DEGRADATION OF PLANT LIGNIN BY *PSEUDOMONAS* SP. STRAIN YS-1P AND *PHANEROCHAETE CHRYSOSPORIUM* RP-78 CO-CULTURES

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Abstract: Delignification is a critical step in the bioconversion of lignocellulosic biomass into useful monomers. Fungi and bacteria both have evolved pathways to degrade lignin and are known to play active roles in decomposition of plant biomass. However, little efforts have been made to study them together as an integrated microbial system. There is limited knowledge about what specific roles they play and what kind of interactions they have during the decomposition process. The major focus of this research is to study lignin degradation using *Pseudomonas* sp. strain YS-1p (bacteria) and *Phanerochaete chrysosporium* RP-78 (fungi) when inoculated as individual cultures as well as in co-cultures in mineral salts medium supplemented with plant biomass: sugarcane bagasse and sorghum bagasse. Flasks were inoculated with strain RP-78 (F) and strain YS-1p (B) at various fungal-to-bacterial ratios including 0:1, 1:0, 1:1, 1:10 and 1:50, respectively. Flasks were sacrificed periodically and monitored for population dynamics. Culture supernatant was assayed for major lignin degrading enzymes including lignin peroxidase, dyp peroxidase and laccase. Additionally, extracellular enzymes were concentrated and subjected to 1-D PAGE-LC-MS/MS for proteome analysis, while recovered plant biomass was used for Py-GC/MS analysis. Our results show that *Pseudomonas* grew best in co-cultures with *P. chrysosporium* whereas the growth of latter was suppressed in the presence of bacteria. This also affected their abilities to produce extracellular enzymes involved in lignocellulose degradation as shown by our results of proteome analysis. Most of the enzymes involved in degradation of lignin, cellulose and hemicellulose were produced by the fungi, while the bacteria produced few enzymes at low levels. This suggests that bacteria were able to derive benefit from fungal enzymes for accessing degradation products and promoting their growth without contributing as much to the degradation process. Enzyme activities of lignin degrading enzymes showed that the two peroxidases: Lip and Dyp were expressed maximum in co-cultures while laccase was expressed in highest amounts in bacterial monocultures. Furthermore, analysis of plant biomass by Py-GC/MS revealed that microbial pretreatment altered the composition of lignocellulose in plant biomass with plant biomass showing significant decrease in lignin derivatives than in untreated controls.

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CHAPTER I

INTRODUCTION

Plant biomass is the most abundant renewable carbon feedstock for cellulosic bio-refineries that has the potential of meeting significant fraction of increasing energy demands (1). Lignocellulose is the major component of plant cell wall and it is mainly composed of cellulose, hemicellulose, and lignin (2). The efficient conversion of plant biomass to biofuel and other chemicals requires a pretreatment step to disintegrate lignin so that cellulose and hemicellulose are available for enzymatic or microbial saccharification and subsequent fermentation. Lignin, a heterogeneous and recalcitrant aromatic structure entraps cellulose and hemicellulose making plant biomass inherently recalcitrant. Therefore, delignification lies as the bottleneck in the bioconversion of plant polysaccharides into biofuels and bioproducts (3).

Current pretreatment technologies use harsh thermal and chemical methods, which are costly, highly energy intensive and produce toxic compounds that inhibit downstream processes. Therefore, lately considerable interest has been given towards microbial breakdown of lignin. To date, most studies on biological pretreatment of lignocellulose have primarily focused

on white rot fungi, which are the most active microbes with respect to lignin degradation (4-6). Fungal lignin degradation has been known to be associated with secondary metabolism, which is triggered only during conditions of nutrient depletion (7, 8). Fungi utilize a wide array of extracellular ligninolytic metalloenzymes such as peroxidases and laccases that oxidize aromatic rings present in lignin for bond scission reactions (6). In addition, they also secrete numerous hydrolytic enzymes responsible for polysaccharide degradation (9, 10). Genome sequencing studies of several white-rot fungi such as *Phanerochaete chrysosporium* (11), *Pleurotus ostreatus* (12), *Trametes versicolor* (13) and *Phlebia radiata* (14) have revealed presence of multiple ligninolytic enzyme systems and offered significant insight into molecular basis of lignin degradation.

Comparatively, lignin metabolism by bacteria is less well characterized even though numerous soil bacteria have been observed to be involved in degradation and modification of lignin (6). Studies show that bacterial lignin degradation is not as efficient as fungal systems with most bacteria being able to metabolize low molecular lignin compounds but with limited ability to metabolize polymeric lignin (15). Filamentous bacteria, actinomycetes, play significant role in processing of organic matter (16, 17) and they are the most extensively studied bacteria with respect to lignin degradation (15, 18). Of particular importance is *Streptomyces viridosporus* T7A, which has numerous ligninolytic peroxidases and which can modify lignin to produce acid-precipitable polymeric lignin (APPL), a high molecular weight (>80000) water-soluble lignin intermediate (19, 20). Several other bacterial genera like *Pseudomonas, Sphingobium, Rhodococcus, Nocardia, Acinetobacter and Arthrobacter* have also been reported to metabolize lignin or lignin-related compounds (15, 21-26). Most of these studies involve mineralization or modification of ^{14}C labeled substrates. Bacterial lignin degradation differs from fungal system in few important aspects. Unlike white-rot fungi which degrade lignin during secondary metabolism

triggered by depletion of nutrients nitrogen, carbon or sulfur (27, 28), bacteria digest lignin as primary metabolic activity (29). In addition, bacterial lignin degradation is more specific than fungi

because one bacterial species can cleave only one type of bond in lignin (30). Moreover, bacterial systems are easier to manipulate *in vitro* for studies involving rDNA technology, protein expression and are more robust to fluctuations of abiotic parameters (31). All of these features make bacteria as attractive candidates for biotechnological applications involving lignin degradation.

In natural environment, microorganisms rarely exist as isolated communities. There are numerous reports, which shed light on close association between fungi and bacteria in natural habitats and thereby infer that a complex process such as lignin degradation necessitates cumulative and active contributions from both fungi and bacteria. Especially, in the context of biomass turnover, multiple groups of phylogenetically distinct microbes appear to coexist as highly interactive and complex communities with dynamic interactions. For example, synergistic interactions between fungi and bacteria were observed in a microcosm experiment involving six species of fungi and four species of bacteria (32). The microbes were grown on fresh beech leaves and both groups were reported to benefit each other's growth. On the contrary, a similar study performed on 1-year-old beech leaves and undefined bacterial and fungal groups revealed antagonistic interactions with respect to carbon mineralization rates and enzyme activities (33). Another study reported both positive and negative interactions between fungal and bacterial communities associated with two different stages of decay in salt marshes (34). In addition, a similar study to our work was published by Schneider et al. in which they assessed contributions of fungi and bacteria to leaf litter decomposition by combining semi-quantitative proteome analyses with qualitative and quantitative analyses of extracellular degradative enzymes (35). More recently, Herve et al (2014) have used microcosm experiments to show that in lignocellulolytic process of wood decomposition, the interactions between fungi and bacteria are so strong that the fungi, *Phanerochaete chrysosporium* shapes the composition of bacterial communities (36). Several lignocellulose degrading microbial consortia isolated from natural sources that were all capable of degrading lignocellulosic substrate have been summarized in a recent review (37). Despite these findings, most of the studies involving biological

pretreatment of lignocellulosic biomass have marginalized bacterial communities and have remarkably focused only on fungal communities.

Therefore, in an effort to advance our understanding of relative contributions of fungi and bacteria to degradation of lignocellulosic biomass within a synthetic microbial consortium, we developed monocultures and co-cultures of *P. chrysosporium* RP-78 and *Pseudomonas* sp strain YS-1p by growing them alone and together on two different plant biomass and studied their lignin degrading abilities. We monitored their population dynamics throughout their growth and performed qualitative and quantitative assessment of major extracellular enzymes involved in lignin degradation. In addition, we also identified the complete set of enzymes produced by the two groups of microorganism at a certain time point, which are involved in depolymerization of lignin and hydrolysis of cellulose and hemicellulose using proteomics approach. Lastly, we performed Py-GC/MS analysis on plant biomass samples to assess compositional changes in plant biomass caused by microbial pretreatment. Our results show that the two groups faced different interactions towards each other. Bacterial growth increased in co-cultures and so their numbers were higher in the presence of fungi. On the other hand, fungal growth was suppressed in co-cultures and their populations thrived better in their monocultures. We also observed that in co-cultures, fungi produced most of the enzymes involved in degradation of lignocellulose, while bacteria produced lower levels of only few relevant enzymes. Our results from Py-GC/MS analysis revealed that microbial treatment was able to decrease lignin derivatives in plant biomass and alter the composition of guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) units.

CHAPTER II

METHODOLOGY

Microorganisms*: Pseudomonas* sp. strain YS-1p was isolated from a lignin degrading enrichment culture developed from rotten wood collected from a mesothermic pond at the Yellowstone National Park, Wyoming. We have maintained this culture on mineral salts medium (MSM) supplemented with 0.2% alkali lignin (Sigma-Aldrich, Co., St. Louis, MO, USA). The composition of MSM (g/L) is: KH_2PO_4, 0.45; K_2HPO_4, 0.9; $MgSO_4$, 0.5; NH_4Cl, 0.5; KCl, 0.3; $CaCl_2$, 0.2; NaCl, 0.9; 1 ml of trace minerals from stock (38) and 1 ml of vitamin mix from stock (39) as described (40). The genome of strain YS-1p has been sequenced and genome analysis has shown to contain several lignin-degrading genes like laccase, Dyp-peroxidase, beta-etherase, vanillate O-demethylase, feruloyl esterase and chloroperoxidase (41). *Phanerochaete chrysosporium* RP-78 (ATCC®MYA-4764™) was obtained from Fungal Genetics Stock Center (http://www.fgsc.net/). The genome of strain RP-78 has been sequenced and shown to contain several genes that code for hydrolytic and oxidative enzymes involved in wood decay (11). The strain was obtained in frozen ampules, which after thawing was cultivated on YMPG agar plates for sporulation and incubated at 37ºC for 1 week. Spores were harvested by flooding the plates with distilled deionized water (DDI) and scraping the fungal mat off the agar plate with a sterile spatula. The spore suspension

was then filtered through a sterile glass wool to remove hyphal fragments and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet containing spores was resuspended in solution containing 10% lactose and 20% glycerol. This suspension was stored at -80^{o}C until further use.

Plant biomass: Sugarcane bagasse and sorghum bagasse were obtained from OSU Agronomy farms. Each plant biomass was ground in a Thomas Wiley® Mini-Mill (Thomas Scientific, Swedesboro, NJ, USA) by passing through a 10-mesh screen. The plant biomass was washed thoroughly several times to remove soluble sugars and other organic substrates prior to using them in our lignin degradation experiments.

Preparation of bacterial and fungal inocula: Strain YS-1p was grown in MSM containing 1/10th strength of LB broth overnight and then plated on LB plates. The plates were incubated at 30^{o}C for 24 hours and colony forming units (CFU) was determined. Strain RP-78spore inoculum was prepared from cryogenically frozen spore stocks (-80^{o}C) by plating them on YMPG agar plates. The spores were counted using a haemocytometer (Hausser Scientific, Horsham, PA, USA).

Lignin degradation experiments set up: To study lignin degradation, 250-ml capacity Erlenmeyer flasks containing 100 mL of MSM and 0.2% plant biomass were sterilized by autoclaving and inoculated with strain YS-1p and *P. chrysosporium* at 0:1, 1:0, 1:1, 1:10 and 1:50; fungi-to-bacteria ratios, respectively. Table 1 shows different flasks/treatments with initial number of bacteria cells and/or fungal spores. Un-inoculated autoclaved control flasks with MSM and 0.2% plant biomass were set up as well. Flasks were incubated at 30^{o} C on a shaker at 100 rpm in the dark. A well-mixed sample (5 ml) was withdrawn from each flask after 0, 4, 7, 14, and 21 days for enzyme assays and determination of bacterial CFU after which the entire content of the flasks was vacuum filtered (Whatman Filter Papers, 55mm Ø) and the filtrate was stored at -20^{o}C. The recovered plant biomass and fungal mycelia from replicate flasks were composited, oven dried and

stored at -80ºC until used for the determination of fungal 5.8 S – ITS 2 DNA copies using Q-PCR. Also, 10 mg of composited dry biomass was weighed and stored separately at -80ºC for Py-GC/MS analysis.

Table 1. Initial *Pseudomonas* sp. YS-1p and *P. chrysosporium* RP-78 populations used in various treatments

Flask	Fungi-to-Bacteria ratio	Strain RP-78 (spores/mL)	Strain YS-1p (CFU/mL)
B	0:1	0	1×10^5
F	1:0	1×10^5	0
X	1:1	1×10^5	1×10^5
Y	1:10	1×10^5	10×10^5
Z	1:50	1×10^5	50×10^5

Population dynamics of bacteria and fungi: In order to monitor the growth of strain YS-1p during the course of lignin degradation, the culture flasks were vigorously shaken with hand and 5 mL aliquots were taken after 0, 4, 7, 14, and 21 days. The strain YS-1p cell number was determined by serial dilutions and plating on LB agar plates. Plates were incubated overnight at 30ºC after which CFUs were determined.

Because fungal cells cannot be accurately determined by plating method, we amplified 5.8S – ITS 2 gene using Q-PCR. We extracted fungal genomic DNA using the method as described by de Lima et al (42). Briefly, the oven dried biomass samples recovered after 4, 7, 14 and 21 days of incubation and frozen at -80ºC were ground in liquid nitrogen using mortar and pestle. 50 mg samples were then transferred to eppendorf tubes and treated with 600µL of lysis buffer (1% SDS with 50mM EDTA). The tubes were heated at 68ºC for 10 minutes and centrifuged at 13,000×g for 5 minutes at room temperature to separate all cell debris. The aqueous phase was transferred to new tubes. Next, 40 µl of 5M potassium acetate (KOAc, pH 4.8) was added to the tubes and after mixing they

were incubated in ice for 30 minutes. This was followed by another round of micro centrifugation at 14000 rpm for 10 minutes to remove excess SDS, dissolved cellular proteins and single stranded DNA. The supernatant was transferred to new tubes and DNA was precipitated using 95% ethanol (2.5 × Volume of supernatant). The resulting pellet was washed with 70% ethanol. Finally, the tubes were inverted on paper towels for 1 minute to drain excess moisture and the pellet was resuspended in 50ul of TE buffer (10mM Tris at pH 8, 1mM EDTA and 1µg/ml RNase A). The extracted DNA samples were quantified using a nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Delaware, USA) and stored at -20^{o}C until used for Q-PCR analysis.

Roche LightCycler 480 Real Time PCR System (Roche Diagnostics, Indianapolis, USA) was used to perform PCR reactions. Amplification of fungal ribosomal DNA region was performed using universal primers, 5.8S-F (5'-GCATCGATGAAGAACGCAGC-3') and ITS 2-R (5'-TCCTCCGCTTATTGATATGC-3') (43). PCR programming and analyses were performed using LightCycler 480 Software Version 1.5. PCR reaction mixes were prepared using LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Each 10µl PCR reaction mix consisted of 5µl 2X LightCycler 480 SYBR Green I Master mix (composed of FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and $MgCl_2$). 1µl forward and reverse primers (each 300nM), 1.5µl of PCR-grade water and 2.5µl of template DNA. The concentration of DNA was normalized to 25ng in each 384 multi-well plate. The PCR parameters (pre-incubation at 95^{o}C for 5 minutes followed by 40 cycles of amplification: denaturation at 95^{o}C for 30s, annealing at 55^{o}C for 30s and extension at 70^{o}C for 1.5 min, and final extension at 40^{o}C for 10 s) were set according to literature for the source of individual primers (44). The amount of DNA was converted to copy number per mL of culture volume based on molecular weight and size of target DNA (45). The average molecular mass of 1 bp dsDNA was taken to be 660 g/mole. All samples were run in duplicates while the negative controls and standards were run in triplicates.

Spectrophotometric enzyme assays: Aliquots (5 mL) from culture flasks collected after 0, 4, 7, 14 and 21 days were centrifuged at 4650×g for 5 minutes. The supernatants were then transferred to new tubes and used for quantitative enzyme assay for three major lignin degrading enzymes; lignin peroxidase (LiP), dye decolorizing peroxidase (DyP) and laccase. The molar extinction coefficients for these enzymes was recalculated for path lengths measured for 96 well microplate (Grenier, CELLSTAR®). Lip activity was measured as the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 and the change in absorbance was measured at A310 as described by Tien and Kirk (46). One unit enzyme activity corresponds to oxidation of one µmol of veratryl alcohol per minute using the molar extinction coefficient of 7328.4 $M^{-1}cm^{-1}$. For Dyp activity, 2, 6-dimethylphenol was used as the substrate and change in absorbance was measured at A_{469} as described (47). One unit enzyme activity corresponds to oxidation of one µmol of 2, 6-dimethoxyphenol per minute using the molar extinction coefficient of 2167 $M^{-1}cm^{-1}$. Laccase activity was determined by oxidation of 2, 2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) at A_{530} as described (48). One unit enzyme activity corresponds to oxidation of one µmol of syringaldazine per minute using the molar extinction coefficient of 51220 $M^{-1}cm^{-1}$. The values of extinction coefficients for all three enzymes were modified from their original papers due to the change in path length associated with going from 3mL cuvette to a 96 well microplate. The path length was changed from 1cm to 0.788cm, which was calculated based on dimensions of the plate as listed on the product's description by the manufacturer and using those numbers to determine the height of volume used for the miniaturized assays. All assays were performed in triplicate and in 96-well plate using an Infinite M200 microplate reader (Tecan, Mannedorf, Switzerland).

Proteome analysis: Proteins in vacuum filtered culture supernatants were concentrated using Vivaspin 20 filters (GE Healthcare Limited, Buckinghamshire, UK). The concentration of protein was determined using Bradford assay (49). Aliquots containing 20µg of proteins were then run on 12% SDS-PAGE gel (50), stained with Coomassie blue and destained for 30 minutes in 10% acetic

acid solution. Protein lanes were then excised, reduced with 5mM Tris, alkylated with 10mM iodoacetamide and subjected to overnight digestion with 4µg/mL trypsin. The resulting peptide mixtures were extracted using 1% trifluoroacetic acid and analyzed by LC-MS/MS using an LTQ-OrbitrapXL mass spectrophotometer (Thermo Fisher Scientific, MA, USA) coupled to a nanoelectrospray ion source (New Objective PV-550) and a chromatography system (Eksigent NanoLC-2D) as described (51). Mascot search engine (v2.2.2, Matrix Science, Boston, MA, USA) was used for database search against translated protein sequences from *P. chrysosporium* as well as YS-1p genome.

The identified proteins/peptides were validated by Scaffold (v4.0.6.1; Proteome Software Inc., Portland, OR, USA) using the ProteinProphet and PeptideProphet algorithms (52). Protein identifications were accepted only at probability thresholds greater than 99% with a minimum of 2 peptides identified, each with 95% certainty. Proteins containing similar peptides which could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The number of spectral counts generated by samples in different time points were compared as a measure of difference in protein expression between those samples (53).

Growth of bacteria on different carbon sources: In order to know if our bacteria can grow on potential products of lignocellulose degradation, we monitored their growth on six different carbon sources; cellulose microcrystalline, carboxymethyl cellulose, cellobiose, glucose, xylan and xylose. The cultures were inoculated with 1 x 10^5 cells/ml of strain YS-1p in flasks containing 100 mL of mineral salts medium (MSM) supplemented with 0.2% of one of the afore mentioned sugars. The flasks were incubated at 30^{o}C for 21 days. 5 ml aliquots were taken from each flask after 0, 4, 7, 14 and 21 days of growth. Bacterial growth was assessed by counting the number of colony-forming units (CFU) on LB agar plates.

Pyrolysis GC-MS analysis: Py-GC/MS analysis was performed using a Pyroprobe 5200 high pressure micropyrolyzer (CDS Analytical Inc., Oxford, PA, USA) connected to a gas chromatograph/mass spectrophotometer (Agilent 7890GC/5975MS). 0.3mg of biomass samples were packed in a quartz tube (25 mm long and 1.9mm inner diameter) and then held in a platinum-heating coil at a constant heating rate of 1000^{o}C/s. Samples were held at pyrolysis temperature for 90s. The volatile mixture was carried into an adsorbent (Tenax-TATM) trap by helium gas where it was maintained at 40^{o}C. The trap captured all the condensed vapors (bio-oil) while the non-condensable permanent gases were purged out with helium. The adsorbed vapors were evaporated by heating the trap to 300^{o}C and subjected to GC/MS through a heated transfer line for compositional analysis. The vapor components were separated in a DB-5 capillary column (30m long, 0.32mm inner diameter, 0.25μm film thickness). The injector was held at 250^{o}C. The column temperature was maintained at 40^{o}C for 2 minutes and then increased to 280^{o}C at the rate of 5^{o}C/min and held for 10 minutes. Helium (99.99% purity) was used as the carrier gas at the flow rate of 1mL/min. The pyrolysis products were identified by comparing their peaks with National Institute of Standard and Technology (NIST) mass spectral library.

CHAPTER III

FINDINGS

Population dynamics of *Pseudomonas* Strain YS-1p and *P. chrysosporium* RP-78

As seen in Figure 1, bacterial cell numbers increased initially for the first 7 days and 14 days for sugarcane bagasse and sorghum bagasse, respectively after which they started to decline. On the other hand, fungal numbers increased gradually and reached maximum on day 21. Highest number of bacterial cells, 1.95×10^{8} CFU/mL and 3.95×10^{8} CFU/mL were measured for sugarcane bagasse and sorghum bagasse, in co-cultures X (F:B = 1:1) after 7 and 14 days of incubation, respectively.

Fungal growth expressed as copy numbers of 5.8S and ITS2 rDNA region per mL of culture was the highest in flasks inoculated with only fungal spores (F) and reached maximun after 21 days for both the plant biomass. In flasks containing sugarcane bagasse, fungal population increased from initial 1×10^{5} to 1×10^{11} gene copy numbers in 21 days, while in flasks containing sorghum bagassse, the fungi increased to 7.46×10^{10} for the same time period. On the other hand, the fungal growth was significantly supressed in all co-cultrure combinations. No fungal growth occurred in control flasks and in bacteria monoculture flasks.

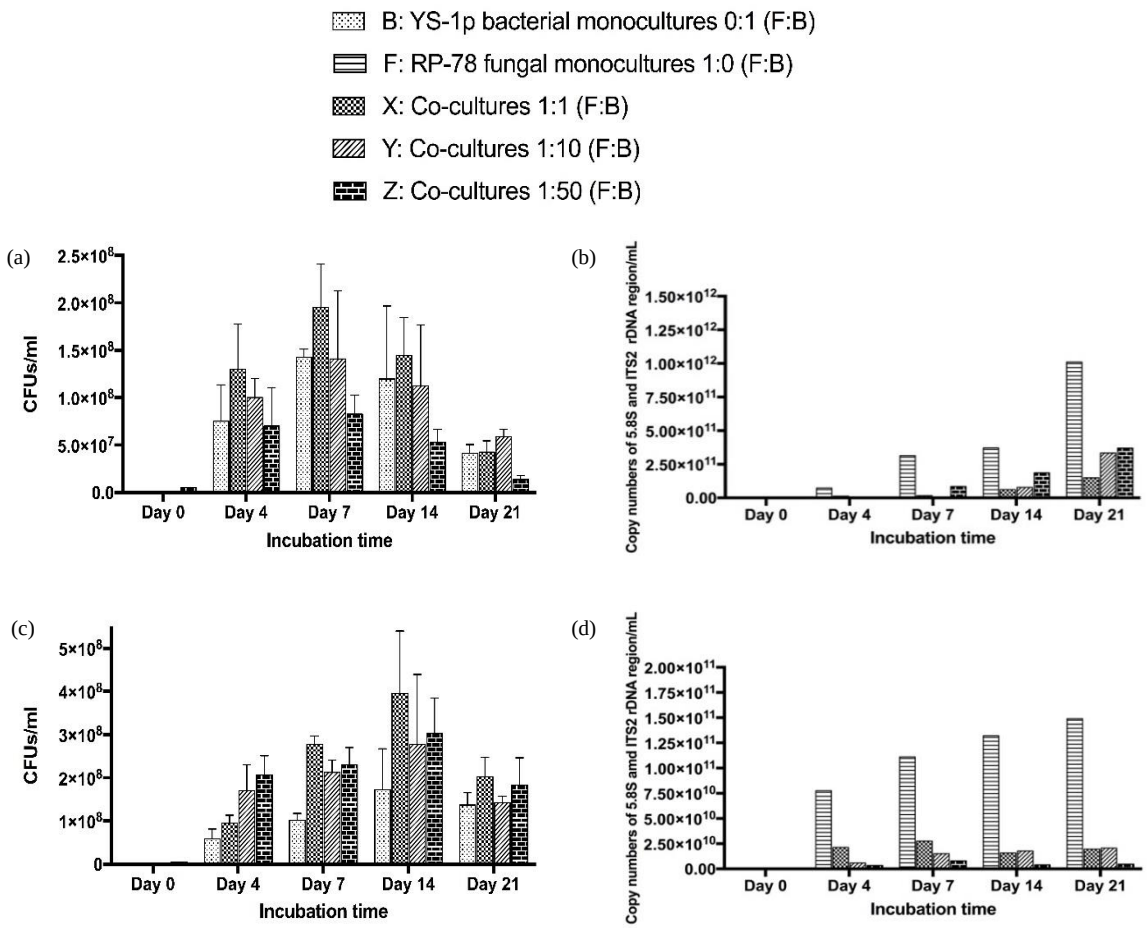
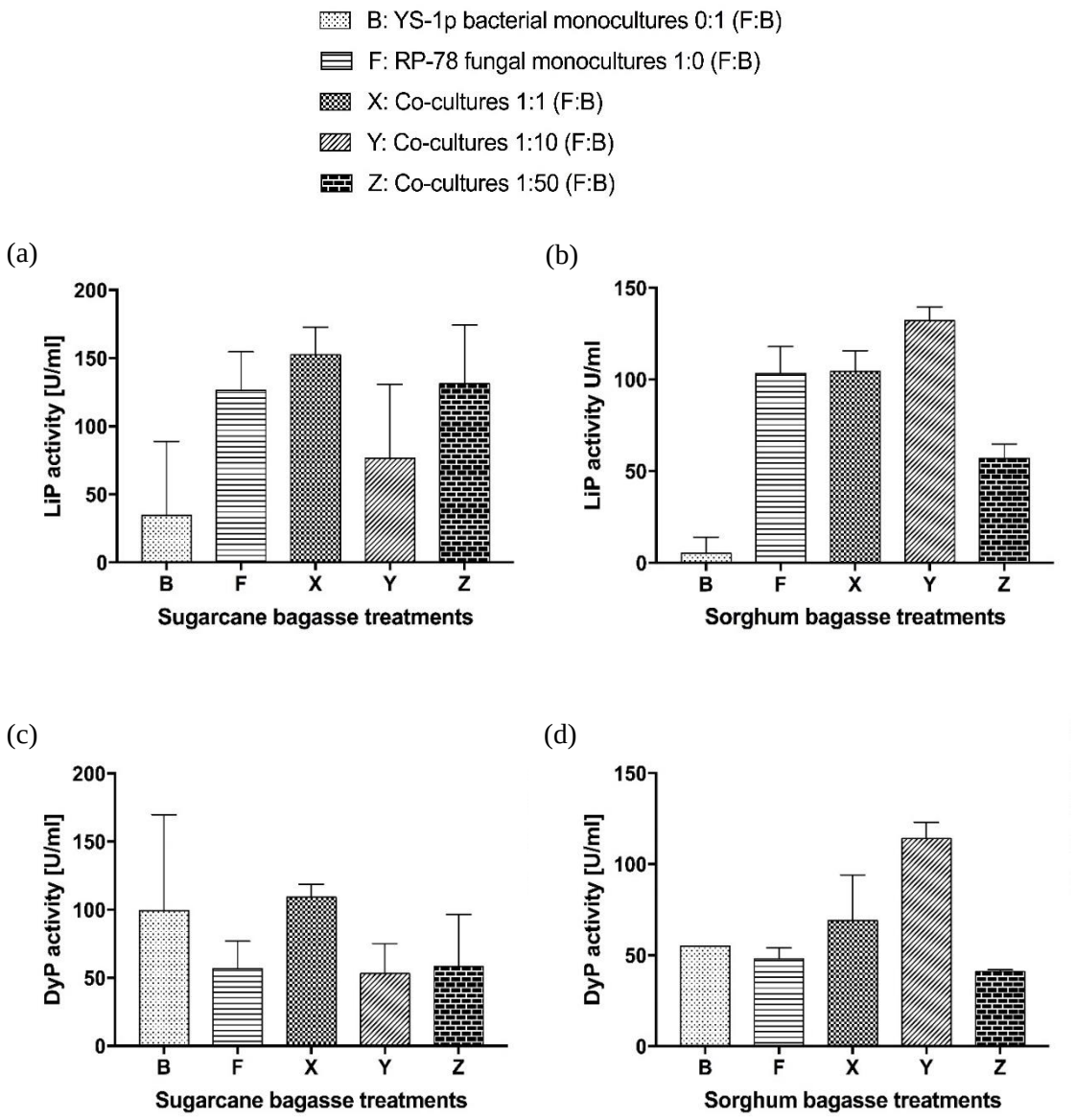


Figure1. Population dynamics of fungi and bacteria when grown as monocultures and co-cultures in flasks with MSM and 0.2% plant biomass. (a) and (b) Growth of *Pseudomonas* sp. YS-1p and *Phanerochaete chrysosporium* RP-78 on sugarcane bagasse cultures, respectively. (c) and (d) Growth of strain YS-1p and strain RP-78 on sorghum bagasse cultures, respectively. Flasks were inoculated with monocultures of strain YS-1p at 1×10^5 cells/mL (B), monocultures of strain RP-78 at 1×10^5 spores/mL (F), and co-cultures of strain YS-1p and strain RP-78 at various ratios including 1:1 (X), 1:10 (Y) and 1:50 (Z) respectively. Aliquots were withdrawn from flasks at different time intervals as described in Methodology section. Growth of strain YS-1p was determined by counting CFU on LB agar plates. Growth of *P. chrysosporium* was measured as copy numbers of 5.8S and ITS2 rDNA region. Data represents means of two independent

experiments. Error bars indicate standard deviation. For assessing fungal growth, biomass samples from two independent experiments were composited for DNA extraction and QPCR analysis.

Activities of extracellular enzymes involved in lignin degradation



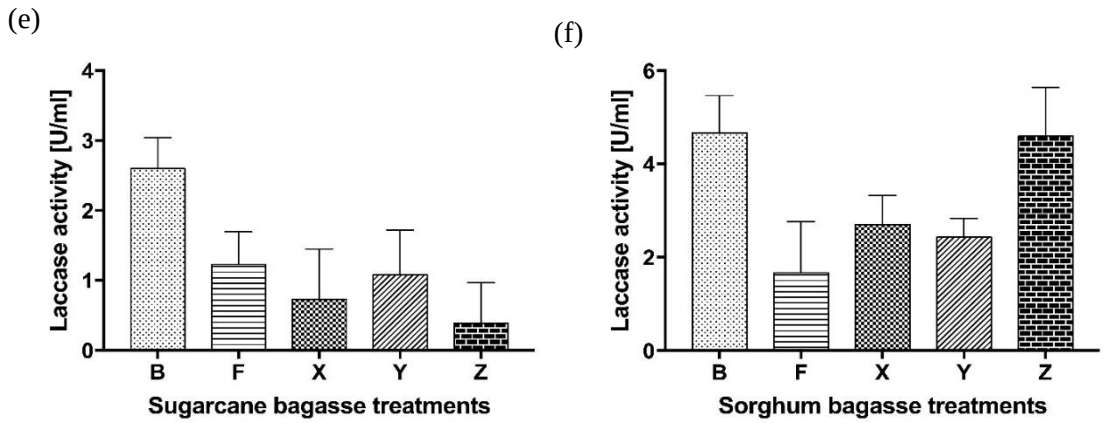


Figure 2. Shown are the highest enzyme activities of LiP, DyP and laccase in samples withdrawn from sugarcane bagasse and sorghum bagasse during 21 days of incubation. (a) and (b) Lignin peroxidase activity observed in culture supernatants of sugarcane bagasse and sorghum bagasse, respectively. The activity was determined by spectrophotometric enzyme assay at A_{310} using veratryl alcohol as the substrate (46). One unit enzyme activity corresponds to oxidation of one µmol of veratryl alcohol to veratraldehyde using the molar extinction coefficient of 7328.4 $M^{-1}cm^{-1}$; (c) and (d) Dyp peroxidase activity observed in culture supernatants of sugarcane bagasse and sorghum bagasse, respectively. The activity was determined by spectrophotometric enzyme assay at A_{469} using 2, 6-dimethoxyphenol as the substrate (47). One unit enzyme activity corresponds to oxidation of one µmol of 2, 6-dimethoxyphenol per minute using the molar extinction coefficient of 2167 $M^{-1}cm^{-1}$. (e) and (f) Laccase activity observed in culture supernatants of sugarcane bagasse and sorghum bagasse, respectively. The activity was determined by spectrophotometric enzyme assay at A_{530} using syringaldazine as the substrate (48). One unit enzyme activity corresponds to oxidation of one µmol of syringaldazine per minute using the molar extinction coefficient of 51220 $M^{-1}cm^{-1}$. Enzyme activities observed in control flasks with sterile medium and plant biomass were subtracted from enzyme activities observed in culture flaks. Data represent means of two independent experiments. Error bars indicate standard deviation.

Analysis showed low Lip activity in flasks inoculated with bacteria only (B) for both sugarcane bagasse and sorghum bagasse for which the activity was recorded at 34 U/mL and 5 U/mL respectively. In comparison to B, fungal monocultures (F) and other co-cultures (X, Y and Z) showed 2-4 folds higher activity for sugarcanes bagasse and 10-25 folds higher activity for sorghum bagasse. The highest enzyme activity of 152 U/mL was observed in co-cultures X (F:B = 1:1) in case of sugarcane bagasse and of 132 U/mL in co-cultures Y (F:B = 1:10) in case of sorghum bagasse, both after 21 days of incubation.

Analysis of DyP activity showed that this enzyme was detected in significant amounts in monocultures (B and F) as well as in co-cultures (X, Y and Z). As observed in LiP analysis, the highest DyP activity was also observed in co-cultures X (F:B = 1:1) at 109 U/mL in case of sugarcane bagasse and in co-cultures Y (F:B = 1:10) at 114 U/mL in case of sorghum bagasse, both after 4 days of incubation.

Analysis of laccase activity showed that this enzyme was detected in lowest amounts in fungal monocultures (F) and in highest amounts in bacterial monocultures (B) for both plant biomass. For sugarcane bagasse, the highest enzyme activity was recorded in B at 2.5 U/mL after 7 days of incubation while for sorghum bagasse it was recorded in B at 4.7 U/mL after 14 days of incubation.

Overall, enzyme assays showed highest LiP and DyP activities in co-cultures X (F:B = 1:1) and co-cultures Y (F:B= 1:10) for sugarcane bagasse and sorghum bagasse, respectively. Laccase activity on other hand was detected to reach maximum value in bacterial monocultures B for both plant biomass.

Proteome analysis

In order to identify secreted extracellular enzymes involved in degradation of plant biomass and to assess individual contribution made by bacteria and fungi in this process, we performed 1-D PAGE-LC-MS/MS analysis on concentrated supernatant samples after 14 days of incubation. Spectral

counts are the total number of tandem spectra assigned to each protein and are representative of relative protein abundance. Previous studies have shown that spectral counts correlate linearly with protein abundance and therefore they can be used as an indicator of the enzyme production rates by respective source organism (54).

A total of 34 fungal proteins and 309 bacterial proteins were identified in the secretome of *P. chrysosporium* RP-78 and *Pseudomonas* sp. YS-1p, respectively, and of these, a total of 31 proteins are related to degradation of lignocellulosic biomass. Table 1 lists the 13 identified proteins related to lignin degradation and Table 2 lists 18 identified proteins related to degradation of cellulose and hemicellulose. Lignin degradation is a complex process that requires multiple enzymes such as peroxidases, laccases and accessory enzymes like aryl oxidases which are involved in generation of hydrogen peroxide required for catalytic activity of peroxidase enzymes. Of the 13 identified proteins (Table 1), 2 alcohol dehydrogenases, 1 chloroperoxidase and 4 peroxidases were produced by strain YS-1p while the rest of the enzymes including 1 alcohol oxidase, 1 cellobiose dehydrogenase, 3 copper radical oxidases and 1 pyranose 2-oxidase- were produced by strain RP-78. Spectral counts showed that fungi produced higher amounts of these enzymes, while bacteria produced lower levels of enzymes. Cellulose hydrolysis requires three different types of enzymes, namely, endoglucanases, cellobiohydrolases and β-glucosidases. Similarly, hydrolysis of hemicellulose also requires multiple enzymes including beta-1, 4-endoxylanases, β-xylosidase, alpha-glucoronidase, alpha-L-arabinofuranosidase and acetyl xylan esterase. All 18 proteins listed in Table 2 are relevant to degradation of cellulose and hemicellulose and are produced by *P. chrysosporium* RP-78. Cellulose degrading enzymes included:

4 endoglucanases, 2 cellobiohydrolases and 3 beta-glucosidases. Similarly, multiple enzymes involved in hemicellulose degradation including 1 acetyl xylan esterase, 1 arabinase, 1 beta-xylosidase, 1 cellobiose dehydrogenase and 2 endo-1, 4-B-xylanase were also detected. Spectral counts showed that the abundance of these proteins was lower in co-cultures than in fungal

monocultures. Overall, bacteria produced enzymes that were mainly involved in lignin degradation while fungi produced enzymes, which were involved in degradation of all three structural components of lignocellulosic biomass- lignin, cellulose and hemicellulose.

Table 2. Identified lignin-degrading proteins and spectral counts in different cultures on Day 14

Identified proteins	Accession number	Source	MW (kDa)	Spectral Counts				
				B	F	X	Y	Z
Alcohol dehydrogenase	Pseudomonas.sp_orf4039	*Pseudomonas* sp. YS-1p	68	453	0	368	336	262
Alcohol dehydrogenase	Pseudomonas.sp_orf2532	*Pseudomonas* sp. YS-1p	44	11	0	6	0	0
Alcohol oxidase	gi|538258879	*P. chrysosporium* RP-78	73	0	365	0	0	0
Cellobiose dehydrogenase	gi|1279638	*P. chrysosporium* RP-78	82	0	447	78	24	26
Chloroperoxidase	Pseudomonas.sp_orf1417	*Pseudomonas* sp. YS-1p	30	10	0	22	20	20
Copper radical oxidase	gi|89113925	*P. chrysosporium* RP-78	68	0	83	0	0	0
Copper radical oxidase	gi|89113933	*P. chrysosporium* RP-78	108	0	56	0	0	0
Copper radical oxidase	gi|90658301	*P. chrysosporium* RP-78	76	0	83	0	0	0
Peroxidase	Pseudomonas.sp_orf3904	*Pseudomonas* sp. YS-1p	17	24	0	26	36	40
Peroxidase	Pseudomonas.sp_orf4519	*Pseudomonas* sp. YS-1p	18	20	0	5	15	13
Peroxidase	Pseudomonas.sp_orf3319	*Pseudomonas* sp. YS-1p	17	6	0	8	3	0
Peroxidase	Pseudomonas.sp_orf3155	*Pseudomonas* sp. YS-1p	17	14	0	12	13	4
Pyranose 2-oxidase	gi|564731201	*P. chrysosporium* RP-78	72	0	676	0	0	0

Table 3. Identified cellulose and hemicellulose degrading proteins and spectral counts in different cultures on Day 14

Identified proteins	Accession number	Source	MW (kDa)	Spectral counts				
				B	F	X	Y	Z
Acetyl xylan esterase	gi|372863982	*P. chrysosporium* RP-78	39	0	75	9	0	0
Arabinanase	gi|394987317	*P. chrysosporium* RP-78	33	0	27	0	0	0
Beta- Glucosidase Bgl1a	gi|145579305	*P. chrysosporium* RP-78	53	0	0	0	0	0
Beta-glucosidase	gi|19352194	*P. chrysosporium* RP-78	83	0	141	7	0	0
Beta-xylosidase	gi|598062278	*P. chrysosporium* RP-78	55	0	180	0	0	0
Cellobiohydrolase Cel7d (Cbh 58)	gi|18158776	*P. chrysosporium* RP-78	46	0	1025	144	60	64
Cellobiohydrolase II	gi|378737110	*P. chrysosporium* RP-78	46	0	84	22	11	10
Cellobiose dehydrogenase	gi|1279638	*P. chrysosporium* RP-78	82	0	271	47	15	16
Cellulose-binding beta-glucosidase	gi|3320413	*P. chrysosporium* RP-78	86	0	93	0	0	0
Endo-1,4-B-xylanase A	gi|12006973	*P. chrysosporium* RP-78	44	0	159	15	0	0
Endo-1,4-B-xylanase B	gi|12006979	*P. chrysosporium* RP-78	30	0	137	14	1	0
Endo-beta-glucanase	gi|241896738	*P. chrysosporium* RP-78	34	0	68	2	0	0
Endoglucanase	gi|58709678	*P. chrysosporium* RP-78	40	0	169	35	4	1
Endoglucanase family 61	gi|21694047	*P. chrysosporium* RP-78	34	0	16	6	0	0
Endoglucanase V-like protein	gi|195547039	*P. chrysosporium* RP-78	21	0	80	1	0	0
Exoglucanase 1	gi|121852	*P. chrysosporium* RP-78	55	0	908	61	20	21
Glycoside hydrolase family 61	gi|370987906	*P. chrysosporium* RP-78	25	0	23	0	0	0
Glycoside hydrolase family 74	gi|162138864	*P. chrysosporium* RP-78	88	0	283	35	5	4

Growth of strain YS-1p on different carbon sources

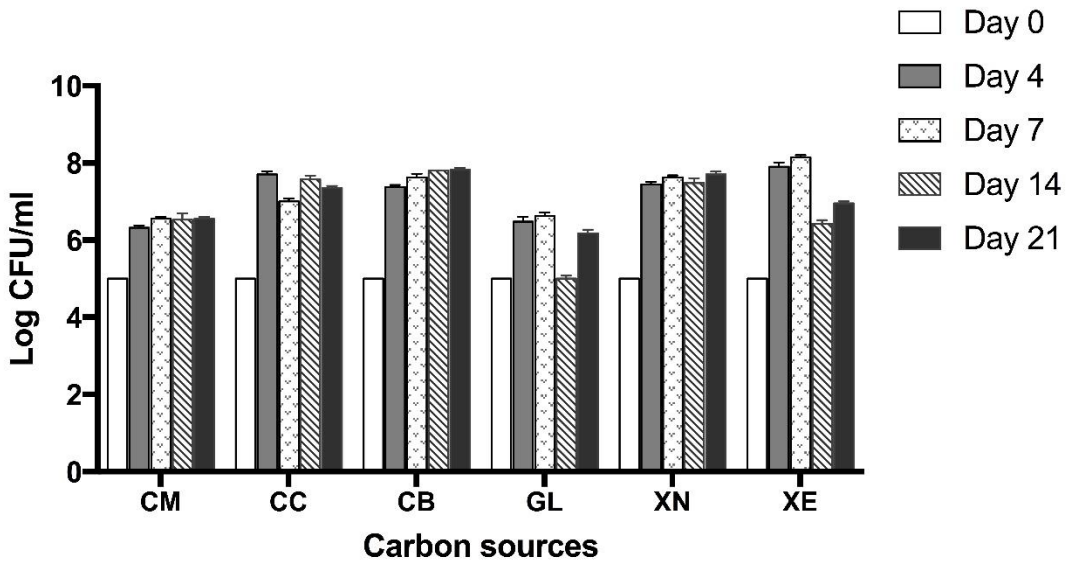
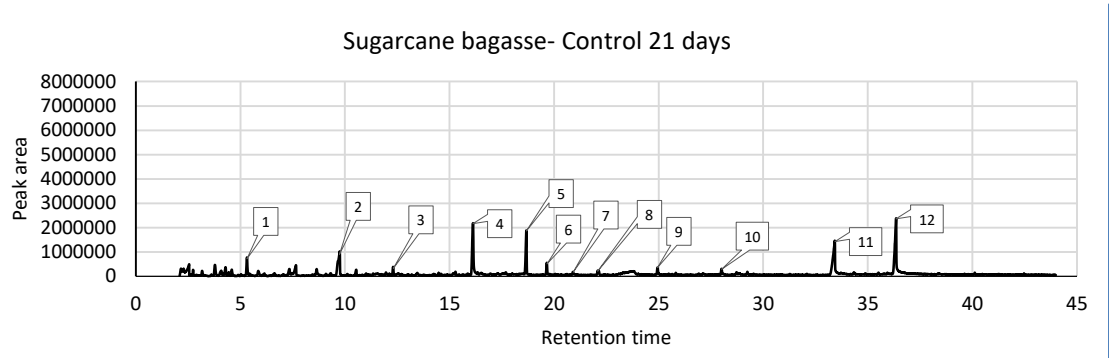


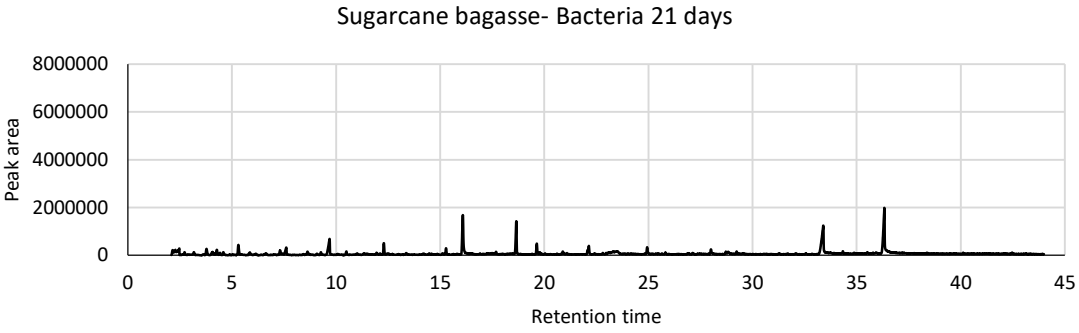
Figure 3. Growth of strain YS-1p on different carbon sources in MSM. (i) CM: Carboxymethyl cellulose; (ii) CC: Cellulose microcrystalline; (iii) CB: Cellobiose; (iv) GL: Glucose; (v) XN: Xylan; (vi) XE: Xylose. Aliquots were withdrawn from flasks at different time intervals as described in Methodology section. Growth was determined by counting CFU on LB agar plates. Data represents means of two independent experiments. Error bars indicate standard deviation.

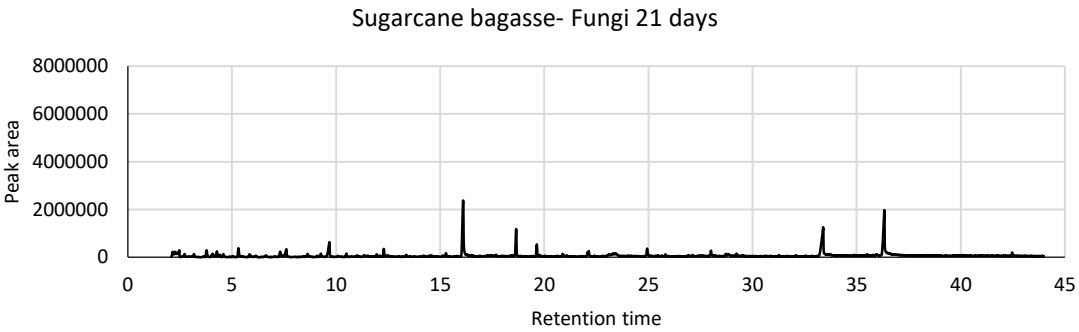
Our results of proteomic analysis showed that *P. chrysosporium* RP-78 produced majority of lignocellulose relevant enzymes in monocultures and in co-cultures, while *Pseudomonas* sp. YS-1p only produced few lignin relevant enzymes and no hemicellulose and cellulose relevant enzymes (Tabel 2 and Table 3). Despite this, the bacteria was able to grow better in co-culture with fungi suggesitng bacteria have benefitted from fungal degradation products. If this is true, then the bacteria should be able to grow on a variety of sugars potentially released due to degradation of lignocellulose. In order to underscore this assumption, we tested the growth of strain YS-1p on different cellulose and hemicellulose carbon substrates. Figure 3 shows the growth of bacteria on

different cellulose and hemicellulsoe substrates. We observed that the bacteria grew well on all the substrates and is able to utilize them as sole source of carbon.

Pyrolysis-GC/MS analysis







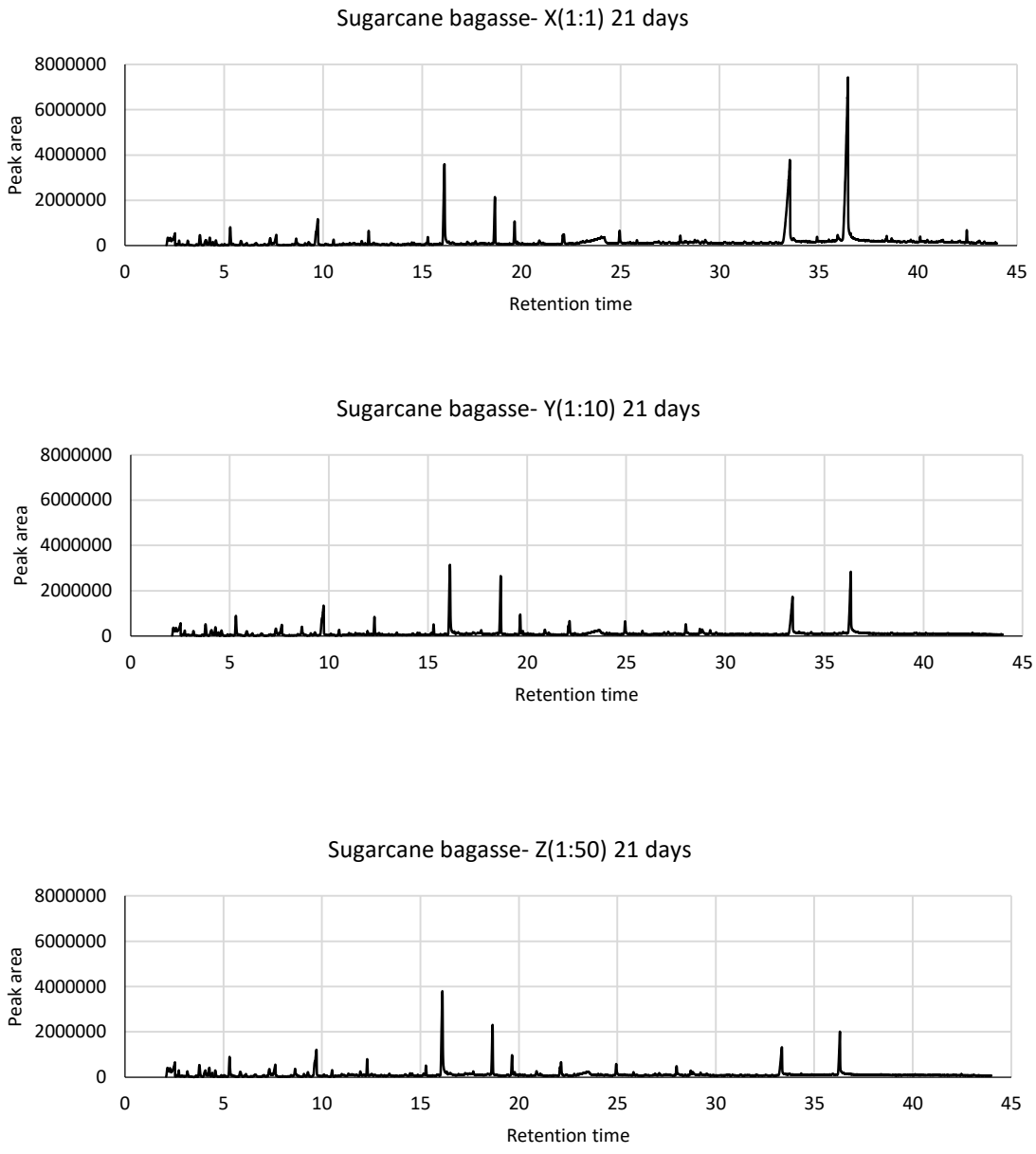
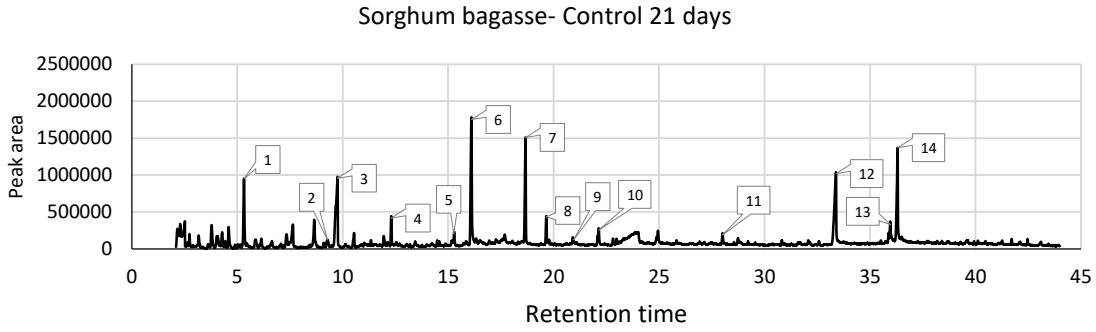
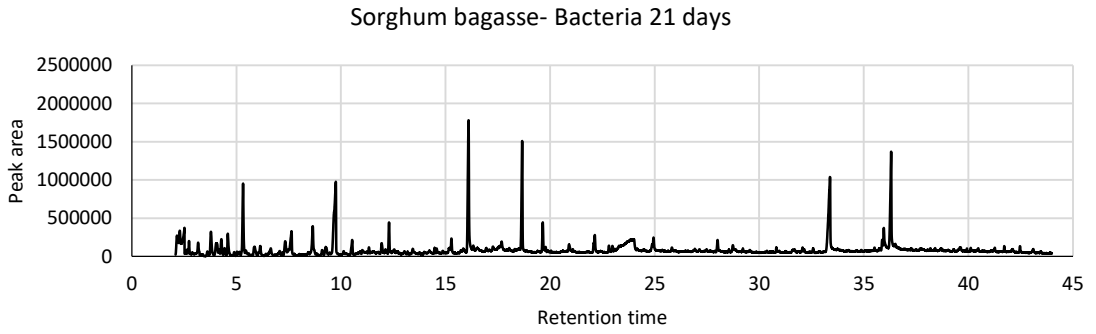
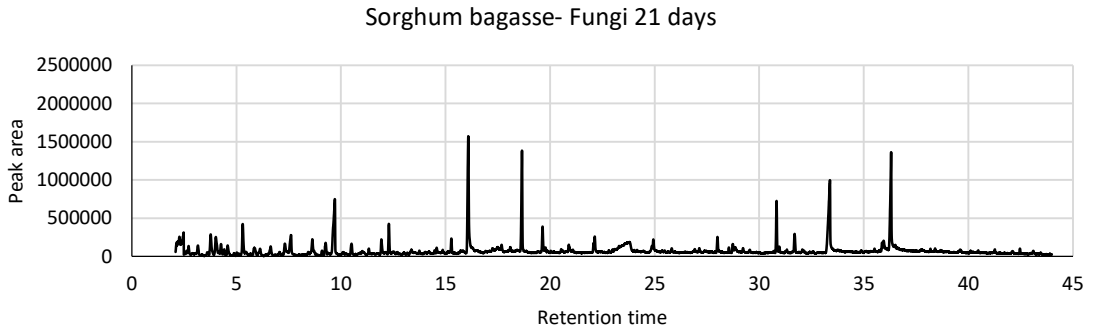


Figure 4. Chromatograms showing pyrolytic products distribution of sugarcane bagasse biomass untreated and treated with strain YS-1p and/or strain RP-78 after 21 days incubation. Main pyrolysis products were detected as: (1) Furfural; (2) 2,4-Dihydro-pyrimidinedione; (3) 2-Methoxyphenol; (4) 2,3-Dihydrobenzofuran; (5) 2-Methoxy-4-vinylphenol; (6) 2,6-Dimethoxyphenol; (7) 3-Hydroxy-4-methoxy-benzaldehyde; (8) 4-Hydroxy-3-methoxy-benzoic

acid; (9) 2,3,5,6-Tetrafluroanisole; (10) 2,6-Dimethoxy-4-(2-propenyl)-phenol; (11) Hexadecanoic acid; (12) Octadecanoic acid







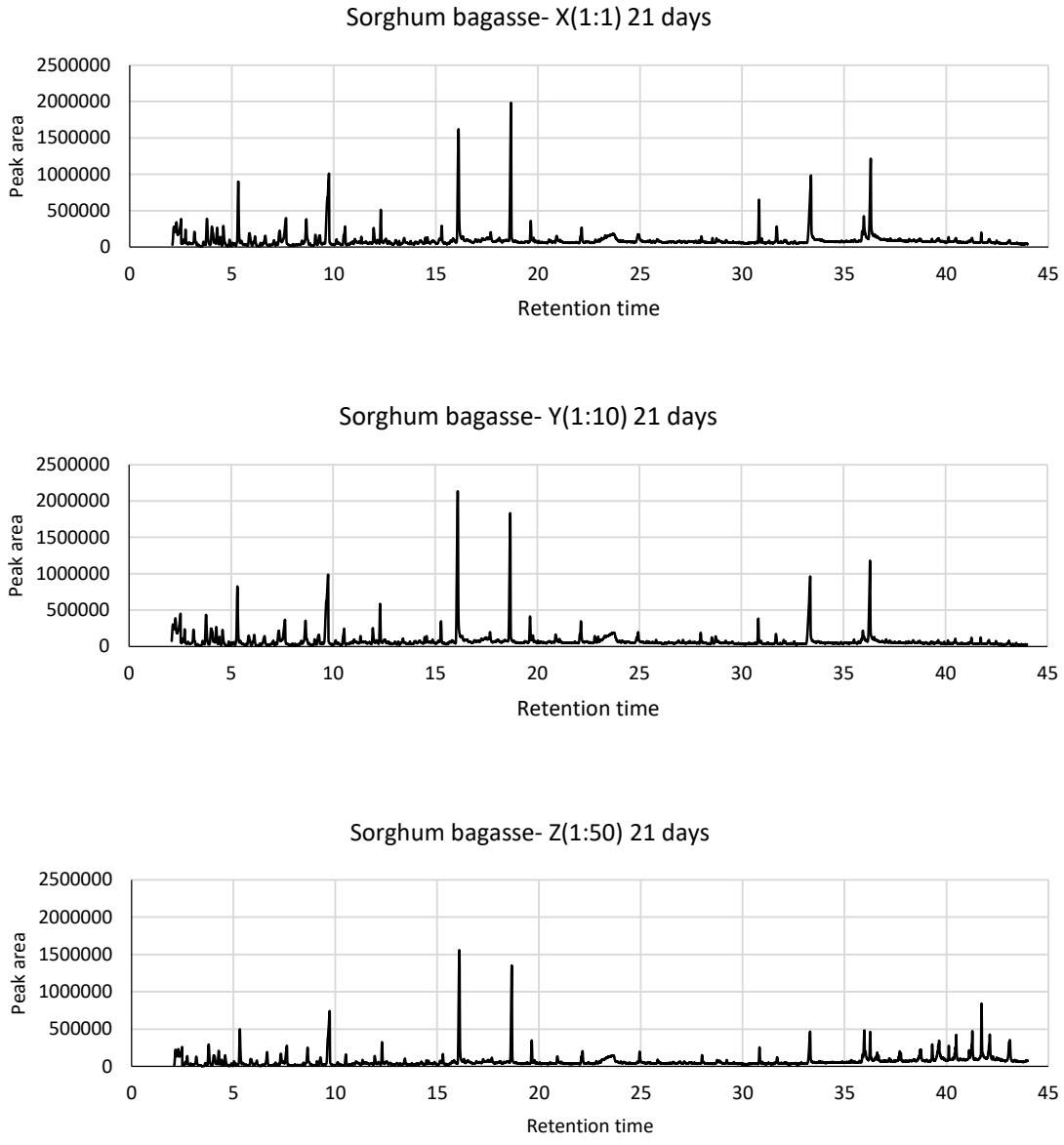


Figure 5. Chromatograms showing pyrolytic products distribution of sugarcane bagasse biomass untreated and treated with strain YS-1p and/or strain RP-78 after 21 days incubation. Main pyrolysis products were detected as: (1) Furfural; (2) Phenol; (3) 2,4-Dihydro-pyrimidinedione; (4) 2-Methoxyphenol; (5) Cresol; (6) 2,3-Dihydrobenzofuran; (7) 2-Methoxy-4-vinylphenol; (8) 2,6-Dimethoxyphenol; (9) Vanillin; (10) Isoeugenol; (11) 2,6-Dimethoxy-4-(2-propenyl)-phenol; (12) Hexadecanoic acid; (13) Vacennic acid; (14) Octadecanoic acid

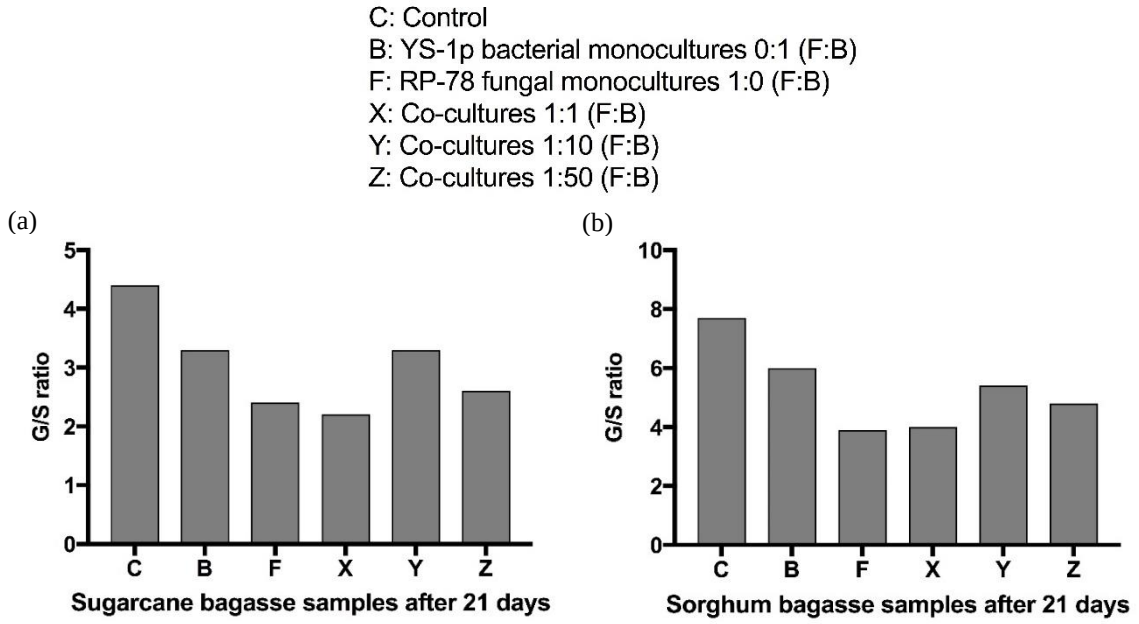


Figure 6. Py-GC/MS peak ratios of 2-Methoxy-4-vinylphenol (G)/2,6-Dimethoxyphenol (S) analyzed for 21 days plant biomass samples of (a) Sugarcane bagasse and (b) Sorghum bagasse

We performed Py-GC/MS analysis of plant biomass at the end of the experiment (21 days) in order to determine compositional changes in plant biomass due to pretreatment with *Pseudomonas* sp. YS-1p and/or *P. chrysosporium* RP-78. Figure 4 and Figure 5 show the Py-GC/MS chromatograms in control and treated sugarcane bagasse and sorghum bagasse, respectively at the end of 21 days. All samples showed the presence of characteristic lignin-derived products. We detected important guaiacyl derivatives like 2-methoxy-phenol, 2-methoxy-4-vinylphenol, 3-hydroxy-4-methoxy-benzaldehyde and 4-hydroxy-3-methoxy-benzoic acid in sugarcane bagasse samples and 2-methoxy-phenol, 4-ethyl-2-methoxy-phenol, 2-methoxy-4-vinyl-phenol, vanillin, 4-hydroxy-3-methoxy-benzoic acid and isoeugenol in sorghum bagasse samples. Similarly, we detected important syringyl derivatives likes 2,6-dimethoxy-phenol and 2,6-dimethoxy-4-(2-propenyl)-

phenol in both sugarcane bagasse and sorghum bagasse samples. Compared to controls, the distribution of pyrolytic products varied in all pretreated samples.

Figure 6 shows the ratio of 2-Methoxy-4-vinyl phenol and 2,6-Dimethoxphenol for different treatments of sugarcane bagasse and sorghum bagasse. 2-Methoxy-4-vinyl phenol and 2,6-Dimethoxyphenol are important derivatives of guaiacyl (G) and syringyl (S) lignin monomers respectively and their ratio, G/S represents compositional changes in plant biomass. The results showed that compared to the controls, this ratio decreased in all plant biomass samples treated with bacteria and/or fungi suggesting pretreated biomass had lower lignin derivatives compared to the untreated biomass.

For sugarcane bagasse, this ratio was lowest for samples in co-culture X in which biomass was inoculated with fungi and bacteria in 1:1 ratio. In this group the G/S ratio decreased from 4.4 to 2.2. This ratio was very comparable to the samples in monocultures F in which biomass was pretreated with fungi alone. The G/S ratio was observed to be 2.4 in this group. For sorghum bagasse, the G/S ratio was lowest in monocultures F in which it decreased from 7.7 to 3.9 and then in co-culture X in which the ratio decreased to 4.0. For both plant biomass, the observed results were consistent in showing that loss of lignin derivatives was highest in plant biomass treated with fungal monocultures F and with co-cultures of fungi and bacteria X.

CHAPTER IV

DISCUSSIONS

Most studies to date involving microbial degradation of lignocellulose have primarily focused on fungi. However, in recent years, the interest has shifted towards bacteria since many bacterial genera have been found to be capable of metabolizing lignin. This infers that both fungi and bacteria play active roles in plant biomass degradation in natural environment. However, studies using both groups as an integrated microbial community in the degradation of lignocellulose is lacking. The main goal of this study was to explore the relative roles of *Pseudomonas* sp. YS-1p and *Phanerochaete chrysosporium* RP-78 in degradation of lignin in sugarcane bagasse and sorghum bagasse.

Our findings show that strain YS-1p grew better in the presence of strain RP-78 on both plant biomass. On the other hand, the growth of strain RP-78 was negatively affected in co-cultures with the bacteria. These findings are consistent with few other reports that have studied interactions between fungi and bacteria during leaf litter decompostion in aquatic model systems (35, 55) as well as in natural soil habitats (56). These studies have shown both synergistic and antagonistic effects of fungi and bacteria on each other's growth. One explanation for this could be that most bacteria are not able to grow efficiently on plant lignin (57).

Our proteome analysis (Table 2 and Table 3) showed that strain YS-1p produced only few lignocellulose-relevant enzymes indicating its limited capability to decompose plant cell wall. On the other hand, strain RP-78 produced many oxidases and hydrolases for lignocellulose degradation. Therefore, it is reasonable assume that bacteria when grown in the presence of fungi, were able to efficiently utilize intermediate of decomposition products released by fungi (58). This is consistent with observed higher bacterial growth in co-cultures (Figure 1). Previous studies have found that bacteria, due to their faster growth rate outcompete fungi for the available organic compounds (35, 59). Gulis et al. had observed delayed fungal sporulation and conidia production in the presence of bacteria which caused antagonistic effect of bacteria on fungal growth (60).

In addition to population dynamics, we also analyzed production of major lignin degrading enzymes by strain YS-1p and strain RP-78 when grown as monocultures or in co-cultures on plant biomass. Enzymes directly affect the transformation of biopolymers into compounds that microorganisms can access and utilize. Therefore such enzyme activity can be linked to degradation efficiency of lignin (61). Genome sequencing of *P. chrysosporium* RP-78 has revealed that it possesses over a dozen different peroxidase genes (ten *lip* genes, five *mnp* genes) but no genes for classical laccase or Dyp-peroxidase (11). Similarly, the genome of Strain YS-1p contains several genes that code for enzymes involved in the degradation of lignin including Dyp-peroxidase, laccase, beta-etherase and chloroperoxidase (41). However, no *liP* (lignin peroxidase) gene was detected in the draft genome. This might be the reason why we detected low LiP activity in flasks inoculated with bacterial monocultures (B) and low laccase and dyp-peroxidase activities in flasks inoculated with fungal monocultures (F) (Figure 2).

As observed in Figure 2, measurement of extracellular lignin degrading enzyme activities for Lip, Dyp and laccase did not show any correlation between population dynamics and enzyme activities. In other words, highest bacterial or fungal populations in monocultures or co-cultures did not

always correspond to the highest enzyme activities. For instance, owing to the lack of *lip* gene in strain YS-1p, we presume

Lip enzyme was only produced by fungi both in monoculture and co-culture conditions. However, as seen in Figure 2, we did not detect maximum Lip activity in fungal monocultures (F) in which their biomass was highest (Figure 1) for both plant biomass. Similarly, Dyp-peroxidase, which was produced only by bacteria, was not detected at maximum levels in those cultures and time points when their populations were highest. Laccase enzyme was detected at low levels (< 6 U/mL) in culture supernatants for both plant biomass. Analysis of laccase activity showed that highest levels of this enzyme was detected in bacterial monocultures (B) and that the enzyme activity decreased in all co-cultures. This indicates that the bacteria did not produce as much enzyme in the presence of fungi as they did when they were growing alone. A possible reason for this could be feedback inhibition of laccase activity by lignin degradation intermediates produced by fungi in co-culture. It is evident that the ability of fungi and bacteria to produce these ligninolytic enzymes was affected when bacteria and fungi were growing together. While production of Lip and Dyp-peroxidase enhanced in co-cultures, laccase activity was observed to decrease. We do not know the exact reason how fungi and bacteria were able to influence their abilities to produce these enzymes.

There have been a few reports on profiling the secretome of basidiomycetes, ascomycetes and bacteria which degrade plant biomass. These studies have identified complete set of lignocellulose degrading enzymes secreted by *Aspergillus nidulans* and *Phanerochaete chrysosporium* growing on sorghum bagasse, *Penicillium echinulatum* growing on sugarcane bagasse and *Erwinia chrysanthemi* growing on *Chrysanthemum* leaves extracts (10, 62-64). But research studying proteomic profiles of multiple microorganisms working together are lacking severely. Schneider et al. were the first to report proteomic analysis of fungi (*Aspergillus nidulans)* and bacteria (*Pectobacterium carotovorum*) growing together to unravel their roles in decomposition of leaf litter (35). In their work they had found that fungi produced most of the extracellular bio-

degradative enzymes such as proteases, cellulases and pectinases regardless of if they were growing alone in monocultures or together with bacteria in co-cultures.

White rot basidiomycete, *P. chrysosporium* is the most extensively studied fungus with sequenced and annotated genome (11, 65). It is known to produce a wide range of enzymes involved in lignocellulose degradation. We identified a total of 34 extracellular proteins in the secretome of the fungi, of which 6 were relevant in lignin degradation and 18 were relevant in degradation of cellulose and hemicellulose (Table 2 and Table 3). Our results show that the fungi produced an extensive collection of hydrolytic and oxidative enzymes to deconstruct lignin, cellulose and hemicellulose. This finding is not surprising owing to the well-known potential of this fungus to degrade lignocellulose. On the other hand, we identified 309 proteins in the secretome of Strain YS-1p, which is almost 10 folds higher than the number of proteins identified in fungal secretome. However, only 7 of them were relevant to degradation of lignocellulose. Abundance of spectral counts revealed that these enzymes were produced by fungi in higher amounts than by bacteria. In addition, we did not detect any cellulose or hemicellulose degrading protein in the bacterial secretome.

It is also worth noting that the abundance of spectral counts for every identified protein was lower in co-cultures, independent of whether it was secreted by bacteria or by fungi. Lower fungal spectral counts in co-cultures could be attributed to lower fungal biomass in co-cultures (Figure 1). However, based on our results, it is not easy to confirm if bacteria, which suppressed fungal growth, directly interfered with fungal secretion of these identified enzymes as well. On the contrary, lower bacterial spectral counts in co-cultures did not correspond to their higher cell numbers in co-cultures. It appears that in co-cultures, bacteria benefitted from fungal machinery to degrade lignocellulose and were able to access products of lignocellulose degradation more rapidly than fungi. This is further supported by our results showing growth of bacteria on different cellulose and hemicellulose substrates which indicates that the bacteria is capable of growing on

lignocellulose degradation products (Figure 3). This might have contributed to their higher cell numbers in co-cultures. Taken together, these results reveal the potential role of fungi as main decomposers while bacteria did not contribute as much to the degradation of plant biomass. In addition, we did not identify any new protein exclusively in secretome of co-cultures. This suggests that neither bacteria nor fungi were triggered to produce new enzymes for lignocellulose degradation when they were growing together.

Finally, our results of Py-GC/MS analysis showed that the distributions of pyrolytic products were highly diverse for plant biomass treated with fungi and/or bacteria than in untreated controls. From this, it is quite evident that microbial treatment of plant biomass physically alters the composition of lignocellulosic biomass, most probably by depolymerizing lignin and hydrolyzing cellulose and hemicellulose. In addition, we also determined ratios of 2-Methoxy-4-vinylphenol (G) to 2,6-Dimethoxyphenol (S) to assess compositional changes of lignin in plant biomass. For both plant biomass, we observed that this G/S ratio was minimum in fungal monocultures (F) and co-cultures inoculated with fungi-bacteria at 1:1 ratio (X). This finding indicated that plant biomass samples in these cultures had lower amount of lignin derivatives in comparison to samples in untreated controls and other cultures. However, we do not know if this corresponds to higher release of fermentable sugars as well.

CHAPTER V

CONCLUSION

Lignin is the second most abundant natural polymer, which is highly recalcitrant to deconstruction. It is strongly intermeshed with cellulose and hemicellulose thereby forming a complex matrix and preventing access to fermentable sugars thus forming a major bottleneck for efficient bioconversion of lignocellulosic biomass into second-generation liquid fuels and other bio-based chemicals. Microbial breakdown of lignin offers a cost-effective way to pretreat plant biomass for efficient downstream applications. However, due to our limited understanding of dynamics and interactions of microbial communities, we still have not been able to engineer and emulate such natural biological systems for applications in biotechnology as much. In this work, we have explored fungi-bacteria interactions when they grow together on plant biomass and investigated their roles in overall process of lignocellulose degradation. Our results showed that the two organisms showed both positive and negative interactions. *Pseudomonas* sp. YS-1p grew better in the presence of *P. chrysosporium* RP-78 than when grew alone. On the other hand, bacteria negatively affected fungi by suppressing fungal growth. In addition, activities of lignin degrading enzymes including lignin peroxidase, Dyp-peroxidase and laccase showed that extent of enzyme activities may not be linked to bacterial cell numbers or fugal biomass. Most importantly, our results shed light on the major role of fungi as decomposers and bacteria as the microbial group which benefitted from fungal

degradation products, at least in our laboratory culture conditions.

This study perhaps is the first to examine fungi-bacteria interactions and their dynamics on lignin degradation; however, additional work is needed to address several limitations posed by our study.

First, we have explored bacterial-fungal interactions by selecting only two groups of model microorganisms while in nature, hundreds of microbial groups work together to accomplish common ecological functions like acquisition of food, growth and survival. Such groups incorporate metabolic sophistication, robustness and stability as their key strengths to perform more efficiently than their individual counterparts. It would be fruitful to explore their interactions and dynamics under conditions of higher microbial diversity by incorporating more microbial groups relevant to lignin degradation. In addition, future research should include proteomic analysis of microbial secretome from multiple time points throughout the study rather than just one. This will be helpful in tracking how protein expression profiles of microbial groups change over time, which can be correlated to microbial mechanisms of degrading lignin and understanding the process of plant cell wall degradation as well.

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VITA

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