Blank**	Average	StDev	**Time [h]**	**Blank**	**Blank**	**Blank**	Average	StDev
0	0.051	0.052	0.055	0.05267	0.0021	0	0.05	0.05	0.05	0.05	8.5E-18
24	0.067	0.075	0.074	0.072	0.0044	8	0.057	0.07	0.066	0.0643	0.006658
48	0.076	0.077	0.083	0.07867	0.0038	20	0.061	0.07	0.068	0.0663	0.004726
72	0.078	0.068	0.077	0.07433	0.0055	32	0.061	0.091	0.069	0.0737	0.015535
						44	0.066	0.075	0.069	0.07	0.004583
						56	0.07	0.108	0.069	0.0823	0.022234
						70	0.07	0.072	0.073	0.0717	0.001528
						94	0.072	0.078	0.071	0.0737	0.003786

Table D6. The OD data as a function of time, the average and standard deviation values are reported for the cultures ST, S42 and the uninoculated control "Blank".

	0.5 g/l glucose & 0.1 g/l KL					0.5 g/l glucose & 0.1 g/l ferulic acid				
Time (h)	FD	FD	FD	Average	StDev	FD	FD	FD	Average	StDev
0	0.051	0.05	0.051	0.050667	0.000577	0.074	0.075	0.065	0.071333	0.005508
10.5	0.06	0.06	0.059	0.059667	0.000577	0.099	0.105	0.097	0.100333	0.004163
24	0.059	0.059	0.06	0.059333	0.000577	0.11	0.113	0.112	0.111667	0.001528
35	0.061	0.06	0.061	0.060667	0.000577	0.118	0.12	0.111	0.116333	0.004726
47	0.06	0.069	0.146	0.091667	0.047269	0.124	0.12	0.115	0.119667	0.004509
60	0.066	0.161	0.144	0.123667	0.050659	0.116	0.123	0.11	0.116333	0.006506
73.5	0.155	0.148	0.142	0.148333	0.006506	0.127	0.132	0.114	0.124333	0.009292
95	0.144	0.139	0.134	0.139	0.005	0.126	0.169	0.119	0.138	0.027074
	0.5 g/l glucose & 0.1 g/l ferulic acid					0.5 g/l glucose				
Time (h)	DO	DO	DO	Average	StDev	STW	STW	STW	Average	StDev
0	0.071	0.071	0.071	0.071	0	0.068	0.068	0.069	0.068333	0.000577
10.5	0.087	0.098	0.094	0.093	0.005568	0.085	0.082	0.084	0.083667	0.001528
24	0.105	0.105	0.103	0.104333	0.001155	0.085	0.089	0.087	0.087	0.002
35	0.1	0.105	0.101	0.102	0.002646	0.091	0.09	0.096	0.092333	0.003215
47	0.096	0.102	0.104	0.100667	0.004163	0.148	0.093	0.146	0.129	0.031193
60	0.108	0.095	0.109	0.104	0.00781	0.142	0.147	0.144	0.144333	0.002517
73.5	0.105	0.115	0.129	0.116333	0.012055	0.136	0.134	0.129	0.133	0.003606
95	0.094	0.112	0.19	0.132	0.051029	0.132	0.12	0.119	0.123667	0.007234
	0.5 g/l glucose & 0.1 g/l ferulic acid					0.5 g/l glucose				
Time (h)	ST	ST	ST	Average	StDev	S42	S42	S42	Average	StDev
0	0.073	0.077	0.072	0.074	0.002646	0.07	0.068	0.069	0.001	0.052
10.5	0.092	0.102	0.094	0.096	0.005292	0.084	0.085	0.087	0.085333	0.001528
24	0.106	0.117	0.102	0.108333	0.007767	0.085	0.083	0.09	0.086	0.003606
35	0.104	0.119	0.107	0.11	0.007937	0.085	0.086	0.093	0.088	0.004359
47	0.108	0.115	0.113	0.112	0.003606	0.096	0.088	0.09	0.091333	0.004163
60	0.108	0.095	0.109	0.104	0.00781	0.145	0.14	0.096	0.127	0.026963
73.5	0.112	0.13	0.114	0.118667	0.009866	0.143	0.132	0.132	0.135667	0.006351
95	0.107	0.128	0.123	0.119333	0.01097	0.135	0.124	0.159	0.139333	0.017898
	0.5 g/l glucose & 0.1 g/l KL					0.5 g/l glucose & 0.1 g/l ferulic acid				
Time (h)	STW	STW	Averag	StDev	STW	STW	STW	Average	StDev	
0	0.052	0.051	0.051	0.051333	0.000577	0.071	0.073	0.073	0.072333	0.001155
10.5	0.062	0.061	0.06	0.061	0.001	0.098	0.098	0.097	0.097667	0.000577
24	0.059	0.063	0.06	0.060667	0.002082	0.107	0.116	0.106	0.109667	0.005508
35	0.063	0.065	0.059	0.062333	0.003055	0.103	0.128	0.115	0.115333	0.012503
47	0.153	0.066	0.061	0.093333	0.051733	0.108	0.123	0.1	0.110333	0.011676
60	0.144	0.113	0.153	0.136667	0.020984	0.114	0.135	0.102	0.117	0.016703
73.5	0.139	0.188	0.143	0.156667	0.027209	0.106	0.148	0.107	0.120333	0.023965
95	0.128	0.178	0.132	0.146	0.027785	0.11	0.213	0.098	0.140333	0.063217

Table D7. The OD data as a function of time, the average and standard deviation values are reported for the cultures MOA, DO, S42, FD and the uninoculated control "Blank".

	0.5 g/l glucose					0.5 g/l glucose & 0.1 g/l KL				
Time (h)	MOA	MOA	MOA	Average	StDev	MOA	MOA	MOA	Average	StDev
0	0.067	0.071	0.07	0.069333	0.002082	0.052	0.049	0.051	0.050667	0.001528
10.5	0.081	0.082	0.082	0.081667	0.000577	0.06	0.058	0.059	0.059	0.001
24	0.08	0.082	0.087	0.083	0.003606	0.061	0.062	0.06	0.061	0.001
35	0.083	0.087	0.098	0.089333	0.007767	0.06	0.058	0.059	0.059	0.001
47	0.078	0.134	0.176	0.129333	0.049166	0.201	0.149	0.061	0.137	0.070767
60	0.106	0.156	0.167	0.143	0.032512	0.188	0.155	0.195	0.179333	0.021362
73.5	0.15	0.147	0.164	0.153667	0.009074	0.18	0.148	0.175	0.167667	0.017214
95	0.135	0.137	0.14	0.137333	0.002517	0.174	0.13	0.178	0.160667	0.026633
	0.5 g/l glucose					0.5 g/l glucose & 0.1 g/l KL				
Time (h)	DO	DO	DO	Average	StDev	DO	DO	DO	Average	StDev
0	0.069	0.069	0.07	0.069333	0.000577	0.053	0.051	0.051	0.051667	0.001155
10.5	0.079	0.083	0.083	0.081667	0.002309	0.06	0.062	0.061	0.061	0.001
22.5	0.083	0.084	0.07	0.079	0.00781	0.061	0.059	0.06	0.06	0.001
35	0.108	0.092	0.097	0.099	0.008185	0.072	0.062	0.068	0.067333	0.005033
47	0.144	0.158	0.142	0.148	0.008718	0.171	0.106	0.162	0.146333	0.035218
60	0.133	0.134	0.13	0.132333	0.002082	0.158	0.174	0.163	0.165	0.008185
73.5	0.128	0.125	0.125	0.126	0.001732	0.155	0.17	0.162	0.162333	0.007506
95	0.111	0.11	0.117	0.112667	0.003786	0.142	0.156	0.157	0.151667	0.008386
0.5 g/l glucose & 0.1 g/l ferulic acid						0.5 g/l glucose & 0.1 g/l ferulic acid				
S42	S42	S42	Averag	StDev	Time (h)	Blank	Blank	Blank	Average	StDev
0.073	0.072	0.073	6E-04		0	0.072	0.072	0.072	0.072	0
0.101	0.104	0.103	0.103	0.001528	10.5	0.099	0.107	0.09	0.098667	0.008505
0.117	0.112	0.116	0.115	0.002646	24	0.099	0.105	0.108	0.104	0.004583
0.127	0.123	0.119	0.123	0.004	35	0.103	0.115	0.117	0.111667	0.007572
0.121	0.118	0.122	0.12	0.002082	47	0.112	0.122	0.117	0.117	0.005
0.115	0.118	0.126	0.12	0.005686	60	0.111	0.122	0.148	0.127	0.019
0.129	0.116	0.132	0.126	0.008505	73.5	0.107	0.116	0.106	0.109667	0.005508
0.135	0.155	0.121	0.137	0.017088	95	0.099	0.113	0.087	0.099667	0.013013
	0.5 g/l glucose & 0.1 g/l ferulic acid					0.5 g/l glucose				
Time (h)	MOA	MOA	MOA	Average	StDev	FD	FD	FD	Average	StDev
0	0.071	0.073	0.073	0.072333	0.001155	0.067	0.068	0.069	0.068	0.001
10.5	0.09	0.084	0.112	0.095333	0.014742	0.08	0.08	0.08	0.08	0
24	0.097	0.109	0.103	0.103	0.006	0.075	0.088	0.083	0.082	0.006557
35	0.099	0.105	0.11	0.104667	0.005508	0.081	0.085	0.09	0.085333	0.004509
47	0.105	0.106	0.119	0.11	0.00781	0.079	0.096	0.09	0.088333	0.008622
60	0.113	0.098	0.116	0.109	0.009644	0.12	0.091	0.165	0.125333	0.037287
73.5	0.133	0.118	0.128	0.126333	0.007638	0.139	0.153	0.167	0.153	0.014
95	0.14	0.113	0.136	0.129667	0.014572	0.133	0.165	0.14	0.146	0.016823

Table D8. The OD data as a function of time, the average and standard deviation values are reported for the cultures ST, S42 and the uninoculated control "Blank".

2/1/2012	**0.5g/L glucose**				
Time [h]	**ST**	**ST**	**ST**	Average	StDev
0	0.07	0.071	0.07	0.070333	0.000577
10.5	0.08	0.08	0.082	0.080667	0.001155
22.5	0.076	0.084	0.085	0.081667	0.004933
35	0.082	0.088	0.088	0.086	0.003464
47	0.082	0.133	0.087	0.100667	0.028113
60	0.145	0.158	0.155	0.152667	0.006807
73.5	0.128	0.151	0.147	0.142	0.012288
95	0.117	0.136	0.126	0.126333	0.009504
2/1/2012	**0.5 g/L glucose**				
Time [h]	**S42**	**S42**	**S42**	Average	StDev
0	0.069	0.07	0.068	0.069	0.001
10.5	0.084	0.085	0.087	0.085333	0.001528
22.5	0.085	0.083	0.09	0.086	0.003606
35	0.085	0.086	0.093	0.088	0.004359
47	0.096	0.088	0.09	0.091333	0.004163
60	0.145	0.14	0.096	0.127	0.026963
73.5	0.143	0.132	0.132	0.135667	0.006351
95	0.135	0.124	0.159	0.139333	0.017898
2/1/2012	**0.5 g/L glucose**				
Time [h]	**Blank**	**Blank**	**Blank**	Average	StDev
0	0.068	0.069	0.069	0.068667	0.000577
10.5	0.081	0.083	0.072	0.078667	0.005859
22.5	0.083	0.082	0.076	0.080333	0.003786
35	0.083	0.085	0.08	0.082667	0.002517
47	0.082	0.086	0.084	0.084	0.002
60	0.088	0.089	0.087	0.088	0.001
73.5	0.095	0.097	0.101	0.097667	0.003055
95	0.136	0.092	0.108	0.112	0.022271

Table D9. The OD data as a function of time, the average and standard deviation values are reported for the cultures ST and the uninoculated control "Blank".

	0.025 g/l TYE + 0.1 g/l KL					**0.025 g/l TYE + 0.1 g/l KL**				
Time [h]	**Blank**	**Blank**	**Blank**	Averag	Stdev	**ST**	**ST**	**ST**	Average	Stdev
0	0.054	0.054	0.054	0.054	0	0.07	0.07	0.069	0.06967	6E-04
16	0.054	0.054	0.054	0.054	0	0.07	0.072	0.069	0.07033	0.002
28	0.054	0.054	0.055	0.054	6E-04	0.092	0.095	0.066	0.08433	0.016
40	0.053	0.082	0.055	0.063	0.016	0.089	0.089	0.079	0.08567	0.006
52	0.053	0.054	0.054	0.054	6E-04	0.09	0.089	0.083	0.08733	0.004
	0.025 g/l TYE + 0.1 g/l Ferulic Acid					**0.025 g/l TYE + 0.1 g/l Ferulic Acid**				
Time [h]	**Blank**	**Blank**	**Blank**	Averag	Stdev	**ST**	**ST**	**ST**	Average	Stdev
0	0.046	0.046	0.047	0.046	6E-04	0.065	0.063	0.064	0.064	0.001
16	0.056	0.057	0.056	0.056	6E-04	0.081	0.081	0.081	0.081	0
28	0.062	0.059	0.068	0.063	0.005	0.079	0.085	0.087	0.08367	0.004
40	0.066	0.066	0.072	0.068	0.003	0.085	0.083	0.082	0.08333	0.002
52	0.07	0.07	0.073	0.071	0.002	0.088	0.087	0.087	0.08733	6E-04
	0.025 g/l TYE + 0.1 g/l Cinnamic Aci					**0.025 g/l TYE + 0.1 g/l Cinnamic Acid**				
Time [h]	**Blank**	**Blank**	**Blank**	Averag	Stdev	**ST**	**ST**	**ST**	Average	Stdev
0	0.044	0.044	0.044	0.044	8E-18	0.059	0.06	0.06	0.05967	6E-04
16	0.05	0.049	0.048	0.049	0.001	0.084	0.086	0.085	0.085	1E-03
28	0.053	0.055	0.06	0.056	0.004	0.083	0.084	0.082	0.083	0.001
40	0.05	0.05	0.049	0.05	6E-04	0.081	0.081	0.08	0.08067	6E-04
52	0.054	0.054	0.054	0.054	0	0.08	0.083	0.085	0.08267	0.003
	0.025 g/l TYE					**0.025 g/l TYE**				
Time [h]	**Blank**	**Blank**	**Blank**	Averag	Stdev	**ST**	**ST**	**ST**	Average	Stdev
0	0.046	0.044	0.044	0.045	0.001	0.061	0.064	0.062	0.06233	0.002
16	0.048	0.047	0.048	0.048	6E-04	0.066	0.072	0.067	0.06833	0.003
28	0.051	0.049	0.051	0.05	0.001	0.083	0.082	0.088	0.08433	0.003
40	0.05	0.051	0.05	0.05	6E-04	0.081	0.084	0.084	0.083	0.002
52	0.05	0.049	0.051	0.05	0.001	0.088	0.091	0.09	0.0905	7E-04
	.025 g/L TYE + 0.1 g/L p-Coumaric aci					**0.025 g/L TYE + 0.1 g/L p-Coumaric acid**				
Time [h]	**Blank**	**Blank**	**Blank**	Averag	Stdev	**ST**	**ST**	**ST**	Average	Stdev
0	0.054	0.054	0.054	0.054	0	0.061	0.061	0.059	0.06033	0.001
16	0.054	0.054	0.054	0.054	0	0.08	0.082	0.076	0.07933	0.003
28	0.054	0.054	0.055	0.054	6E-04	0.082	0.081	0.081	0.08133	6E-04
40	0.053	0.082	0.055	0.063	0.016	0.081	0.083	0.083	0.08233	0.001
52	0.053	0.054	0.054	0.054	6E-04	0.082	0.083	0.082	0.08233	6E-04

Table D10. The OD data as a function of time, the average and standard deviation values are reported for the culture S42.

	0.025 g/l TYE + 0.1 g/l KL				
Time [h]	**S42**	**S42**	**S42**	Average	Stdev
0	0.066	0.069	0.069	0.068	0.001732
16	0.1	0.102	0.107	0.103	0.003606
28	0.093	0.101	0.104	0.099333	0.005686
40	0.095	0.1	0.101	0.098667	0.003215
52	0.093	0.095	0.098	0.095333	0.002517
	0.025 g/l TYE + 0.1 g/l KL				
Time [h]	**S42**	**S42**	**S42**	Average	Stdev
0	0.062	0.064	0.062	0.062667	0.001155
16	0.105	0.112	0.107	0.108	0.003606
28	0.118	0.129	0.132	0.126333	0.007371
40	0.127	0.14	0.138	0.135	0.007
52	0.134	0.142	0.147	0.141	0.006557
	0.025 g/l TYE + 0.1 g/l KL				
Time [h]	**S42**	**S42**	**S42**	Average	Stdev
0	0.06	0.062	0.061	0.061	0.001
16	0.097	0.082	0.098	0.092333	0.008963
28	0.098	0.106	0.106	0.103333	0.004619
40	0.098	0.101	0.099	0.099333	0.001528
52	0.096	0.105	0.108	0.103	0.006245
	0.025 g/l TYE				
Time [h]	**S42**	**S42**	**S42**	Average	Stdev
0	0.061	0.061	0.061	0.061	0
16	0.095	0.092	0.094	0.093667	0.001528
28	0.092	0.093	0.095	0.093333	0.001528
40	0.093	0.094	0.093	0.093333	0.000577
52	0.088	0.091	0.09	0.089667	0.001528
	0.025 g/L TYE + 0.1 g/L p-Coumaric acid				
Time [h]	**S42**	**S42**	**S42**	Average	Stdev
0	0.059	0.061	0.059	0.059667	0.001155
16	0.106	0.098	0.101	0.101667	0.004041
28	0.095	0.102	0.099	0.098667	0.003512
40	0.098	0.102	0.101	0.100333	0.002082
52	0.091	0.097	0.096	0.094667	0.003215

Table D11. The OD data as a function of time, the average and standard deviation values are reported for the cultures MOA, DO, FD, and STw.

	0.5 g/l glucose blank					0.5 g/l glucose & 0.1 g/l KL				
Time (h)	MOA	MOA	MOA	Average	StDev	MOA	MOA	MOA	Average	StDev
0	0.054	0.057	0.055	0.055333	0.001528	0.05	0.05	0.051	0.050333	0.000577
24	0.074	0.084	0.079	0.079	0.005	0.067	0.066	0.067	0.066667	0.000577
48	0.088	0.077	0.162	0.109	0.046228	0.172	0.137	0.152	0.153667	0.017559
72	0.15	0.134	0.149	0.144333	0.008963	0.152	0.165	0.162	0.159667	0.006807
96	0.129	0.122	0.147	0.132667	0.012897	0.151	0.161	0.153	0.155	0.005292
120	0.121	0.111	0.138	0.123333	0.01365	0.151	0.146	0.158	0.151667	0.006028
144	0.114	0.111	0.113	0.112667	0.001528	0.13	0.138	0.146	0.138	0.008
	0.5 g/l glucose blank					0.5 g/l glucose & 0.1 g/l KL				
Time (h)	DO	DO	DO	Average	StDev	DO	DO	DO	Average	StDev
0	0.055	0.06	0.062	0.059	0.003606	0.072	0.053	0.053	0.059333	0.01097
24	0.084	0.083	0.089	0.085333	0.003215	0.117	0.067	0.133	0.105667	0.034429
48	0.135	0.143	0.138	0.138667	0.004041	0.135	0.158	0.17	0.154333	0.017786
72	0.127	0.123	0.121	0.123667	0.003055	0.151	0.142	0.161	0.151333	0.009504
96	0.116	0.112	0.126	0.118	0.007211	0.107	0.152	0.142	0.133667	0.023629
120	0.106	0.101	0.108	0.105	0.003606	0.133	0.118	0.139	0.13	0.010817
144	0.091	0.093	0.109	0.097667	0.009866	0.13	0.135	0.17	0.145	0.021794
	0.5 g/l glucose blank					0.5 g/l glucose & 0.1 g/l KL				
Time (h)	FD	FD	FD	Average	StDev	FD	FD	FD	Average	StDev
0	0.058	0.059	0.06	0.059	0.001	0.054	0.054	0.055	0.054333	0.000577
24	0.076	0.077	0.074	0.075667	0.001528	0.068	0.068	0.067	0.067667	0.000577
48	0.078	0.081	0.078	0.079	0.001732	0.067	0.122	0.069	0.086	0.031193
72	0.138	0.147	0.136	0.140333	0.005859	0.166	0.148	0.158	0.157333	0.009018
96	0.112	0.133	0.123	0.122667	0.010504	0.15	0.154	0.148	0.150667	0.003055
120	0.111	0.127	0.112	0.116667	0.008963	0.131	0.122	0.13	0.127667	0.004933
144	0.098	0.14	0.089	0.109	0.027221	0.129	0.145	0.138	0.137333	0.008021
	0.5 g/l glucose blank					0.5 g/l glucose & 0.1 g/l KL				
Time (h)	STW	STW	STW	Average	StDev	STW	STW	STW	Average	StDev
0	0.055	0.058	0.056	0.056333	0.001528	0.052	0.053	0.055	0.053333	0.001528
24	0.08	0.079	0.083	0.080667	0.002082	0.067	0.066	0.064	0.065667	0.001528
48	0.131	0.146	0.148	0.141667	0.009292	0.148	0.116	0.161	0.141667	0.023159
72	0.124	0.134	0.135	0.131	0.006083	0.131	0.135	0.147	0.137667	0.008327
96	0.113	0.145	0.145	0.134333	0.018475	0.14	0.134	0.134	0.136	0.003464
120	0.113	0.139	0.15	0.134	0.019	0.13	0.134	0.136	0.133333	0.003055
144	0.102	0.142	0.155	0.133	0.027622	0.131	0.131	0.148	0.136667	0.009815

Table 3. The OD data as a function of time, the average and standard deviation values are reported for the cultures ST and S42.

	Corn Stover					Corn Stover				
Time (h)	ST	ST	ST	Average	STDEV	S42	S42	S42	Average	STDEV
0	0.047	0.048	0.05	0.04833	0.00153	0.048	0.047	0.048	0.047667	0.000577
4.5	0.055	0.058	0.057	0.05667	0.00153	0.06	0.055	0.058	0.057667	0.002517
19.5	0.054	0.058	0.057	0.05633	0.00208	0.057	0.057	0.057	0.057	0
30	0.057	0.06	0.058	0.05833	0.00153	0.055	0.057	0.059	0.057	0.002
43.5	0.063	0.066	0.055	0.06133	0.00569	0.054	0.054	0.054	0.054	0
91.5	0.171	0.081	0.223	0.15833	0.07184	0.209	0.134	0.212	0.185	0.044193
139.5	0.161	0.195	0.201	0.18567	0.02157	0.178	0.177	0.202	0.185667	0.014154
	Corn Cob					Corn Cob				
Time (h)	ST	ST	ST	Average	STDEV	S42	S42	S42	Average	STDEV
0	0.053	0.054	0.054	0.05367	0.00058	0.054	0.055	0.054	0.054333	0.000577
4.5	0.058	0.06	0.061	0.05967	0.00153	0.059	0.061	0.063	0.061	0.002
19.5	0.059	0.061	0.06	0.06	0.001	0.06	0.061	0.061	0.060667	0.000577
30	0.059	0.061	0.061	0.06033	0.00115	0.06	0.062	0.062	0.061333	0.001155
43.5	0.058	0.06	0.061	0.05967	0.00153	0.061	0.061	0.061	0.061	0
91.5	0.062	0.063	0.063	0.06267	0.00058	0.063	0.063	0.063	0.063	0
139.5	0.194	0.073	0.191	0.15267	0.06901	0.066	0.069	0.065	0.066667	0.002082
	Lodge Pole Pine					Lodge Pole Pine				
Time (h)	ST	ST	ST	Average	STDEV	S42	S42	S42	Average	STDEV
0	0.046	0.046	0.046	0.046	8.5E-18	0.046	0.046	0.047	0.046333	0.000577
4.5	0.096	0.098	0.097	0.097	0.001	0.076	0.094	0.087	0.085667	0.009074
19.5		0.097	0.095	0.096	0.00141	0.076	0.094	0.068	0.079333	0.013317
30		0.109	0.108	0.1085	0.00071	0.101	0.097	0.082	0.093333	0.010017
43.5		0.105	0.101	0.103	0.00283	0.097	0.11	0.112	0.106333	0.008145
91.5	0.158	0.187	0.206	0.18367	0.02417	0.165	0.247	0.193	0.201667	0.041681
139.5	0.151	0.164	0.181	0.16533	0.01504	0.142	0.229	0.19	0.187	0.043578

Table D13. The OD data as a function of time, the average and standard deviation values are reported for the uninoculated control "Blank".

	Corn Stover				
Time (h)	Blank	Blank	Blank	Average	STDEV
0	0.043	0.044	0.043	0.043333	0.000577
4.5	0.05	0.051	0.052	0.051	0.001
19.5	0.052	0.052	0.053	0.052333	0.000577
30	0.051	0.052	0.057	0.053333	0.003215
43.5	0.049	0.051	0.05	0.05	0.001
91.5	0.062	0.071	0.064	0.065667	0.004726
139.5	0.07	0.074	0.069	0.071	0.002646
	Corn Cob				
Time (h)	Blank	Blank	Blank	Average	STDEV
0	0.049	0.05	0.05	0.049667	0.000577
4.5	0.054	0.057	0.058	0.056333	0.002082
19.5	0.056	0.058	0.057	0.057	0.001
30	0.059	0.059	0.06	0.059333	0.000577
43.5	0.055	0.057	0.056	0.056	0.001
91.5	0.059	0.064	0.061	0.061333	0.002517
139.5	0.061	0.065	0.067	0.064333	0.003055
	Lodge Pole Pine				
Time (h)	Blank	Blank	Blank	Average	STDEV
0	0.047	0.046	0.047	0.046667	0.000577
4.5	0.066	0.07	0.074	0.07	0.004
19.5	0.074	0.066	0.065	0.068333	0.004933
30	0.062	0.073	0.073	0.069333	0.006351
43.5	0.069	0.071	0.071	0.070333	0.001155
91.5	0.084	0.062	0.072	0.072667	0.011015
139.5	0.083	0.062	0.071	0.072	0.010536

Protein Data: Bradford Protein Concentration and Enzymatic Activity Assays

Table 4. The bradford assay protein concentration data generated during the extraction process and used for the activity assays.

12/20/2011							
t=0m	MOA	DO	ST	FD	STw	S42	
S1	0.081	0.084	0.079	0.077	0.078	0.078	
S2	0.075	0.073	0.073	0.072	0.073	0.071	
S3	0.105	0.102	0.191	0.086	0.097	0.097	
t=2m	MOA	DO	ST	FD	STw	S42	
S1	0.081	0.079	0.085	0.074	0.078	0.077	
S2	0.073	0.073	0.072	0.072	0.073	0.069	
S3	0.109	0.104	0.188	0.086	0.097	0.101	
t=2m-0m	MOA	DO	ST	FD	STw	S42	
S1	0	0.005	-0.006	0.003	0	0.001	
S2	0.002	0	0.001	0	0	0.002	
S3	-0.004	-0.002	0.003	0	0	-0.004	
				blank	0.081		
1/30/2012							
t=0m	MOA	DO	ST	FD	STw	S42	
S1	0.1	0.099	0.101	0.09	0.102	0.101	
S2	0.101	0.103	0.101	0.1	0.102	0.099	
S3	0.102	0.105	0.123	0.11	0.105	0.101	
S4	0.095	0.099	0.098	0.099	0.101	0.096	
t=2m							
S1	0.098	0.097	0.098	0.096	0.098	0.1	
S2	0.097	0.096	0.098	0.096	0.097	0.098	
S3	0.095	0.101	0.099	0.094	0.095	0.103	
S4	0.096	0.097	0.097	0.099	0.091	0.093	
S1	0.002	0.002	0.003	-0.006	0.004	0.001	
S2	0.004	0.007	0.003	0.004	0.005	0.001	
S3	0.007	0.004	0.024	0.016	0.01	-0.002	
S4	-0.001	0.002	0.001	0	0.01	0.003	
	Protein				Laccase Assay		
3/20/2012	S42	ST	2.5195		S42		
S1	0.782	0.764	0.773	0.3387316	0.126	0.315	
S2	0.539	0.54	0.5395		0.122	0.142	
S3	1.221	1.193	1.207		0.167	0.165	
0.0 mM	0.5 mM	0.05 mM		S42		ST	
	S42	ST	Average	1/6/2012	1/30/2012	1/6/2012	1/30/2012
S1	0.094074	0.0907407	0.0924074	0.1592593	-0.015555556	0.277777778	0.003
S2	0.049074	0.0492593	0.0491667	0.0422222	-0.00962963	0.056296296	0.003
S3	0.070148	0.0680741	0.0691111	0.1414815	0.16962963	0.165925926	0.024
	0.213296	0.2080741	0.0036927	0.342963	0.144444444		
			0.2106852				

Table D15. Protein concentration and laccase assay data.

S42	S42	S42	ST	ST	ST	ST	
3/20/2012	1/6/2012	1/30/2012	3/20/2012	1/6/2012			
0.05	0.001	0.001	0.129	0.119	-0.01	0	
0.02	0.001	0.001	0.125	0.116	-0.009	0	
-0.002	0.003	-0.002	0.117	0.153	0.036	0.002	
		S42					
1/30/2012	1/6/2012	3/20/2012	1/30/2012				
-0.0148148	0.006279	0.5314961	-0.064286				
-0.0074074	0.023684	0.4075472	-0.103846				
0.07407407	0.021204	-0.028511	-0.01179				

1/6/2012							
t=0m	MOA	DO	ST	FD	STw	S42	
S1	0.087	0.097	0.094	0.093	0.087	0.088	
S2	0.088	0.088	0.087	0.087	0.086	0.087	
S3	0.088	0.097	0.093	0.094	0.087	0.111	
t=2m	MOA	DO	ST	FD	STw	S42	
S1	0.085	0.095	0.094	0.092	0.086	0.087	
S2	0.087	0.091	0.087	0.087	0.086	0.086	
S3	0.086	0.096	0.091	0.089	0.088	0.108	
t=2m-0m	MOA	DO	ST	FD	STw	S42	
S1	0.002	0.002	0	0.001	0.001	0.001	
S2	0.001	-0.003	0	0	0	0.001	
S3	0.002	0.001	0.002	0.005	-0.001	0.003	
Bradford	OD595						
S1	0.272	0.268	0.272	0.276	0.269	0.271	
S2	0.275	0.278	0.282	0.279	0.284	0.279	
S3	0.507	0.512	0.392	0.477	0.492	0.521	
S4	0.313	0.311	0.307	0.312	0.323	0.319	
S1	-0.014815	-0.017778	-0.0148148	-0.011852	-0.017037	-0.015556	
S2	-0.006296	-0.01037	-0.0074074	-0.00963	-0.005926	-0.00963	
S3	0.1592593	0.162963	0.0740741	0.137037	0.1481481	0.1696296	
S4	0.0038889	0.0035185	0.0027778	0.0037037	0.0057407	0.005	
	0.142037	0.1383333	0.0546296	0.1192593	0.1309259	0.1494444	0.1224383
			0.0518519				0.0347743

Table D16. Protein concentration data and a standard curve used to correlate absorbance to protein concentration.

1/6/2012-blank								
t=0m	MOA	DO	ST	FD	STw	S42		
S1	0.006	0.016	0.013	0.012	0.006	0.007		
S2	0.007	0.007	0.006	0.006	0.005	0.006		
S3	0.007	0.016	0.012	0.013	0.006	0.03		
t=2m	MOA	DO	ST	FD	STw	S42		
S1	0.004	0.014	0.013	0.011	0.005	0.006		
S2	0.006	0.01	0.006	0.006	0.005	0.005		
S3	0.005	0.015	0.01	0.008	0.007	0.027		
Bradford	OD595							
29-Dec	MOA	DO	ST	FD	STw	S42		
S1	0.717	0.619	0.67	0.694	0.619	0.317		
S2	0.468	0.415	0.427	0.459	0.409	0.29		
S3	1.087	0.892	1.111	0.837	0.954	0.511		
S4	0.356	0.404	0.383	0.443	0.363	0.351		
S1	0.314814815	0.242222222	0.28	0.297778	0.242222	0.018519		
S2	0.065185185	0.045555556	0.05	0.061852	0.043333	-0.00074		
S3	0.588888889	0.444444444	0.606667	0.403704	0.49037	0.162222		
S4	0.011851852	0.020740741	0.016852	0.027963	0.013148	0.010926		
	6-Jan	MOA	DO	ST	FD	STw	S42	
	S1	0.511	0.719	0.667	0.647	0.496	0.507	
	S2	0.387	0.487	0.444	0.468	0.387	0.406	
	S3	0.538	0.533	0.516	0.63	0.469	0.483	
	S4	0.701	0.636	0.652	0.697	0.689	0.351	
	S1	0.162222222	0.316296	0.277778	0.262963	0.151111	0.159259	
	S2	0.035185185	0.072222	0.056296	0.065185	0.035185	0.042222	
	S3	0.182222222	0.178519	0.165926	0.25037	0.131111	0.141481	
	S4	0.075740741	0.063704	0.066667	0.075	0.073519	0.010926	
		0.45537037	0.630741	0.566667	0.653519	0.390926	0.353889	0.508519
				0.5				
		Standard Curve						
	0	0.288	0.284	0.287	0.286333	0.002082		
	12.5	0.331	0.328	0.332	0.330333	0.002082		
	25	0.352	0.378	0.37	0.366667	0.013317		
	37.5	0.406	0.388	0.39	0.394667	0.009866		
	50	0.433	0.424	0.419	0.425333	0.007095		
	62.5	0.447	0.453	0.442	0.447333	0.005508		
	75	0.459	0.466	0.441	0.455333	0.012897		

Statistical Analysis of Growth on Lignin Like Compounds

Table D175. T-tests assuming un-equal variances to confirm the statistical significance of the growth on the nutrient sources compared to the lignin associated compound.

ST	TYE	KL	ST	TYE	Ferulic	ST	TYE	Cinnamic	ST	TYE	pCoumaric			
	Variable 1	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*			
Mean	0.019667	0.017667	Mean	0.019667	0.023333	Mean	0.019667	0.023	Mean	0.019667	0.022333			
Variance	0.000184	1.03E-05	Variance	0.000184	3.33E-07	Variance	0.000184	4E-06	Variance	0.000184	3.33E-07			
Observati	3	3	Observati	3	3	Observati	3	3	Observati	3	3			
Hypothesi	0		Hypothesi	0		Hypothesi	0		Hypothesi	0				
df	2		df	2		df	2		df	2				
t Stat	0.248282		t Stat	-0.46735		t Stat	-0.4207		t Stat	-0.33989				
P(T<=t) or	**0.413541**		**P(T<=t) or**	**0.343113**		**P(T<=t) or**	**0.357434**		**P(T<=t) or**	**0.383159**				
t Critical o	2.919986		t Critical o	2.919986		t Critical o	2.919986		t Critical o	2.919986				
P(T<=t) tw	**0.827083**		**P(T<=t) tw**	**0.686226**		**P(T<=t) tw**	**0.714867**		**P(T<=t) tw**	**0.766318**				
t Critical t	4.302653		t Critical t	4.302653		t Critical t	4.302653		t Critical t	4.302653				

ST	**Glc**	**KL**	ST	Glc	CS	**ST**	**Glc**	**CC**	**ST**	**Glc**	**LPP**	ST	Glc	Ferulic
	Variable 1	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*
Mean	0.056	0.099	Mean	0.056	0.1485	Mean	0.056	0.009	Mean	0.056	0.137667	Mean	0.056	0.045333
Variance	8.1E-05	0.000109	Variance	8.1E-05	0.001201	Variance	8.1E-05	0	Variance	8.1E-05	0.000584	Variance	8.1E-05	9.63E-05
Observati	3	3	Observati	3	2	Observati	3	3	Observati	3	3	Observati	3	3
Hypothesi	0		Hypothesi	0		Hypothesi	0		Hypothesi	0		Hypothesi	0	
df	4		df	1		df	2		df	3		df	4	
t Stat	-5.40322		t Stat	-3.69336		t Stat	9.045154		t Stat	-5.48385		t Stat	1.387376	
P(T<=t) or	**0.00284**		**P(T<=t) or**	**0.084166**		**P(T<=t) or**	**0.006002**		**P(T<=t) or**	**0.005963**		**P(T<=t) or**	**0.11881**	
t Critical o	2.131847		t Critical o	6.313752		t Critical o	2.919986		t Critical o	2.353363		t Critical o	2.131847	
P(T<=t) tw	**0.00568**		**P(T<=t) tw**	**0.168333**		**P(T<=t) tw**	**0.012003**		**P(T<=t) tw**	**0.011927**		**P(T<=t) tw**	**0.23762**	
t Critical t	2.776445		t Critical t	12.7062		t Critical t	4.302653		t Critical t	3.182446		t Critical t	2.776445	

S42	TYE	KL	**S42**	**TYE**	**Ferulic**	**S42**	**TYE**	**Cinnamic**	**S42**	**TYE**	**pCoumaric**			
	Variable 1	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*			
Mean	0.028778	0.027333	Mean	0.028778	0.078333	Mean	0.028778	0.042	Mean	0.028778	0.036			
Variance	1.81E-06	2.33E-06	Variance	1.81E-06	4.23E-05	Variance	1.81E-06	0.000031	Variance	1.81E-06	0.000001			
Observati	3	3	Observati	3	3	Observati	3	3	Observati	3	3			
Hypothesi	0		Hypothesi	0		Hypothesi	0		Hypothesi	0				
df	4		df	2		df	2		df	4				
t Stat	1.228385		t Stat	-12.918		t Stat	-3.99788		t Stat	-7.45601				
P(T<=t) or	**0.143318**		**P(T<=t) or**	**0.00297**		**P(T<=t) or**	**0.028623**		**P(T<=t) or**	**0.000864**				
t Critical o	2.131847		t Critical o	2.919986		t Critical o	2.919986		t Critical o	2.131847				
P(T<=t) tw	**0.286635**		**P(T<=t) tw**	**0.005939**		**P(T<=t) tw**	**0.057246**		**P(T<=t) tw**	**0.001729**				
t Critical t	2.776445		t Critical t	4.302653		t Critical t	4.302653		t Critical t	2.776445				

S42	**Glc**	**KL**	S42	Glc	CS	**S42**	**Glc**	**CC**	**S42**	**Glc**	**LPP**	S42	Glc	Ferulic
	Variable 1	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*		*Variable 1*	*Variable 2*
Mean	0.074667	0.092667	Mean	0.074667	0.137333	Mean	0.074667	0.008667	Mean	0.074667	0.155333	Mean	0.074667	0.064333
Variance	6.53E-05	3.03E-05	Variance	6.53E-05	0.001902	Variance	6.53E-05	3.33E-07	Variance	6.53E-05	0.001746	Variance	6.53E-05	0.000276
Observati	3	3	Observati	3	3	Observati	3	3	Observati	3	3	Observati	3	3
Hypothesi	0		Hypothesi	0		Hypothesi	0		Hypothesi	0		Hypothesi	0	
df	4		df	2		df	2		df	2		df	3	
t Stat	-3.18752		t Stat	-2.44693		t Stat	14.10692		t Stat	-3.28258		t Stat	0.968277	
P(T<=t) or	**0.016648**		**P(T<=t) or**	**0.067101**		**P(T<=t) or**	**0.002494**		**P(T<=t) or**	**0.040803**		**P(T<=t) or**	**0.202164**	
t Critical o	2.131847		t Critical o	2.919986		t Critical o	2.919986		t Critical o	2.919986		t Critical o	2.353363	
P(T<=t) tw	**0.033295**		**P(T<=t) tw**	**0.134201**		**P(T<=t) tw**	**0.004987**		**P(T<=t) tw**	**0.081606**		**P(T<=t) tw**	**0.404329**	
t Critical t	2.776445		t Critical t	4.302653		t Critical t	4.302653		t Critical t	4.302653		t Critical t	3.182446	

APPENDIX E

FUTURE WORK

Ultrafiltration of Kraft Lignin

The ability to utilize waste lignin from industrial processes is a goal of this project. Industrial processes digest the lignin molecule to different extents, so the isolates were grown on kraft lignin and the impact of the bacteria on the concentration and structure of kraft lignin in the cell free supernatant was visualized on a spectrophotometer. To add a level of complexity to the dataset, the supernatant was ultrafiltered through a 1, 10, 50 and 100 kDa filter prior to measurement on the spectrophotometer. The ultrafiltration device was used to separate out the supernatant before and after enrichment with the isolates by molecular weight. A standard curve was performed with the kraft lignin used in the growth medium to find the absorbance maxium for the exact batch and composition of kraft lignin used during the experiment. A standard curve showed that the absorbance maximum is 286±2 nm; typically an absorbance maximum of 280nm is expected for lignin, although pH and side chain substitutions can shift the wavelength (Herzfeld 1940; Chandra 2007). This method does not give a very specific picture of what is happening to the kraft lignin present in the medium, a slight absorbance increase is shown in all molecular weight fractions after being enriched with the isolates. Figure A.11 shows a graph to represent the absorbance at 287 nm, which correlates to the concentration of kraft lignin.

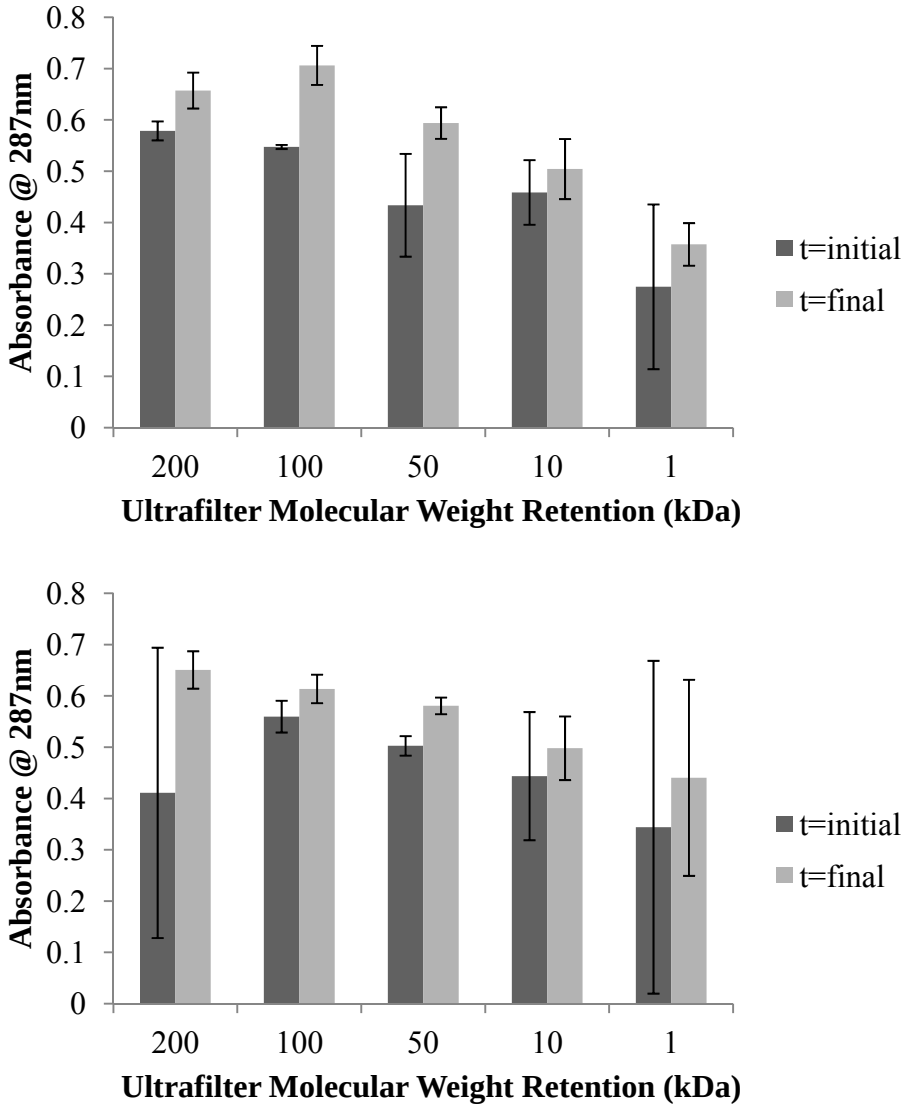

Figure E1. A graph to represent the absorbance at 287 nm, which correlates to the concentration of kraft lignin. The ultrafiltration device was used to separate out the supernatant before and after enrichment with the isolates *T. Thermophilus* ST (top) and S42 (bottom) by molecular weight.

The increase in absorbance at the final growth point when compared to the initial reading could be caused by a shift in pH, the change in the concentration of xylans, or change in the absorption of syringyl units (Vermerris 2006). Excessive degradation of carbohydrates still associated with kraft lignin even after the pulping process and the

change in water molecules associated with the lignin molecules can also contribute to a shift in absorption (Fukushima 2001). To confirm the initial and final supernatant samples were at pH 9, they were diluted with sodium borate buffer (pH 9) and re-run on the spectrophotometer, the results were consistent with the results presented in Figure 11 (Chandra 2007).