ABSTRACT

MATHEWS, STEPHANIE LAMBETH. Biodegradation and Bioconversion of Lignocellulosic Pulping Waste Streams by *Paenibacillus glucanolyticus*. (Under the direction of Dr. Amy Grunden and Dr. Joel Pawlak.)

Lignocellulose is the most abundant renewable carbon source. It makes up the cell wall of plants and contains cellulose, hemicellulose, and lignin. The paper and pulp mill industry uses wood, a lignocellulosic material, as the feedstock for the production of paper. This process requires separation of the secondary plant cell wall components and produces solid fibers primarily composed of cellulose and a liquid stream abundant in lignin and hemicellulosic carbohydrates and waste pulping chemicals (i.e. black liquor). Cellulose fibers have a number of uses including production of paper, textile fibers, protective materials for liquid crystal displays, composites, and can be degraded into sugars for biofuels and chemical precursors. Currently the soluble lignin and hemicellulose fraction is not used for significant commercial applications as it is highly variable, difficult to degrade, and of varying molecular weight. This work sought to isolate bacteria from black liquor and determine the potential of isolates to degrade lignin and black liquor into more useful chemical and starting raw materials. The bacterium, *Paenibacillus glucanolyticus* SLM1, was isolated from black liquor and identified by 16 S rDNA sequencing. Growth curves were used to determine the ability of this bacterium to grow on black liquor and lignocellulose components, and bacterial growth supernatants were analyzed by gas chromatography-mass spectrometry to determine metabolic products of growth on black liquor. Bacterial lignin degradation was further examined with gel permeation chromatography. Next-generation sequencing of *P. glucanolyticus* was also used to identify genes encoding enzymes involved in the degradation of lignocellulose components.

Growth studies indicated that *P. glucanolyticus* SLM1 can grow on black liquor, cellulose, hemicellulose and lignin as the sole carbon source under both aerobic and anaerobic conditions. *P. glucanolyticus* SLM1 produces several organic acids after growth on black liquor under anaerobic conditions including lactic, butyric, propanoic, and succinic acids. The physiology of *P. glucanolyticus* SLM1 was also compared to that of the type strain, *P. glucanolyticus* 5162. Strain SLM1 grows optimally at pH 9 while strain 5162 grows optimally at pH 7. Both strains can grow on black liquor and lignocellulose components as well as on hardwood and softwood flour as the sole carbon source in minimal media. Gel-permeation chromatography revealed that strain SLM1 was able to degrade lignin under both aerobic and anaerobic conditions, while strain 5162 was not able to degrade lignin under aerobic conditions. Genomic sequencing of these strains identified several enzymes that could be involved in the degradation of lignocellulose.

Since few bacteria have been shown to degrade lignin, and no single bacterium has previously been demonstrated to degrade lignin under anaerobic conditions, the results presented here that *P. glucanolyticus* SLM1 could be used to convert lignocellulose and lignocellulosic industrial waste into valuable chemical building blocks and biofuels represent a significant advance.

Biodegradation and Bioconversion of Pulping Waste Streams
by *Paenibacillus glucanolyticus*

by
Stephanie Lambeth Mathews

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DEDICATION

To my husband, Colton, for his never ending patience, understanding and, support.

To Dr. Ann Matthysse for her invaluable mentorship and directing my path towards graduate school.

BIOGRAPHY

Stephanie Lambeth Mathews was born in 1989. She spent much of her childhood with her twin sister exploring the living things on their grandfather's farm in Winston-Salem, North Carolina. Stephanie received her bachelor's degree at the University of North Carolina, Chapel Hill in 2010. In addition to meeting her husband here, Stephanie also began working in Dr. Ann Matthysse's lab as a work study student in 2008. She diligently washed the dishes and made media until she was given the opportunity to begin her own research project: identification of the genes in *Agrobacterium tumefaciens* which encode plant cell wall degrading enzymes. In addition to learning more about microbiology and the process of research, she was given the opportunity to teach other students. With persuasion from Dr. Matthysse, Stephanie entered the Ph. D. program in Microbiology and Forest Biomaterials at North Carolina State University working with Dr. Amy Grunden and Dr. Joel Pawlak. In this program she was given the opportunity to work on a uniquely collaborative project isolating a bacterium from the pulp mill waste. Stephanie also purified and characterized an extremophilic endoglucanase from *Sulfolobus solfataricus*, assisted with the growth and structural characterization of bacterial cellulose, and began preliminary work isolating lignocellulose degrading bacteria from insects. She participated in the Preparing the Professoriate Program, Microbiology Graduate Student Association and, University Graduate Student Association. Stephanie continues to be fascinated with all of the things microorganisms can do and plans to continue research and teaching.

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Preface

This manuscript outlines the research performed by Stephanie L. Mathews with guidance from her advisors, Dr. Amy Grunden and Dr. Joel Pawlak for the completion of her Ph. D. dissertation. Stephanie completed this degree with majors in Microbiology and Forest Biomaterials. As such, the research is an interdisciplinary study combining both fields in the characterization of the ability of a bacterium to grow on and degrade lignocellulosic components. The works first began with a challenge: determine if bacteria are present in black liquor. Black liquor is an industrial waste produced by the paper and pulp industry. It is characterized by high pH, dark color, and an abundance of lignin: a recalcitrant aromatic compound. This environment was believed to be inhospitable to microorganisms because of the process used to produce the black liquor: high heat and use of sodium hydroxide and sodium sulfide, as well as the components of black liquor which include several aromatic compounds and hydroxide radicals produced as a result of thermochemical lignin degradation.

Paenibacillus glucanolyticus SLM1 was isolated from a storage tank of black liquor present in the pulping laboratory at North Carolina State University. The bacterium was characterized using growth studies, gas chromatography mass spectrometry, gel permeation chromatography, and genomic sequencing. This research identified a bacterium which could be used to transform the industrial waste black liquor into valuable chemical building blocks through the use of enzymes which break down the components of lignocellulose: cellulose, hemicellulose and lignin.

CHAPTER 1

Literature Review

Bacterial Biodegradation and Bioconversion of Industrial Lignocellulosic Streams

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Abstract

Lignocellulose is a term for plant materials that are composed of matrices of cellulose, hemicellulose, and lignin. Lignocellulose is a renewable feedstock for many industries. Lignocellulosic materials are used for the production of paper, fuels, and chemicals. Typically, industry focuses on transforming the polysaccharides present in lignocellulose into products resulting in the incomplete use of this resource. The materials that are not completely used make up the underutilized streams of materials that contain cellulose, hemicellulose, and lignin. These underutilized streams have potential for conversion into valuable products. Treatment of these lignocellulosic streams with bacteria, which specifically degrade lignocellulose through the action of enzymes, offers a low energy and low cost method for biodegradation and bioconversion. This review describes lignocellulosic streams and summarizes different aspects of biological treatments including the bacteria isolated from lignocellulose containing environments and enzymes which may be used for bioconversion. The chemicals produced during bioconversion can be used for a variety of products including adhesives, plastics, resins, food-additives and petrochemical replacements.

Keywords: bacteria, biodegradation, cellulose, hemicellulose, lignin, and lignocellulose

1.1 Lignocellulose

Lignocellulose refers to plant material that is predominantly composed of the plant cell wall. The plant cell wall is a heterogeneous complex of cellulose, hemicellulose, and lignin (Mosier et al. 2005). Lignocellulose also contains other structural polymers including waxes and proteins (Malherbe and Cloete 2002). Lignocellulosic polymers are the most abundant biopolymer on the earth (Emtiazi et al. 2007). Many industries use lignocellulose as a raw material for the production of a variety of products including pulp, paper, chemicals, and fuels. Use of lignocellulose is advantageous in that it is not a significant human food source and thus industrial use does not conflict with agricultural use (Durre 2007; Kumar et al. 2008). Many industrial processes that use lignocellulose only use a portion of the biomass resulting in an underutilized lignocellulose stream. These streams have great potential for the production of energy and chemicals. Lignocellulose can be converted to fuels, chemicals, and other renewable materials using a number of different types of processing. Thermal, mechanical, chemical, and biological processes are the main types of conversion technologies that are currently being used in lignocellulosic industries. Thermal, mechanical, and chemical conversion is extensively reviewed in Bridgwater (2006), in Sun and Cheng (2002), and in Hendriks and Zeeman (2009). Biological treatment uses microorganisms for the degradation and conversion of a material. This article will focus on biological conversion of lignocellulose streams because it offers a method by which specific products can be produced with minimal energy input.

The plant cell wall composition differs between types of plants. Table 1-1 lists the typical chemical composition of non-woody plants (grasses) and woody plants (hardwood

Table 1-1

Composition of lignocellulosic materials (Betts et al. 1997; Malherbe and Cloete 2002)

Raw Material	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Hardwoods	18-25	45-55	24-40
Softwoods	25-35	45-50	25-35
Grasses	10-30	25-40	25-50

and softwood). Softwood typically contains the highest percent lignin, while hardwood and grasses typically contain higher percentages of polysaccharides. Both hardwoods and softwoods have a similar secondary wall structure. Wood cell wall structure is shown in Figure 1-1A. The wood cell wall is made up of a middle lamella and a primary and secondary cell wall. The middle lamella is thin but is a lignin-rich layer which connects the wood cells. The middle lamella contains the highest concentration of lignin. The primary cell wall contains cellulose, hemicellulose and pectin (Sticklen 2008). The secondary cell wall (Figure 1-1B) can be divided into three separate parts (S1, S2, S3) based on the structure and composition of the polymers present (Wiedenhoeft 2013). While the exact chemical analysis of each secondary cell wall layer has not been determined for all wood cell wall types, typically the S1 layer has the greatest concentration of lignin in the secondary cell wall while the S2 layer contains more lignin than the S1 layer because of the S2 layer thickness (Daniel 2009). The S2 layer also contains the greatest amount of cellulose and hemicellulose (Daniel 2009; Panshin and de Zeeuw 1980). Cellulose chains are linked together in the secondary cell wall by hydrogen bonding forming several parallel sheets that make up the microfibril

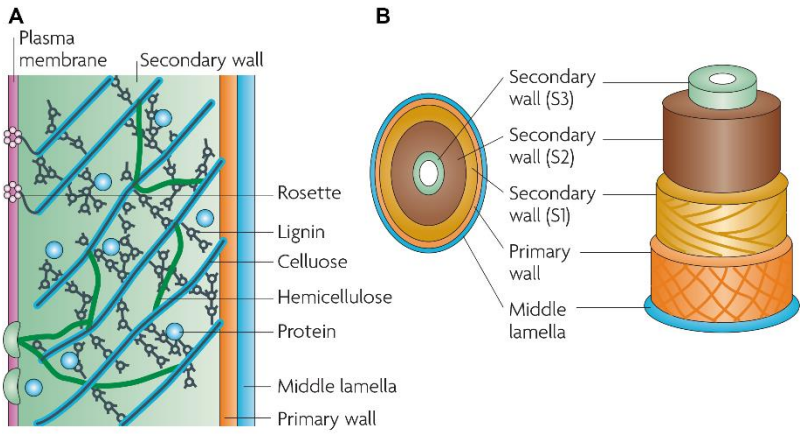

Figure 1-1 Structure of the wood cell wall. A. Structure of a wood cell wall made up of the middle lamella, primary cell wall, and the secondary cell wall (Sticklen 2008). B. Structure of the secondary cell wall containing cellulose fibers bound to lignin and hemicellulose (Sticklen 2008). Figure 1 was reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews] (Sticklen 2008).

structure (Beguin and Aubert 1994). The S3 layer forms the boundary of the lamella and has the least amount of lignin.

1.1.1 Cellulose. Cellulose is a linear polymer of 500-15,000 glucose units that are linked by β-1,4 bonds (Figure 1-2A). It makes up 45% of the dry weight of biomass (Perez et al. 2002). Compact fibrils of cellulose are formed by hydrogen bonds which link chains of cellulose into fiber strands. The cellulose structure within the polymer can differ. Crystalline cellulose refers to the organized and compact fibrils while amorphous cellulose is not organized in this manner (Perez et al. 2002). The structural differences between these types of cellulose impact the degradation of the polymer as crystalline cellulose is less susceptible to degradation, especially by the action of enzymes. While native structure and arrangement of cellulose is a debated topic, recent work by Graham et al. (2013) suggests that organization of crystalline and amorphous regions can be disrupted by sample preparation (base, proteinase K, and heat) (O'Sullivan 1997). Thus, cellulose fibers encountered in industrial applications are typically altered from their native structure. Cellulose fibers are used in the paper and pulp mill and for the production of textiles. Cellulose has also been used for the production of ethanol, lactic acid, furfural, and other chemicals (Reddy and Yang 2005). Cellulose derivatives like cellulose acetate have been used to produce films and membranes (Fischer et al. 2008).

1.1.2 Hemicellulose. Hemicellulose is the second most abundant renewable biomass (Kumar et al. 2008). Hemicellulose is composed of several sugars: xylan, mannan, galactan, and arabinan. Xylan is the major component of hemicellulose and is linked with β-1,4 glycosidic bonds (Figure 1-2B) (Collins et al. 2005). Xylan makes up 25-50% of the total dry

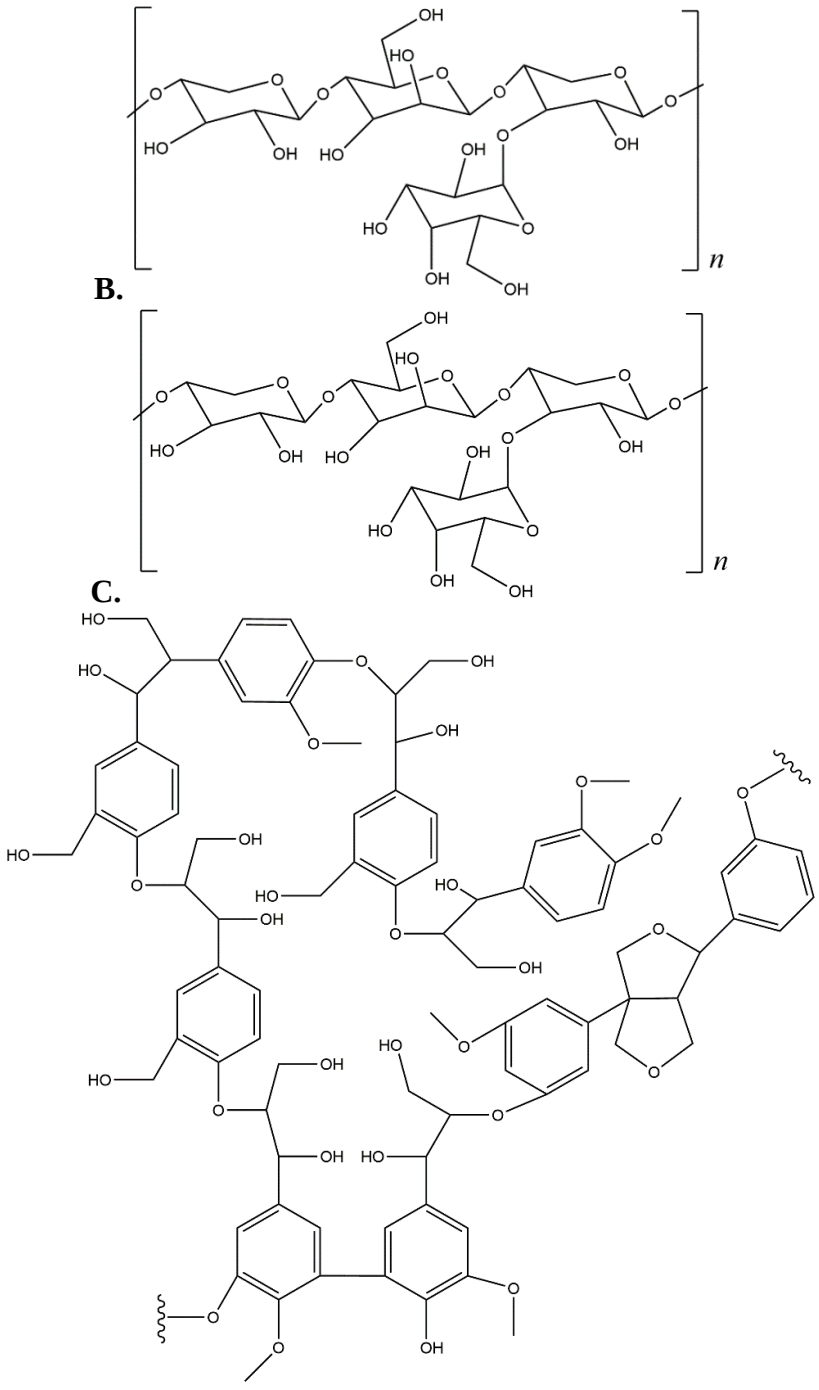

Figure 1-2 Chemical structure of lignocellulose components. A. Structure of cellulose. B. Structure of hemicellulose. C. Structure of lignin (modified from Perez et al. 2002)

weight in non-woody grasses, 15-30% in hardwood species and 7-10% in softwood species (Wong et al. 1988). The other five-carbon sugars associated with hemicellulose are connected to xylan by various types of linkages including α-1,2 and α-1,3 bonds. Hemicellulose is linked to cellulose in the plant cell wall by hydrogen bonding and linked to lignin by ionic interactions (Beg et al. 2001). Hemicellulose is used for production of ethanol and lactic acid (Reddy and Yang 2005; Saha 2003).

1.1.3 Lignin. Lignin is an amorphous highly branched polymer present in vascular plants and accounts for up to 30% of the dry weight of the plant biomass (Bugg et al. 2011b). It is also the most common naturally occurring aromatic polymer (Pandey and Kim 2010). Lignin is present in the soil as a result of plant decay. In the plant cell wall, lignin provides structural support and resistance against microbial attack and oxidative stress (Crawford and Crawford 1976). The lignin polymer is composed of phenylpropane units (Perez et al. 2002). There are three types of monolignols which polymerize to form the lignin polymer: coniferyl alcohol with an aryl-OCH3 group (known as guaiacyl), sinapyl alcohol with two aryl-OCH3 groups (syringyl), and p-coumaryl alcohol with no OCH_3 groups (p-hydroxyphenyl) (Crawford 1981; Chakar and Ragauskas 2004; Bugg et al. 2011a). These phenylpropane units are linked by β-aryl ether, di-aryl propane, biphenyl, diaryl ether, phenylcoumarane, spirodienone, and pinoresinol bonds. Lignin dimers are then polymerized to form the lignin polymer which is shown in Figure 1-2C. The composition of lignin differs between plants in respect to the types and amounts of monolignols present. Softwood lignin is predominantly composed of coniferyl alcohols (80%), while hardwoods are 56% coniferyl alcohols and 40% sinapyl alcohols. Grass lignin contains more p-coumaryl alcohol than softwoods and

hardwoods (up to 10%) and equal amounts of coniferyl and sinapyl alcohols (Bugg et al. 2011a; Dhillon et al. 2012). Lignin present in wood is normally burned or slowly converted into organic matter in the soil by microbiological and climatological agents (Gottlieb and Pelczar 1951). Lignin has also been used in the production of adhesives, phenolic compounds, and food additives (Reddy and Yang 2005).

1.2 Industrial Waste Streams

Lignocellulosic streams are produced as a result of those industries which use lignocellulosic biomass as the feedstock. These industrial processes utilize cellulose and hemicellulose for the production of pulp and paper or degrade these polysaccharides into sugars to produce fuel. Before using cellulose and hemicellulose, these industries employ treatments designed to remove lignin from the biomass. These pretreatments require energy and chemical input resulting in increased manufacturing cost; however, pretreatment is necessary to ensure the quality of these products made from polysaccharides. For paper production, the presence of lignin leads to a lower quality paper. Lignin decreases paper strength and can lead to decreased brightness. For fuel production, lignin prevents access of the carbohydrate degrading enzymes from binding which limits product yield (Malherbe and Cloete 2002). The pretreatment process enhances the ability to degrade lignocellulose. There are many types of pretreatment strategies which use chemicals, heat, or mechanical force to separate these plant cell wall components and have been reviewed in Hendriks and Zeeman (2009) and Zakzeski et al. (2010). Mechanical pretreatment decreases particle size and the degree of polymerization which increases the surface area for enzymatic or chemical

reactions. Thermal treatments solubilize cellulose, hemicellulose and lignin while also resulting in hydrolysis of hemicellulose. Acid pretreatments result in the solubilization of hemicellulose and the precipitation of lignin. Alkaline pretreatment solubilizes lignin. After pretreatment, the desired components can be isolated by screening or filtering. In the pulp mill, lignin is solubilized by alkaline pretreatments and the polysaccharides are collected by screening the resulting slurry. The lignin rich liquid stream is separated from the polysaccharide-rich solids. Therefore, lignin-containing lignocellulose streams are commonly produced as a byproduct of industries which use lignocellulose as the feedstock. Lignocellulose residues have been produced from wood, grasses, and a variety of agricultural residues (corn stover, wheat straw), forest residues (sawdust, mill waste) and municipal waste (Sanchez 2009).

The major producer of industrial lignocellulose streams is the pulp and paper industry. Figure 1-3 depicts the process diagram for a typical kraft pulping operation. The objective of any chemical pulping process is to remove lignin and separate cellulosic fibers, as a result a lignocellulose stream is produced (Chakar and Ragauskas 2004). The primary chemical pulping method is the sulfate process. The sulfate process was developed by C. F. Dahl who found that sulfide accelerated delignification of lignocellulose for the production of a stronger pulp (Smook 1992). This process was patented in 1884 and has since become the most widely used pulping technique worldwide (Zakeski et al. 2010). This process, commonly referred to as kraft pulping, uses chemicals (sodium hydroxide and sodium sulfide), heat (up to 180˚C), and high pH (10-14) to treat wood chips for up to two hours for the production of pulp (Zakeski et al. 2010). During kraft cooking it is estimated that 80% of

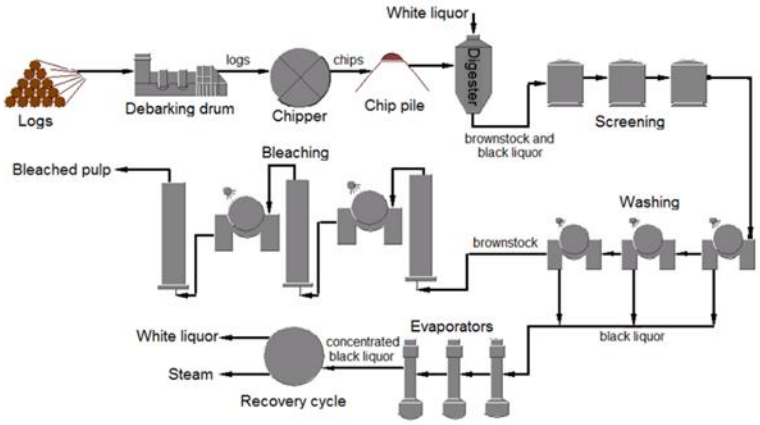

Figure 1-3 Process diagram of pulp paper industry lignocellulose stream production.

the lignin, 50% of the hemicellulose and 10% of the cellulose and 90% of the extractives are dissolved and present in the black liquor (Smook 1992). Aryl-alkyl bonds are cleaved and side chains are modified as hydroxide and hydrosulfide react with lignin (Chandra et al. 2008; Chakar and Ragauskas 2004). This results in the production of smaller water and alkali-soluble lignin fragments. Kraft lignin is considered to be highly irregular, making it difficult for many organisms to degrade due to the variety of compounds generated by the non-specific chemical reactions of the kraft process.

Black liquor produced by the pulp and paper mill is a dark colored alkaline liquid waste containing lignin, cellulose, hemicellulose, phenolics, resins, fatty acids and tannins (Chandra and Abhishek 2011). After cooking, the pulp is washed to separate any residual liquor during a phase known as brownstock washing. Black liquor is generally “disposed of”

by burning in the recovery boiler to create steam energy and recover chemicals used in pulping. Because the recovery boiler is a major capital expense of a mill, increasing paper production (and therefore black liquor) is typically limited by the capacity of the recovery boiler. An alternative or additional use of black liquor would benefit the pulp and paper industry. Pulp and paper mills are the largest producers of lignin as an industrial byproduct generating between 40 and 50 million tons of lignin each year as a result of the pulping process (Zakeski et al. 2010). Mead Westvaco and Metso pulp and paper companies commercially produce kraft lignin as a dried powder (Zakeski et al. 2010). Currently, only 2% of the lignin produced is used in valuable industrial processes, the rest is burned to generate energy and to recover chemicals (Gosselink et al. 2004; Zakeski et al. 2010).

In addition to black liquor, the pulp and paper industry generates a large amount of wastewater from brownstock washing and bleaching. This wastewater contains lignin, cellulose, phenolics, resins, fatty acids, tannins, chlorine and sulfates (Pokhrel and Viraraghavan 2004; Santos et al. 2009). Effluents are also characterized by dark colorization, suspended solids, and high pH. These characteristics make pulp and paper mill effluent toxic to zooplankton and fish and carcinogenic if not properly treated for disposal (Santos et al. 2009). Resin acids which result from pulping pine, spruce, and fir trees are particularly toxic to fish. A large portion of the resin acids are removed by the recovery boiler, but Liss et al. (1997) have detected toxic levels in pulp mill effluents ranging from 1000 to 50 ppm. Resin acids are difficult to degrade because a number of structural changes (isomerization, dehydrogenation, oxidation and chlorination) occur abiotically. However some microorganisms have been isolated from pulp effluents which are capable of growth on (and

degradation of) resin acids (Liss et al. 1997). Pulp and paper mill wastewater is treated to reduce toxic environmental effects by removing suspended solids (bark particles and fiber debris) with screening, enzymatic treatment, chemical oxidation, or with the addition of microorganisms to remove phenolics (Pokhrel and Viraraghavan 2004).

The textile industry also produces wastewater which contains toxic compounds like azo dyes and could be treated with lignocellulose-degrading bacteria. Azo dyes make up half of the dyes which are produced annually and are characterized by a nitrogen-nitrogen double bond (Stolz 2001). Bacterial treatment of textiles wastes would be beneficial over fungal treatment because the dyes contained in these effluents are highly variable and bacteria may continue to actively produce enzymes which degrade these dyes without great specificity for growth conditions or dye substrate (Anwar et al. 2014; Stolz 2001).

Environmental sources may also contain lignocellulose. While not the focus of this review, biological treatment of water, soil, and compost piles requires bacteria that produce many of the same enzymes as those used for treating these industrial lignocellulosic streams. During composting, microorganisms transform organic matter including plant material or plant-derived material containing cellulose, hemicellulose, and lignin (Tuomela et al. 2000; Lopez-Gonzalez et al. 2014).

While there are many sources of lignocellulosic streams lignocellulose pretreatment streams like black liquor, industrial wastewater from pulping and textiles industries, and compost piles vary widely in composition and structure. This is due to the varied composition of biomass used (softwood, hardwood, or grasses) and also due to the different pretreatments used. Industrial lignin sources are vastly modified as these processes use heat,

pressure and/or chemicals to separate lignin from cellulose and hemicellulose (Crawford 1981).

1.3 Lignocellulose Degrading Fungi

The biological treatment of lignocellulose streams has focused on a few genera of white rot fungi which produce enzymes that degrade lignin and phenolics present in this waste by non-specific degradation, and the most widely studied lignocellulose degrading microorganism is the white-rot fungus *Phanerochaete chrysosporium* (Leonowicz et al. 1999; Chandra and Abhishek 2011). Fungi are the most rapid degraders of lignocellulose, for this reason they have been most widely used for industrial waste treatment and the production of lignocellulose degrading enzymes (Sanchez 2009). *P. chrysosporium, Trichoderma reesei, Pleurotus ostreatus, Pycnoporus cinnabarinus,* and *Sporotrichum pulverulentum* have been used for biological treatment of lignocellulosic material or used to produce commercial enzymes for lignocellulose degradation (Sanchez 2009; Sun and Cheng 2002). Fungal lignocellulose degradation occurs through the action of extracellular enzymes which include polysaccharide degrading enzymes and oxidative lignin degrading enzymes. These fungal enzymes have a higher rate of activity than characterized bacterial enzymes but are typically not very active under different environmental conditions like high pH, oxygen limitation or high lignin concentration which is characteristic of these lignocellulose streams (Chandra and Abhishek 2011). Even though fungal lignin degradation has been studied since 1980, there have been limited commercial biocatalytic processes developed for lignocellulose depolymerization due to the requirements for additional energetic and chemical input for efficient fungal enzymatic activity. Because bacteria can grow in conditions characteristic of

this waste (high pH and oxygen limitation), it may be that use of appropriate bacteria could lead to the development of an efficient biological treatment scheme.

1.4 Lignocellulose Degrading Bacteria

Kraft pulping is the most widely used pulping process worldwide, and because of this there is interest among researchers to isolate bacteria directly from pulp and paper mill industrial waste for use in biological treatment. This work is summarized in Table 1-2. Bacteria have been isolated from soil, wastewater, lignocellulose, and black liquor. These bacteria are capable of utilizing pulp and paper mill lignocellulosic waste to provide a variety of benefits including decolorizing pulp and paper mill effluent, producing low molecular weight compounds, and producing lignocellulose degrading enzymes (Anwar et al. 2014; Chandra 2001; Chandra et al. 2007; Chandra et al. 2008; Chandra and Abhishek 2011; Chandra et al. 2011; Chandra and Singh 2012; Chen et al. 2012; Ko et al. 2007; Mathews et al. 2014; Morri et al. 1995; Raj et al. 2007a; Raj et al. 2007b; Singh et al. 2009; Shi et al.

Table 1-2

Bacteria isolated from industrial lignocellulose streams with lignocellulose-degrading capacity.

Bacterial Strain	Source of isolation	Lignocellulose substrates supporting growth	Relevant characteristics	Growth conditions (on lignocellulose substrates)	Reference
Aneurinibacillus aneurinilyticus (AY856831)	Pulp paper mill effluent	Kraft lignin	Decolorize kraft lignin produce low molecular weight compounds	Facultative anaerobe/ Microaerophilic	Chandra et al. 2007; Raj et al. 2007a
Azotobacter	Soil		Decolorize and solubilize lignin	Aerobic	Morri et al. 1995

Table 1-2 [continued]

Bacillus cereus	Pulp paper mill effluent	Phenol (with glucose)	Degrade phenol and pentacholorophenol pollutants	Aerobic	Singh et al. 2009
Bacillus megatarium	Soil		Decolorize and solubilize lignin		Morri et al. 1995
Bacillus sp. (AY952465)	Pulp and paper sludge	Kraft lignin	Decolorize kraft lignin produce low molecular weight compounds	Facultative anaerobe, 10% NaCl	Chandra et al. 2007; Raj et al. 2007a
Citrobacter freundii	Pulp paper mill effluent	10% Black liquor	Decolorize lignin	Microaerophilic	Chandra and Abhishek 2011
Citrobacter sp.	Pulp paper mill effluent	10% Black liquor	Decolorize lignin	Microaerophilic	Chandra and Abhishek 2011
Citrobacter sp.	Rayon grade pulp black liquor	10% Black liquor		Requires oxygen	Chandra et al. 2011
Cupriavidus basilensis B-8	Bamboo slips	Kraft lignin	Degrade kraft lignin, produce low molecular weight compounds, Ligninolytic enzymes	Aerobic	Shi et al. 2013
Enterobacter	Soil	Lignin model compounds	Oxidative ligninolytic enzymes	Requires 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Yadav et al. 2014
Escherichia coli	Soil	Lignin model compounds	Oxidative ligninolytic enzymes	Requires ABTS	Yadav et al. 2014
Halomonas campisalis, Bacillus licheniformis, Bacillus pumilus,Bacillus subtilis, Bacillus megatherium, Bacillus cerus (consortium)	Wheat straw black liquor storing pool	Vanillin	Produce acetic lactic and formic acid		Yang et al. 2008

Table 1-2 [continued]

Klebsiella pneumoniae	Rayon grade pulp black liquor	10% Black liquor		Requires oxygen	Chandra et al. 2011
Novosphingobium sp. B-7	Eroded bamboo slips	Kraft lignin, guaiacol, vanillin, p-coumaric acid, cinnamic acid, ferulic acid, sinapic acid, vertraldehyde	Growth on kraft lignin as sole carbon source. Produced low molecular weight compounds. MnP	Aerobic	Chen et al. 2012
Paenibacillus campinasensis	Black liquor	Cellulose, hemicellulose	Xylanase, cellulase, pectinse, cyclodectrin glucanotransferase		Ko et al. 2007
Paenibacillus glucanolyticus	Black liquor	Black liquor, cellulose, hemicellulose, lignin	Growth at pH 9	Facultative anaerobe, pH 9	Mathews et al. 2014
Paenibacillus sp. (AY952466)	Pulp and paper sludge	Kraft lignin, phenol (with glucose)	Decolorize kraft lignin produce low molecular weight compounds	Facultative anaerobe, 3% NaCl	Chandra et al. 2007; Raj et al. 2007a
Pandoraea sp. B-6	Bamboo slips	Kraft lignin	Decolorize, reduce COD, produce low molecular weight compounds, ligninolytic enzymes	Aerobic, pH 10	Shi et al. 2013
Pantoea sp.	Pulp paper mill effluent		Decolorize, reduce COD and BOD, degrade lignin and chlorophenol, ligninolytic enzymes	Aerobic, pH 7	Chandra and Singh 2012
Providencia rettgeri	Pulp paper mill effluent		Decolorize, reduce COD and BOD, degrade lignin and chlorophenol, ligninolytic enzymes	Aerobic, pH 9	Chandra and Singh 2012

Table 1-2 [continued]

Pseudochrobactrum glaciale	Pulp paper mill effluent		Decolorize, reduce COD and BOD, degrade lignin and chlorophenol, ligninolytic enzymes	Aerobic, pH9	Chandra and Singh 2012
Pseudomonas putida	Pulp paper mill effluent		Remove color, phenolics and sulfide	Aerobic	Chandra 2001
Serratia	Soil	Lignin model compounds	Oxidative ligninolytic enzymes	Requires ABTS	Yadav et al. 2014
Serratia marcescens	Soil; rayon grade pulp black liquor	10% Black Liquor	Decolorize and solubilize lignin	Requires oxygen	Morri et al. 1995; Chandra et al. 2011
Unidentified (8 strains)	Pulp paper effluent sludge	Kraft lignin	Produce phenolics and low molecular weight acids. Phenol oxidase activity	Aerobic	Chandra et al. 2008

2013a; Shi et al. 2013b; Yadav et al. 2014). The majority of the bacteria isolated from pulp paper mill effluent are γ-proteobacteria (*Acinetobacter, Azotobacter, Citrobacter, Enterobacter, Escherichia, Halomonas, Klebsiella, Pantoae, Providencia, Pseudomonas,* and *Serratia)*. Some of these strains belong to the *Enterobacteriaceae* family composed of gram-negative facultative anaerobic bacteria which can ferment sugars to produce a variety of end products including lactic acid. Other gram-negative bacteria, which were isolated from pulp paper mill effluent, belong to the α-*Proteobacteriaceae* family (*Novosphingobium* and *Pseudochrobactrum*) and are characterized by some relevant metabolic capabilities:

degradation of aromatic compounds. Two β-proteobacteria, which are also gram-negative, were isolated from bamboo (*Cupriavidus* and *Pandoraea).* Additional bacteria isolated from pulp paper mill effluent belong to the *Bacillaceae* family (*Aneuribibacillus, Bacillus,* and *Paenibacillus*) which is composed of gram-positive spore-forming bacteria that are also facultative anaerobes.

Isolation of bacteria from other industrial streams may also provide organisms of value for the biological treatment of lignocellulose. Bacteria belonging to the *Flavobacterium, Bacteroides, Eubacterium, Clostridium, Bacillus* and *Sphingomonas* genera as well as strains of *Proteus vulgaris* and *Streptococcus faecalis* have been shown to degrade azo dyes present in textile industry wastewaters (Stolz 2001). These dyes have also been reported to be degraded by ligninolytic fungi using enzymes involved in lignin degradation (LiP and MnP) suggesting the possibility that the dye-degrading bacteria may also degrade lignin. Compost piles may also be a source of bacteria which can degrade lignocellulose. Members of the genera *Pseudomonas, Klebsiella, Nocardia, Streptomyces, Thermoactinomyces, Micromonospora* and *Bacillus* have been isolated from compost piles and shown to be capable of degrading cellulose in the presence of lignin (Tuomela et al. 2000). While the reported isolations have identified a variety of bacteria which can grow in the presence of lignocellulose and components of these industrial waste streams (phenolic compounds, sulfide, and dyes), it is of particular interest to design future studies to isolate those bacteria which can specifically degrade the more recalcitrant components such as lignin and aromatic lignin degradation products.

1.4. 1 Growth Conditions. The growth conditions for bacteria which can degrade and convert industrial lignocellulosic streams is also important. Future work should also focus on isolation of bacteria which can grow in industrially relevant conditions (low oxygen, high lignocellulose concentration, basic pH, and requires few additional nutrients).

The amount of oxygen present is an important factor for growth of bacteria. Facultative organisms can grow in both aerobic and anaerobic environments. All of the fungi currently used for biological treatment of lignocellulose require oxygen. Anaerobic treatment of lignocellulose may be preferable because costs for supplementation of oxygen are eliminated and microorganisms are able to successfully remove high strength organic effluents under anaerobic conditions (Pokhrel and Viraraghavan 2004). Chandra and Abhishek (2011) isolated *C. freundii* and *Citrobacter sp.* from pulp paper mill effluent and demonstrated that these bacteria could also decolorize effluent (10% black liquor) under aerobic and microaerophilic conditions (< 0.2 mg L^{-1} oxygen). *Paenibacillus* sp. (AY952466), *A. aneurinilyticus* (AY856831), and *Bacillus* sp. (AY952465) were isolated from pulp paper mill effluent and shown to degrade kraft lignin under microaerophilic conditions (Chandra et al. 2007). *P. glucanolyticus* was isolated from black liquor and shown to be capable of growth on black liquor under both aerobic and anaerobic conditions (Mathews et al. 2014).

The pH at which these bacteria grow may provide an additional benefit over fungal biological treatment. While most fungi grow in neutral or slightly acidic pH, bacteria can grow in a wide range of pH. Black liquor and other industrial wastes vary in pH. Black liquor is extremely basic, ranging from pH 10-14. Lignin, the predominant component of black liquor, also increases in solubility at higher pH allowing for more efficient substrate access

by enzymes (Shi et al. 2013a). *P. glucanolyticus, Pandoraea* sp. B-6, *P. rettgeri,* and *P. glaciale* were shown to be capable of growth at pH 9 and 10, which is potentially important because development of bacterial treatments methods for using bacteria which can grow in un-neutralized lignocellulosic streams could be very cost effective (Mathews et al. 2014, Shi et al. 2013a; Chandra and Singh 2012).

Bacteria which do not need additional nutrients for growth beyond what is provided by the lignocellulosic waste stream would also be advantageous in terms of minimizing costs for biological treatment. White-rot fungi, which have been used for biological treatment require glucose or polysaccharides for growth. However, some bacteria have been shown to use lignocellulose components as the sole carbon source requiring no additional sugars or polysaccharides. *Novosphingobium* sp. B-7 was isolated by Chen et al. (2012) from eroded bamboo slips and can use kraft lignin as the sole carbon source for growth. This strain was also capable of growing on lignin-degradation products including guaiacol, vanillin, p-coumaric acid, cinnamic acid, ferulic acid, sinapic acid, and vertraldehyde. *P. glucanolyticus* isolated from black liquor by Mathews et al. (2014) was shown to be capable of growth on black liquor, cellulose, hemicellulose, and lignin as the sole carbon source. In addition to isolating bacteria that can degrade lignin or phenolics, bacteria can also be isolated from paper pulp mill effluent which metabolize polysaccharides. Those bacteria that can use both cellulose and hemicellulose (hexose and pentose sugars) are especially applicable for biological pretreatment. Utilization of mixed sugars is a unique metabolic property characteristic of some bacteria including *Escherichia coli, Klebsiella, Erwinia, Lactobacillus, Bacillus,* and *Clostridia* (Saha 2003). Microorganisms such as *Saccharomyces* and

Xymomonas have been modified to introduce pentose fermentation characteristics (Saha 2003). Ko et al. (2007) isolated *Paenibacillus campinansensis* from black liquor in the brownstock washers and found it could degrade saccharides and polysaccharides. Enzymes which could degrade these sugars were also detected including xylanase, cellulases, pectinase and cyclodextrin glucotransferase.

1.4.2 Metabolic Products. Biological treatment of lignocellulosic streams provides a way to not only dispose of this effluent but also to transform it into valuable products. Development of a lignin degradation process could provide a replacement for petroleum-derived chemicals (Raj et al. 2007b). The bioconversion of lignocellulosic streams to chemicals by bacterial treatment offers a renewable and profitable way for these industries to transform low-value products derived from lignocellulose. Biotransformation is possible because bacteria have versatile pathways of enzymes which degrade complex substances including recalcitrant aromatic substances ranging from simple phenols to the highly complex lignin polymer (Chen et al. 2012). Production of low molecular weight compounds provides evidence of the degradation of lignin and other components of the lignocellulose stream. Cinnamic acid and guaiacol are thought to be produced by cleavage of ester linkages in guaiacyl and p-hydroxyphenyl units in the lignin polymer (Shi et al. 2013b). The presence of low molecular weight aromatic compounds (3,5-dimethylbenzaldehyde, ferulic acid, vanillin, and vanillic acid) are evidence of lignin degradation because these compounds are considered to be the main structural units of lignin (Chen et al. 2012). Some bacteria have been isolated from pulp paper mill effluent which can degrade lignocellulosic components and produce valuable metabolic products. The low molecular weight metabolic products can

be sold for use as chemical feedstocks or additives. *A. aneurinilyticus* and *Bacillus sp.* produced acetoguiacone, gallic acid, ferulic acid, cinnamic acid, and benzaldehyde when grown in media containing kraft liquor (Raj et al. 2007b). Production of ethanol, and oxalic, lactic, hexanoic, acetic, butyric, propanoic, succinic, and vanillic acids by *P. glucanolyticus* was detected after growth on black liquor (Mathews et al. 2014). *C. basilensis* produced cinnamic acid and 3-5-dimethylbenzaldehyde after growth on kraft lignin (Shi et al. 2013a), while *Novosphingobium* sp. B-7 generated ethanediol and 3-methyl-2-butanol (Chen et al. 2012), and *Pandoraea* sp. B-6 produced veratryl alcohol, cinnamic acid, ethylguaiacol, ferulic acid, 3-5-dimethylbenzaldehyde, and lignophenols when grown on kraft lignin (Shi et al. 2013b). Others have isolated a consortium of bacteria which can degrade lignocellulosic components and produce valuable metabolic products. Under these conditions it may be that some bacteria produce enzymes for degradation while other bacteria convert these degradation products into organic compounds. Chandra and Singh (2012) analyzed the ability of a bacterial consortium to decolorize and detoxify pulp paper mill effluent finding that the maximum effect was detected when these three strains were used together. Bacterial consortia isolated from pulp paper mill effluent sludge produced phenolic monomers and guaiacol, as well as acetic, formic, lactic, propanoic, butanoic, and hexanoic acids when grown in media with kraft lignin (Chandra et al. 2008; Yang et al. 2008).

1.5 Lignocellulose Degrading Enzymes

Biological treatment of lignocellulose occurs through the specific action of enzymes produced by microorganisms which degrade the components of lignocellulose: cellulose,

hemicellulose, and lignin. The enzymes involved in lignocellulose degradation are described below and listed in Table 1-3. Because these major lignocellulose components are linked together, degradation requires enzymes which can act in concert. Often the presence of lignin prevents access of the carbohydrate degrading enzymes from binding to their substrates which is why many of the lignocellulosic streams are produced using a pretreatment method which liberates carbohydrates for downstream fermentation (Malherbe and Cloete 2002).

1.5.1 Cellulose. Enzymatic degradation of cellulose occurs by the action of three different proteins: cellobiohydrolase (exocellulase), endocellulase and β-glucosidase (Sun and Cheng 2002). Cellulases can exist as a complex of enzymes in a cellulosome. Free cellulose degrading enzymes also exist and are secreted by the bacterium. Aerobic cellulase systems are comprised of several secreted enzymes whereas anaerobic cellulase systems feature cellulosomes: surface attached multi-enzyme complexes (Fontes and Gilbert 2010; Pason et al. 2006). Most cellulose in the environment is degraded aerobically but 5-10% is degraded anaerobically in soils, compost piles, animal rumens, insect guts, and aquatic environments (Leschine 1995). Cellulase systems are characterized by a number of

Table 1-3

Lignocellulose degrading enzymes (Leonowicz et al. 1999; Hatakka et al. 2001)

Enzyme	**Substrate**	**Product**
Cellulose		
endo-1,4-β-glucanase	1,4-β-glucose linkages	cellulose
exo-1,4-β-glucanase (cellobiohydrolase, glucohydrolase)	Terminal 1,4-β-glucose linkages	cellobiose
β-glucosidase	1,4-β-glucose linkages	glucose
Hemicellulose		
endo-1,4-β-xylanase	1,4-β-xylose linkages	hemicellulose
β-xylosidase	1,4-β-xylose linkages	xylose
α-glucuronidase	α—D-glucan, H_2O	Glucan, alcohol
α-L-arabinofuranosidase	α-L-arabinose residue	α-L-arabinose
acetylesterase	Acetic ester, H_2O	Acetate, alcohol
endo-1,4-β-mannanase	1,4-β-mannose linkages	Mannans
β-mannosidase	Terminal 1,4-β-mannose linkages	mannose
α-galactosidase	Terminal α-D-galactose residue	galactose
Lignin		
lignin peroxidase	Alkyl side chains, C-C, aromatic ring	aldehydes
manganese-dependent peroxidase	Aromatic structures, H_2O_2, Mn^{2+}	Cleave Cα-Cβ bond
Laccase	Phenolic compounds, aromatic amines	Cleave C-C bond (vanillin)
horseradish peroxidase	Phenolic compounds, aromatic amines	Cleave C-C bond
protocatechuate 3,4-dioxygenase	2,4-Dihydroxybenzoate, O_2	3-carboxy-*cis, cis* muconate
catechol 1,2 dioxygenase	Catechol	*cis, cis* muconate
Superoxide dismutase	$O_2^{\cdot -}$, $H^{\cdot}$	H_2O_2, $O_2^{\cdot}$
aryl alcohol oxidase	Aromatic primary alcohol, O_2	Aromatic primary aldehyde, H_2O_2

properties: multiple enzyme components which contain a substrate-binding site, catalytic site, and are associated with the cellular surface (Beguin and Aubert 1994). Cellulase enzymes have a variety of industrial applications which include textile, laundry, paper and biofuel increasing water absorbency which can be used for biostoning of jeans and biopolishing of industries. The textile industry uses cellulase enzymes for softening cellulosic fabrics and cotton (stone washing). The laundry industry has made use of cellulases by adding them to detergents (Singh et al. 2007). Pawlak (2009) describes the use of cellulases as biochemical additives and processing agents in the pulp and paper industry. Cellulose is commercially available in several different forms (cotton, filter paper, avicel, carboxymethyl cellulose) which can be used to assay the efficiency of the enzymes responsible for cellulose degradation. These commercial substrates differ in crystallinity, surface area, pore size and solubility (Beguin and Aubert 1994).

A few bacterial species have been isolated from environments containing lignocellulose and have been determined to produce cellulolytic enzymes. A *Paenibacillus* species isolated from soil was capable of degrading carboxymethyl cellulose and filter paper by the production of extracellular enzymes (Emtiazi et al. 2007). *Paenibacillus curlandolyticus* B6 is a facultative anaerobe capable of adhering to xylan and cellulose and producing enzymes that degrade these polysaccharides (Pason et al. 2006). *P. curlandolyticus* B6 was also shown to depolymerize sugars in different types of lignocellulosic waste: corn husk, sugarcane bagasse and rice straw (Pakdeedachakiat et al. 2008). Shi et al. (2010) isolated a bacterial multifunctional xylanase from corn ensilage which can bind to and degrade xylan and β-glucan at a pH range of 6-10.

1.5.2 Hemicellulose. Degradation of hemicellulose requires multiple enzymes including xylanases, β-xylosidase, endo-1,4-β-xylanase, and debranching enzymes: acetylxylan-esterase, α-glucuronidase, and α-arabinofuranosidase (Shi et al. 2010; Kumar et al. 2008). Xylanase enzymes can exist as secreted enzymes that act individually but can also exist as xylanosomes which are "multifunctional, multi-enzyme complexes found on the surface of several microorganisms" (Beg et al. 2001). A complete review of xylanase enzymes can be found in Collins et al. (2005) and a list of microbial xylanases can be found in Beg et al. (2001). Xylanases have been used in animal feed, brewing, food processing, waste treatment, and pulp and paper industries. Xylanases reduce viscosity in animal feed results in higher nutrition. The brewing industry uses xylanases to reduce viscosity and improve filtration rate (Shi et al. 2010). The pulp and paper industry has also made use of xylanases for selective degradation in cellulosic pulps to remove residual xylans in brownstock holding tanks and also in deinking (Singh et al. 2007; Yang et al. 1995). Xylanases can also improve bread quality by increasing volume, enhance clarification of juices, facilitate extraction of coffee plant oils and starch, and degumming flax, hemp or jute fibers (Beg et al. 2001).

One bacterium has been isolated from an environment containing lignocellulose which produces hemicellulose-degrading enzymes. This bacterium can also grow at high pH which is characteristic of many lignocellulosic industrial waste streams. Few xylan degrading alkaliphiles have been identified; however, *Bacillus* sp (V1-4) isolated from hardwood kraft pulp, produced xylanase at pH 9 under mesophilic temperatures and aerobic conditions

(Yang et al. 1995). This enzyme is promising for use in biological treatment of lignocellulose streams.

1.5.3 Lignin. Lignin depolymerization is problematic due to its structure. Many bacteria are able to degrade single-ring aromatic compounds. This metabolic capability is commonly found in soil bacteria such as *Pseudomonas, Alcaligenes, Arthrobacter, Nocardia* and *Streptomyces* (Vicuna 1988). However, few bacteria have been shown to degrade polymeric lignin. Many different preparations of lignin have been used for the growth of microorganisms which has added to the confusion about the action of bacterial and fungal and enzymes on lignin (Gottlieb and Pelczar 1951). Degradation of the lignin polymer is slow and unlike cellulose and hemicellulose, lignin is amorphous and its aromatic structures are difficult to degrade (Bungay 1992). However, some microorganisms (fungi and bacteria) produce enzymes that degrade lignin during carbon limitation (Vicuna 2000; Bugg et al. 2011a). Laccase, lignin peroxidase, and manganese peroxidase are the best studied enzymes involved in lignin degradation. These enzymes introduce hydroxyl groups into aromatic substrates with the help of mediators and by the reduction of oxygen, after which the aromatic ring is cleaved (Boll et al. 2002). There is also evidence of microbial anaerobic lignin degradation, but the enzymatic mechanism of this process has not been identified (Bungay 1992; Healy and Young 1979; Zeikus et al. 1982). Laccases can be used in the pulp and paper industry to enhance bleaching of kraft pulp, but chemical mediators are required. There is great potential for use of lignin in lignocellulose industries which would add value to these industrial processes. Lignin could be used as a source of renewable chemicals for the

food and flavor industry and for fine chemicals and materials synthesis (vanillin, acid-precipitable polyphenolic polymeric lignin, and ferulic acid) (Bugg et al. 2011a).

Some bacteria are capable of lignin degradation: *Aneurinibacillus aneurinilyticus, Bacillus* sp., *Bacillus cereus* C10-1, *Critobacter* sp., *Nocardia, Novosphingobium* sp. B-7, *Paenibacillus* sp., *P. glucanolyticus, P. norimbergensis, P. jessinii* PS06, *P. putida, R. jostii* RHA1, and *S. marcescens,* (Bandounas et al. 2011; Bugg et al. 2011b; Chandra et al. 2011; Chen et al. 2012; Mathews et al. 2014; Raj et al. 2007b; Zimmerman 1990). Many of these bacteria can grow on lignin as a sole carbon source, reduce the presence of lignin in the growth media, or produce lignin monomers as a result of bacterial growth. However, very few bacterial lignin-degrading enzymes have been characterized. *Sphingomonas paucimobilis* SYK6 is the bacterial lignin degrader which has been studied most extensively. This bacterium is able to cleave the most abundant linkage (β-aryl ether bonds) through the action of the glutathione-dependent β-etherase enzyme (Masai et al. 1989). *Amycolatopsus* sp. 75iv2 and *Streptomyces griseus* 75vi2 were isolated from soil and oxidative lignin-degrading enzymes were produced extracellularly. These bacteria also contain oxidative enzymes which can break bonds present in the lignin polymer through hydroxylation or demethylation which are distinct from the canonical fungal enzymes (Brown and Chang 2014). Lignin degrading enzymes have also been shown to have other activities involved in physiological processes such as pigmentation, sporulation and metal resistance (Kellner et al. 2008). Future research should focus on determining the enzymatic mechanism by which these bacteria degrade lignin, especially those which degrade lignin under industrially relevant conditions (Kawakami and Shumiya 1983).

1.5.4 Techniques for Identifying Novel Enzymes from Lignocellulose-containing Environments.

Lignocellulose is an abundant renewable resource that could be transformed into valuable products by the action of enzymatic degradation and conversion. New enzymes with unique properties are of interest because of their application in industrial processes. Major enzymes for lignin degradation have only been thoroughly investigated in fungi, thus, unique lignin degrading enzymes with unique properties in other domains are also of interest. Sources for these new enzymes are bacteria growing in environments with lignocellulose. Prokaryotes are of particular interest because they can grow at extreme conditions (pH, temperature, salinity, in the presence of toxic compounds). Researchers are looking for these organisms and enzymes in a number of environments. Forest soils are a rich source of prokaryotes which can degrade lignocellulose because this environment is rich in humic matter (degraded plant material). Amazon rainforest soil is rich in organic matter and biochar. Nakamura et al. (2014) isolated *Pseudomonas, Arthrobacteria, Paenibacillus, Stenthrophomonas* and *Bacillus* from phrenathrene enrichment cultures. Woo et al. (2014) isolated *Burkholderia* sp. strain LIG30 from Luqillo rainforest (Puerto Rico) soil and demonstrated its ability to use alkali lignin as the sole carbon source. The sequenced genome revealed 22 putative peroxidases and catalases that may be responsible for lignin-degradation. Soil environments can range in the level of oxygen availability. Many of these environments are anaerobic. Lignocellulose degrading bacteria can also be isolated from anaerobic soil sediments and animal rumens (Zimmermann 1990). Aquatic samples are also a source for lignocellulose-degrading bacteria and enzymes. Zeikus et al. (1982) found that lignin-related aromatic substrates are being biodegraded in anoxic aquatic ecosystems;

however, the specific microorganisms responsible for this activity have not been identified. Examples of these ecosystems in which lignin-related substrates are found are aquatic sediments and sewage sludge digesters. *Paenibacillus* species were isolated from an aquatic sample and demonstrated the ability to degrade polycyclic aromatic hydrocarbons: naphthalene, biphenyl, fluorene, and phenanthrene (Daane et al. 2001). Liu et al. 2014 isolated a bacterium from surface seawater samples identified as *Rhizobium* sp. MGL06 and was capable of decolorizing the aromatic dye malachite green (Liu et al. 2014).

There are also a variety of tools which can be used to identify microorganisms with the metabolic capacity to degrade lignocellulose. Adav et al. (2012) outlined a method using iTRAQ (isobaric tags for relative and absolute quantification) which can tag peptides for analysis and quantification. The secretomes of *P. chrysosporium* and *T. fusca* were analyzed to determine the presence of cellulase, xylanase and lignin depolymerizing enzymes (Adav et al. 2012; Adav et al. 2010). Similar techniques should be used for bacteria to identify lignocellulose-degrading enzymes. Zymograms are another tool which can be used to determine the specific activity of proteins. This assay uses native gels and dyes to determine the presence of lignocellulose-degrading enzymes. Microarrays have been employed to analyze mRNA collected from soil communities for the presence of lignin-degrading genes (Bailey et al. 2010). Lignin-related substrates can also be included in growth studies to determine the ability of these organisms to degrade lignin. Ahmad et al. (2010) used a protocol in which lignin-degradation can be measured using altered lignin substrates which contain a fluorophore or specific light absorbance properties. Dyes have also been utilized as a colorimetric indicator of the activity of lignin degrading enzymes (Bandounas et al. 2011).

1.5.5 Modification and Recombinant Expression of Lignocellulose Degrading Enzymes.

Isolation and characterization of novel lignocellulose degrading organisms or enzymes are valuable especially for biological treatment of industrial lignocellulose streams. It may be that while the enzymes produced by these bacteria can degrade components of the industrial lignocellulose streams and are active under industrially relevant conditions, the bacteria which produce these enzymes do not grow quickly or produce large quantities of these enzymes. Other bacteria could also be genetically modified to enhance or introduce lignocellulose degrading properties. The modification of bacteria capable of degrading polysaccharides and the heterologous expression of these enzymes in hosts which can produce high titers of enzymes has been reviewed in Lynd et al. (2005). While many fungi can produce highly efficient lignin-degrading enzymes, heterologous overexpression of these enzymes at industrially relevant concentrations has proven to be difficult. Expression and modification of bacterial lignin-degrading enzymes may be more easily achieved because of the molecular biology tools available (Santhanam et al. 2011). Successful modification of a bacterial lignin-degrading enzyme has been demonstrated by Brissos et al. (2009) and Gupta and Fanias (2010) in which a CotA from *B. subtilis,* was optimized to improve reduction potential and substrate affinity. Recombinant hosts used to successfully produce high titers of bacterial lignocellulose degrading enzymes and the tools to transform these organisms have also been well studied. Piscitelli et al. (2010) has reviewed heterologous laccase production. Lactic acid bacteria are commonly used in the food industry for the production of dairy products and are characterized by their ability to grow at low pH. Boguta et al. (2014) screened lactic acid bacteria for their potential use in pretreatment of lignocellulosic

feedstocks. Application of these strains for biological treatment would require modifications to increase resistance against inhibitors. Boguta et al. (2014) isolated lactic acid bacteria with high resistance towards furfuryl alcohol, vanillin, and vanillin alcohol.

1.6 Biodegradation and Bioconversion Technology

The isolation and characterization of bacteria, which can degrade industrial lignocellulosic streams, has great potential for application. These waste streams have not been widely used because they contain toxic compounds, low levels of fermentable sugars and have a high pH (Kirk et al. 1983). These conditions have not favored growth of fungi, but bacteria have been isolated, which can degrade lignocellulose components under these conditions. The most obvious application of this technology is the biodegradation and bioconversion of underutilized industrial lignocellulosic streams through biological pretreatment. Ideally, this biological treatment technology would use unmodified lignocellulosic streams and bacteria for the production of fuels and chemicals. This scheme is similar to that of a petroleum refinery in which many products are produced from one feedstock (Figure 1-4). Kamm and Kamm (2004) suggest that the future of a pulp and paper mill is that of a biorefinery. The potential number of products which could be made from lignocellulose is great (Heiningen 2006; Zakzeski et al. 2010). The ideal biorefinery would utilize lignocellulosic streams and require limited additional input for bacterial growth. The lowest energy and chemical input would require bacteria which can grow at high pH, degrade lignocellulosic components at high concentrations, and require limited oxygen input. Each major component of lignocellulose has a variety of valuable products which could be produced as a result of microbial biodegradation and bioconversion of the renewable feedstock lignocellulose.

Polysaccharides can be converted into glycerol, lipids, acetone, *n*-butanol, butanedioic acid, lactic acid, isopropanol, acetic acid, and succinic acid. Succinic acid is a building block for biodegradable plastic (polybutylene succinate), polyester polyols, plasticizers, polyurethane, and 1,4-butanediol. An estimated 30,000-50,000 ton/year is produced in petro-chemical refineries and lignocellulose could provide a source for production of this chemical with less environmental impact than petroleum through microbial biodegradation and bioconversion (Jansen and van Gulik 2014). Lignin is the most predominant and recalcitrant component of pulp and paper mill underutilized lignocellulosic streams. If efficient bacterial enzymes are identified, lignin could be converted into many industrially-relevant chemicals including phenols, aromatics and olefins (Wyman and Goodman 1993; Hatakeyama 2002; Feldman 2002). These chemicals could be used in existing applications such as the production of wood adhesives (phenol-formaldehyde resins), plastics and resins (phenol), commercial food sweeteners (cinnamic acids), additives for fragrances, and precursors for pharmaceuticals from lignin degradation products (Philbrook et al. 2013; Lee and Lee 1998). Vanillin is also produced from lignin and serves as a food flavoring agent (Bandounas et al. 2011). Ferulic acid produced from lignin is used as an anti-oxidant and food additive (Philbrook et al. 2013). The structure of lignin also suggests that it could be employed as a source to produce organic chemicals which would offer a renewable solution to the current process which uses petroleum to produce these chemicals. It is estimated that 90% of industrial chemicals are produced from petroleum which generates over $1.5 trillion annually in revenue (Witcoff and Reuben 1996; Clark and Deswarte 2008). Lignocellulose offers a cheap and sustainable feedstock for these products.

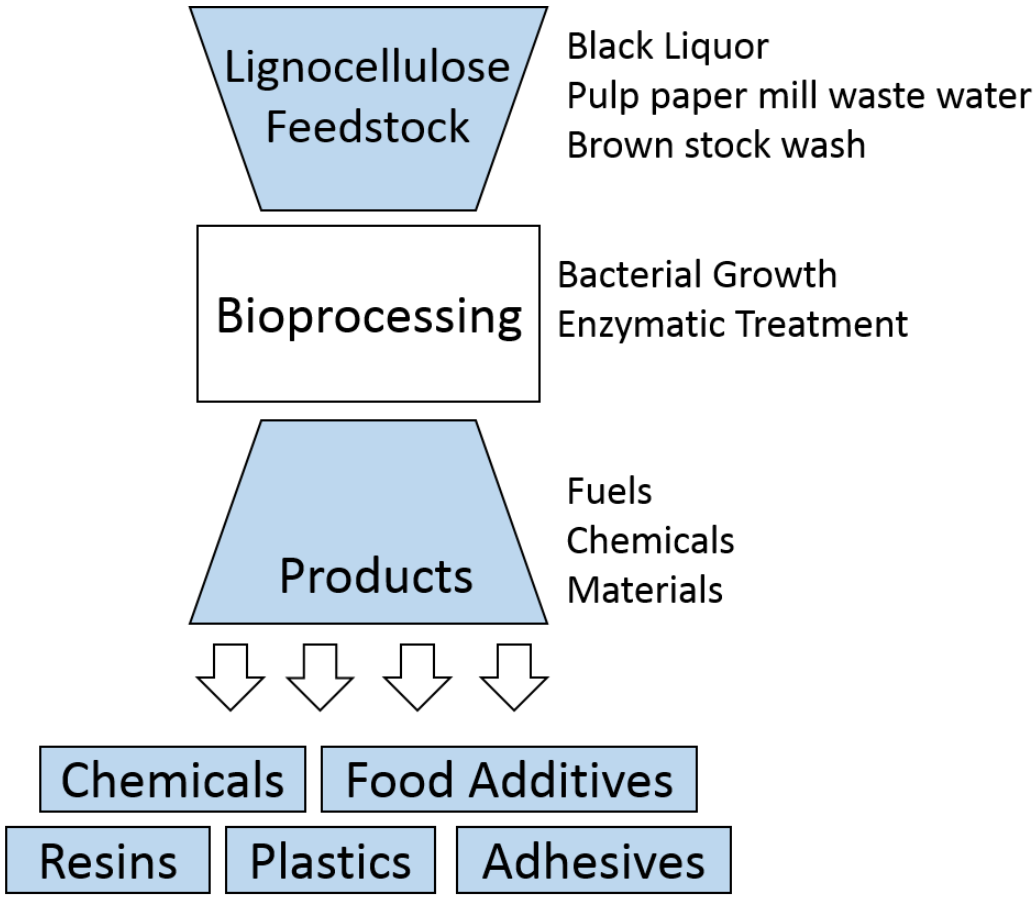

Figure 1-4 Potential application of lignocellulose-degrading bacteria

These bacteria or the enzymes produced by these bacteria could be used for additional processes including pulping, pretreatment of lignocellulose, and bleaching. The pulp paper industry has already used enzymes for bleaching, biopulping, and pretreatment to lower energy requirements and produce a higher quality product as a result of lignin removal through microbial degradation (Kirk et al. 1983). Enzymatic bleaching has been performed with the addition of lignin and hemicellulose degrading enzymes from white rot fungi

(Pawlak 2009). Other enzymes have been used in the pulp paper industry for deinking, refining, elimination of extractives and lipids, removal of stickies, debarking, degradation of hemicellulose before bleaching and, enhancement of lignin degradation during bleaching and pulping (Pawlak 2009). The enzymes produced from the bacteria isolated from lignocellulosic streams may also be used for these processes and have advantages over enzymes currently employed because of their ability to degrade lignocellulose components under industrially relevant conditions. Biomass pretreatment is another process for which these enzymes could be used. Biomass pretreatment is largely applied to the production of bioethanol from lignocellulose. Biomass pretreatment is the most expensive step of this process (Philbrook et al. 2013). Lignin-specific enzymes would be of great value for this process as they would lower pretreatment cost and increase downstream polysaccharide degradation enzyme efficiency.

1.7 Conclusion

Lignocellulose is a renewable resource that is used by the pulp and paper, biofuel, and chemical industries. These industries use the polysaccharides for conversion into paper, ethanol, and a variety of chemicals resulting in underutilized lignocellulosic streams. Industrial lignocellulosic streams vary in composition but typically contain cellulose, hemicellulose and lignin. These compounds have great potential for conversion into valuable products; however, lignocellulosic streams have not been widely used because they contain toxic compounds, low levels of fermentable sugars, and high pH. Biological treatment is one way in which these lignocellulose streams could be degraded and converted into valuable products. This process is advantageous over other treatment schemes because the organisms

which degrade and convert these compounds into valuable chemicals may require little energy and/or chemical input. Biological treatment of lignocellulosic streams should use organisms which can degrade the components of lignocellulose. Fungi and bacteria have been shown to degrade lignocellulose. Use of bacteria is advantageous over fungi because of their increased tolerance to harsh industrial conditions. More than 25 bacterial species have been isolated directly from these industrial lignocellulosic streams. These bacteria have been shown to be capable of degrading lignocellulose components and converting these components into valuable chemicals such as acetoguiacone, benzaldehyde, and ethanol as well as acetic, butyric, cinnamic, ferulic, gallic, lactic, propanoic, succinic, and vanillic acids. Therefore these bacteria or the enzymes they produce may be applied directly to the industrial lignocellulosic stream for degradation and conversion. Future work should focus on the characterization of the enzymes involved in these bacterial metabolic processes to aid in development of efficient biological treatment schemes.

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CHAPTER 2

Methods for Facilitating Microbial Growth on Pulp Mill Waste Streams and Characterization of the Biodegradation Potential of Cultured Microbes

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Abstract

The kraft process is applied to wood chips for separation of lignin from the polysaccharides within lignocellulose for pulp that will produce a high quality paper. Black liquor is a pulping waste generated by the kraft process that has potential for downstream bioconversion. However, the recalcitrant nature of the lignocellulose resources, its chemical derivatives that constitute the majority of available organic carbon within black liquor, and its basic pH present challenges to microbial biodegradation of this waste material. Methods for the collection and modification of black liquor for microbial growth are aimed at utilization of this pulp waste to convert the lignin, organic acids, and polysaccharide degradation by-products into valuable chemicals. The lignocellulose extraction techniques presented provide a reproducible method for preparation of lignocellulose growth substrates for understanding metabolic capacities of cultured microorganisms. Use of gas chromatography-mass spectrometry enables the identification and quantification of the fermentation products resulting from the growth of microorganisms on pulping waste. These methods when used together can facilitate the determination of the metabolic activity of microorganisms with potential to produce fermentation products that would provide greater value to the pulping system and reduce effluent waste, thereby increasing potential paper milling profits and offering additional uses for black liquor.

Keywords: biodegradation (bacterial degradation), pulp mill waste, black liquor, kraft lignin, lignocellulose extraction, GC-MS

2.1 Introduction

The pulping of wood is a chemically intensive process that has been optimized over many years to create a system with minimal waste. However, some outputs of this process could be used to produce higher value product(s). Black liquor is one such example. It is generated from the kraft process which is the dominant chemical pulping method, representing 85% of world lignin production (Tejado et al. 2007). The kraft process (Figure 2-1) uses temperature (160-200 ˚C), pressure (120 psig), and the chemicals contained in white liquor (sodium hydroxide and sodium sulfide) to dissolve the lignin from the wood fibers (Brannvall 2009; Biermann 1996). Black liquor contains lignin, organic acids, and polysaccharide degradation by-products (Sjöström 1924). It is incinerated to produce steam and recover chemicals in the recovery boiler that provides thermal energy for downstream papermaking and pulping processes. The volume of black liquor generated by pulping can exceed the amount that the recovery boiler can effectively process. Disposing of the black liquor as effluent negatively affects aquatic flora and fauna and thus is not an option. Application of microbial organisms that could use black liquor for growth would be beneficial in terms of increasing chemical recovery and generation of value-added product(s) that would improve the overall life cycle analysis of the pulping system. Chemical and biological conversion of lignin derived monomers has successfully produced vanillin and cinnamic acid for use as food sweeteners and fragrance additives, phenol used for plastic and resins, and cyclohexane which could be used for fuel (Philbrook et al. 2013).

Previous work on biodegradation of this pulp waste has been focused on lignin depolymerization. The International Lignin Institute (ILI) reports that between 40 and 50

million tons of lignin is produced each year (http://www.ili-lignin.com/aboutlignin.php). Only 1.5% of that lignin is used for commercial industrial processes (Sanchez et al. 2011). Lignin depolymerization by laccase and peroxidase enzymes produced by white rot fungi of the *Phanerochaete* and *Trametes* genera has been studied at length (Font et al. 2003). Soil bacteria known to degrade aromatic compounds such as *Nocardia* and *Rhodococcus* (Zimmerman 1990), *Pseudomonas putida* mt-2 (Ahamad et al. 2010), and *Streptomyces viridosporus* T7A (Ramchandra et al. 1998) have also been shown to be capable of lignin degradation. Bacterial degradation of pulping waste is promising because some bacteria can thrive in the saline and alkaline (pH 10-14) conditions that characterize the pulping waste effluents (Mishra and Thakur 2012). While lignin is the main component of black liquor, microorganisms may also degrade the other components that make up black liquor. These techniques do not exclusively identify lignin degrading microorganisms, but serve to identify microorganisms that can be applied directly to the pulping waste black liquor instead of its further processed constituents.

Black liquor was collected and modified for microbial growth through neutralization and filter-sterilization. Microbial growth requirements were identified for an environmental microbial isolate by minimal media growth experiments on lignocellulosic components produced by a novel lignocellulose extraction protocol. Growth media were analyzed by gas chromatography-mass spectrometry (GC-MS) to determine the metabolic products of the environmental microbial isolate when grown on black liquor as the sole carbon source. The combination of these techniques provides an assessment tool to determine the metabolic capacity of a microorganism when grown on pulp mill wastes such as black liquor. Use of

such techniques also offers insight into the value of application of specific microorganisms to pulp mill waste for the generation of by-products.

2.2 Protocol Text

The materials used for these experiments are listed in table 2-1.

2.2.1 Collection of black liquor and Preparation of black liquor for growth cultures

1.1) Collect a black liquor sample from the outlet valve attached to the kraft digester in a sterile glass bottle and allow it to cool to room temperature before proceeding with the following steps.

Neutralization

1.2) Add 100 ml of black liquor sample to a 500 ml beaker equipped with a stir bar.

1.3) Place the beaker on a stir plate and adjust the speed to medium.

1.4) Slowly add drops of phosphoric acid until the black liquor becomes viscous (viscosity similar to glycerol).

1.5) Aliquot all of the black liquor solution by slowly pouring into 50 ml conical tubes and centrifuge at 9,300 x g for 30 minutes.

1.6) Place the supernatant into the beaker and measure the pH using pH test ribbons. The pellet of precipitated lignin can be discarded and treated as nonhazardous solid waste.

1.7) Continue to add phosphoric acid and centrifuge the solution until the pH strips indicate a neutral pH of the supernatant.

Sterilization

1.8) Pass the solution through a 0.22 μm filter to remove any contaminating bacteria and place the filtered solution into a sterile container for storage. Store at room temperature.

2.2.2 Lignocellulose extraction

Carbohydrate Separation

2.1) Place 5 g of switchgrass, pine, or Bermuda grass (milled to 1 mm or smaller particle size) into a 500 ml Erlenmeyer flask. Then add 200 ml of distilled or deionized water, 7.5 g of $NaClO_2$, 2.5 ml of glacial acetic acid and a stir bar to the flask.

2.2) Place the flask in an oil bath placed on top of a stir plate at 80 ˚C for 1 hour. Adjust the speed of the stir plate to medium.

2.3) Add an additional 7.5 g of $NaClO_2$ and 2.5 ml of glacial acetic acid to the flask. Continue stirring at 80 ˚C in the oil bath for 30 minutes.

2.4) Repeat the previous step 2 times (Note: a total of 30 g of $NaClO_2$ and 10 ml of glacial acetic acid is added to the flask containing the lignocellulosic biomass). Cool the solution to room temperature.

2.5) Place a sheet of filter paper into a Buchner funnel. Place the funnel on top of a side arm flask and attach it to a vacuum.

2.6) Slowly pour the solution into the funnel.

2.7) Wash the solid material 3 times with 100 ml of distilled or deionized water. Discard the filtrate after washing the solid material.

2.8) Reserve 1 gram of the solid material and place it onto a clean sheet of filter paper.

2.9) Dry this solid material overnight in a 50 ˚C oven. After removing the sample from the oven place into a storage vial labeled ‘holocellulose’.

2.10) Add the remaining solid material to 250 ml of 10% NaOH (v/v) in a 500 ml Erlenmeyer flask. Cap the flask with a stopper.

2.11) Place the solution in an incubator at 70 ˚C for 1 hour shaking at 250 rpm.

2.12) Cool the solution for 30 minutes at room temperature.

2.13) Remove the solid material using filter paper and Buchner funnel attached to a side arm flask.

2.14) Transfer the solid material on a clean sheet of filter paper to a 50 ˚C oven and allow it to dry overnight.

2.15) After the residue has dried, place it in a storage vial and label it ‘cellulose’.

2.16) Transfer the filtrate from step 2.13 to a 500 ml Erlenmeyer flask and add 30 ml of acetic acid and 250 ml of isopropyl alcohol. Cap the flask and place the solution on the bench top for at least 8 hours at room temperature.

2.17) Transfer all of the solution to 50 ml conical tubes and centrifuge the solution at 9,300 x g for 30 minutes. Carefully pipette the solution out of the conical tube and discard.

2.18) Resuspend the pellet in 30 ml of distilled or deionized water by vortexing.

2.19) Repeat steps 2.17-2.18 15 times to thoroughly wash the pellet.

2.20) Remove the supernatant and save the pellet for freeze drying. Once freeze-dried, collect the sample and place it in a vial labeled ‘hemicellulose’.

2.2.3 Lignin separation

Lignin separation protocol based on Karaaslan et al. (2010).

2.21) Add 5 g of switchgrass, pine, or Bermuda grass (milled to 1 mm or smaller particle size) to 500 ml of a solution of 0.25 M NaOH and 30% ethanol. Place the slurry in an incubator at 75 ˚C with shaking at 250 rpm for 2 hours.

2.22) Cool the solution to room temperature.

2.23) Place a sheet of filter paper in a Buchner funnel on top of a side arm flask. Attach to a vacuum and slowly pour the solution into the funnel.

2.24) Discard the solid material.

2.25) Add a stir bar to the flask containing the filtrate and place the flask on top of a stir plate adjusted to medium speed. Add concentrated hydrochloric acid dropwise to the solution until a pH of 2.0 is obtained (approximately 20 ml).

2.26) Allow the acidified solution to sit overnight at room temperature.

2.27) Centrifuge the solution at 9,300 x g for 30 minutes in conical tubes. Carefully pipette the solution out of the conical tube and discard.

2.28) Resuspend the pellet in 30 ml of distilled or deionized water by vortexing.

2.29) Repeat steps 2.27-2.28 15 times to thoroughly wash the pellet.

2.30) After centrifugation, remove the supernatant and save the pellet for freeze drying. Once freeze-dried, place the sample in a vial labeled ‘lignin’.

2.2.3 Preparation of microbial growth cultures

Agar Plates

3.1) Add 0.2% (w/v) sterile lignocellulose extraction products to M9 minimal media agar before pouring plates (Lech and Brent 1992).

3.2) Streak solidified plates with approximately 200 uL of a bacterial culture grown in M9 minimal media with an O.D. of approximately 2.0.

3.3) Incubate plates at 37℃ and monitor growth.

Liquid Cultures

3.4) Add 50 ml of sterile Luria-Bertani (LB) media or M9 minimal media to a sterile 125 ml flask (Lech and Brent 1992). Add black liquor at 10% (v/v) or 0.2% (w/v) of lignocellulose extraction products.

3.5) Inoculate the media with 0.1% (v/v) of a bacterial culture at an O.D. of approximately 2.0.

3.6) Incubate the cultures at 37 ˚C with shaking at 200 rpm.

3.7) Assess growth every 6 hours during the culturing period by measuring optical density (λ=600 nm). Place a 1 ml aliquot of the culture into a cuvette. Determine optical density of culture blanked against uninoculated media.

3.8) Transfer bacteria to an anaerobic serum bottle at mid log phase.

3.8.1) To prepare the anaerobic serum bottle: seal the serum bottle with a butyl rubber stopper and an aluminum crimp top under aerobic conditions and autoclave. Do not modify the headspace within the serum bottle after sterilization.

3.8.2) Transfer the entire 50 ml culture from the 125 ml flask into the sealed sterile serum bottle using a syringe.

3.8.3) Incubate at 37 ˚C with shaking at 200 rpm.

2.2.4 Sample preparation for GC-MS

Prepare samples according to Raj, Reddy and Chandra (2007). Negative control used for comparison consists of M9 minimal media with 10% black liquor and is also referred to as the uninoculated sample.

4.1) Centrifuge uninoculated and inoculated bacterial cultures (50 ml) at 9,300 x g for 30 minutes after 450 hours of incubation.

4.2) Transfer 10 ml of the supernatant into a glass test tube. (Note: Plastic is incompatible with the chemicals in the following steps.)

4.3) Acidify the supernatant with concentrated HCl to pH 1-2.

4.4) Add 3 volumes of ethyl acetate (30 ml), cap the tube and mix by inverting 4-6 times.

4.5) Place a small amount of anhydrous Na_2SO_4 (1-2 g) in a clean glass test tube.

4.6) Carefully collect the organic layer (top) from the ethyl acetate solution by pipetting with a disposable, glass Pasteur pipette.

4.7) Place the organic layer in the test tube with anhydrous Na_2SO_4.

4.8) Dewater the organic layer over anhydrous Na_2SO_4 by gently tapping the test tube to mix. Gradually add small amounts of anhydrous Na_2SO_4 and mix until there are no large clumps.

4.9) Place a sheet of filter paper into a Buchner funnel on top of a side arm flask attached to a vacuum.

4.10) Pour the solution into the funnel. Discard the solid material.

4.11) Transfer the solution to an evaporating flask and evaporate the filtrate using a rotary evaporator.

4.12) Place 3 mg of ethyl acetate extraction residue into a 2 ml amber chromatography vial.

4.13) Dissolve the residue with the addition of 100 µl of 1,4-dioxane and 10 µl pyridine.

4.14) Add 50 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylcholorsilane 99:1% (BSTFA). Then place in an incubator at 60°C with shaking for 15 minutes.

4.15) Store samples at 4 ˚C until use.

2.2.5 GC-MS

Equip the gas chromatograph (GC) interfaced with a mass spectrometer (MS) with a non-polar capillary column and helium as the carrier gas with a flow rate of 1 ml min^{-1}.

5.1) After the oven temperature is stabilized at 50 ˚C, inject 1µl of the sample at a 1/50 split ratio.

5.2) Hold the column at 50 °C for 5 minutes and increase to 280 °C at 5 °C min^{-1}. Maintain the final temperature of 280 ˚C for 20 minutes.

5.3) Maintain the transfer line between the gas chromatograph and mass spectrometer at 300 °C.

5.4) Select a solvent delay of 15 minutes.

5.5) Record electron ionization mass spectra between the range of 10-500 (*m/z*) at electron energy of 70 eV.

5.6) Derivatize and chromatograph standard as above. Identify compounds by comparing retention times of purchased standards or data in the National Institute of Standards and Technology (NIST) mass spectral database.

Table 2-1

Materials

Name	**Company**	**Catalog Number**	**Notes**
1,4-dioxane (Certified ACS)	Fisher	D111	Flammable, preoxidizable chemical
Black Liquor	Department of Forest Biomaterials at North Carolina State University	N/A	pH 12.72, 13.67% solids
Ethanol (Microbiology Grade)	Fisher	BP2818	
Ethyl Acetate (ACS reagent grade)	EMD	EX0240-9	Flammable
Glacial acetic acid (Certified ACS)	EMD	AX0073	Corrosive
HP-5 capillary column	Agilent	19091J-577	
Hydrochloric acid (Certified ACS)	EMD	HX0603P	
N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylcholorsilane 99:1% (BSTFA)	Fluka	15238	Flammable, causes skin burns and eye damage with contact
Na_2SO_4 (FCC Grade)	VWR	BDH8026	
$NaClO_2$ (80%)	Sigma Aldrich	244155	Flammable, Toxic

Table 2-1 [Continued]

NaOH (Certified ACS)	EMD	1.06498.1000	Corrsosive
Phosphoric Acid (Certified ACS)	Fisher	A242	
Pyridine (99%)	Alfa Aesar	A12005	Flammable, toxic in contact with skin
Switchgrass	Cherry Research Farm Goldsboro, NC	N/A	Harvested August 2011
Whatman no. 1	Whatman	1001-150	

2.3 Representative Results

The collection and modification of black liquor generated in the kraft pulping process (Figure 2-1) will allow one to use this pulping waste to determine the biodegradation capacity of a single bacterial isolate or mixed culture. Figure 2-2 shows aerobic growth measured by optical density of the microbial environmental isolate cultured in LB media alone and LB media supplemented with 10% neutralized black liquor. These results indicate that this bacterium grows in the presence of neutralized black liquor. The growth of the environmental isolate without black liquor initially grows more quickly (with a generation time of 20 hours during exponential phase) but growth is not sustained beyond 62 hours gowth of the microbial environmental isolate in the presence of black liquor is slow (with a generation time of 35 hours during exponential phase); however, the culture continues to increase in optical density until after 230 hours of growth. The growth curve of the microbial environmental isolate grown in the presence of black liquor also depicts

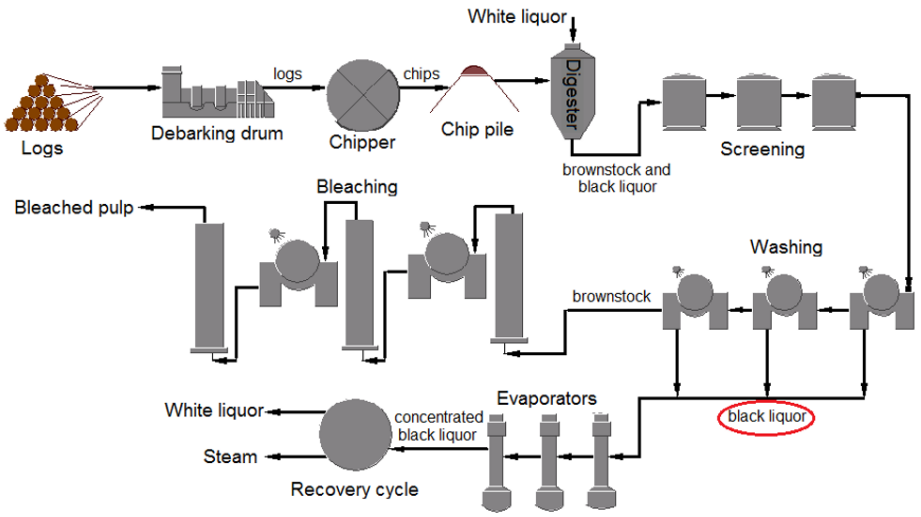

Figure 2-1 Flow diagram for a kraft pulp mill. The image depicts the processing of wood into pulp. The kraft process aims to separate lignin from its polysaccharide constituents resulting in the production of bleached wood pulp and black liquor (circled in red).

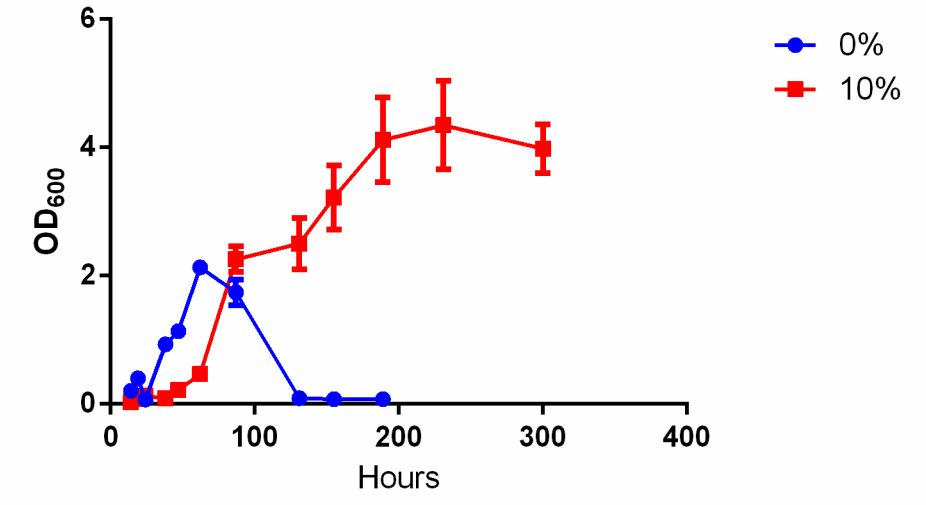

Figure 2-2 Growth of the environmental microbial isolate. Growth studies conducted aerobically in LB media (blue circles) or in LB media supplemented with 10% neutralized black liquor (v/v) (red squares) at 37 ˚C with shaking at 200 rpm. n=3

biphasic growth which suggests that the black liquor contains more than one carbon source that can be used by the microbial environmental isolate and that there is a preference of nutrient utilization exhibited by the bacterium. Further experiments are necessary to determine its ability to utilize black liquor for growth requirements. One may also determine growth of a bacterium on non-neutralized black liquor or determine the effect of increased concentrations of black liquor on growth.

After demonstrating growth in the presence of black liquor, one may determine carbon source utilization by minimal media growth experiments. Commercial substrates that make up the components of lignocellulose can be used as carbon sources in the minimal media. The lignocellulose extraction protocol presented here provides a model for determining the growth requirements of the bacterium. Figure 2-3 shows growth of the environmental microbial isolate on M9 minimal media agar supplemented with each component of the lignocellulose extraction. The presence of colonies on the minimal media agar plates indicates the ability of the environmental isolate to degrade holocellulose, cellulose, hemicellulose, and lignin. Colonies were present on M9 agar (without any carbon source added) and this may suggest the ability of the environmental isolate to degrade agar. Instead of plates, minimal media growth curves for liquid cultures could also be used to determine the specific rate of growth on each lignocellulose fraction so that agar, which could be a potential carbon source, would not have to be added.

The analysis of extracted and derivatized bacterial cultures by GC-MS reveals that metabolic products are produced and black liquor components are degraded. Figure 2-4A

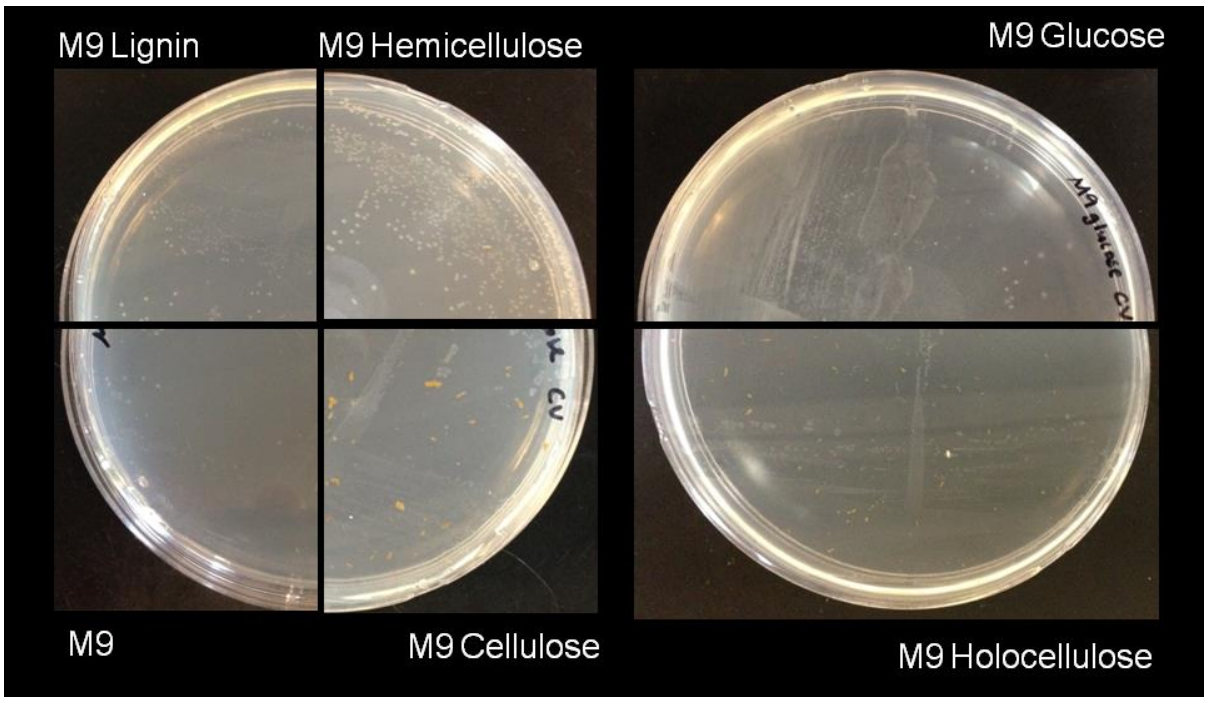

Figure 2-3 Determination of the ability of an environmental microbial isolate to use specific wood -derived carbon sources for growth. An overnight culture of an environmental microbial isolate was plated on M9 minimal media agar supplemented with lignocellulose extraction products. M9 minimal media supplemented with glucose served as a positive control while M9 minimal media without a carbon source served as a negative control. The environmental microbial isolate, producing white semi-translucent colonies, can use holocellulose, cellulose, hemicellulose, and lignin as the sole carbon source for growth. M9 holocellulose and cellulose plates exhibit opaque yellow-brown particles which are the insoluble fraction of the holocellulose and cellulose added to the media.

shows the spectra of TMS derivatives present in the uninoculated sample. Figure 2-4B shows the spectra of TMS derivatives produced by the environmental microbial isolate when grown anaerobically in M9 minimal media with 10% non-neutralized black liquor. Three replicates of each type of sample were run and produced similar results; the spectra shown in Figure 2-4 represent one of the replicates. Comparison of the two spectra reveals differences in the components present in the media, suggesting the presence of fermentation products and degradation of black liquor components. Figure 2-4 shows that compounds are produced as a result of microbial growth. The peak with a retention time of 39.34 appears only in the inoculated sample. The peak with a retention time of 21.84 increased in the inoculated sample compared to the uninoculated sample. The peak with a retention time of 25.79 decreased in the inoculated sample. These results can then be compared with mass spectrometry libraries or standard compounds prepared the same way to determine the identity of the compounds produced by microbial fermentation. Figure 2-4C shows the spectra of a guaiacol standard which was used to identify the peak with a retention time of 25.79 minutes.

2.4 Discussion

This protocol describes a combination of techniques that aims to identify microorganisms that can degrade pulping waste, the carbon sources utilized during growth on pulping waste, and the microbial metabolic products produced when grown on pulping waste. We have shown the success of this protocol with the microbial environmental isolate: a

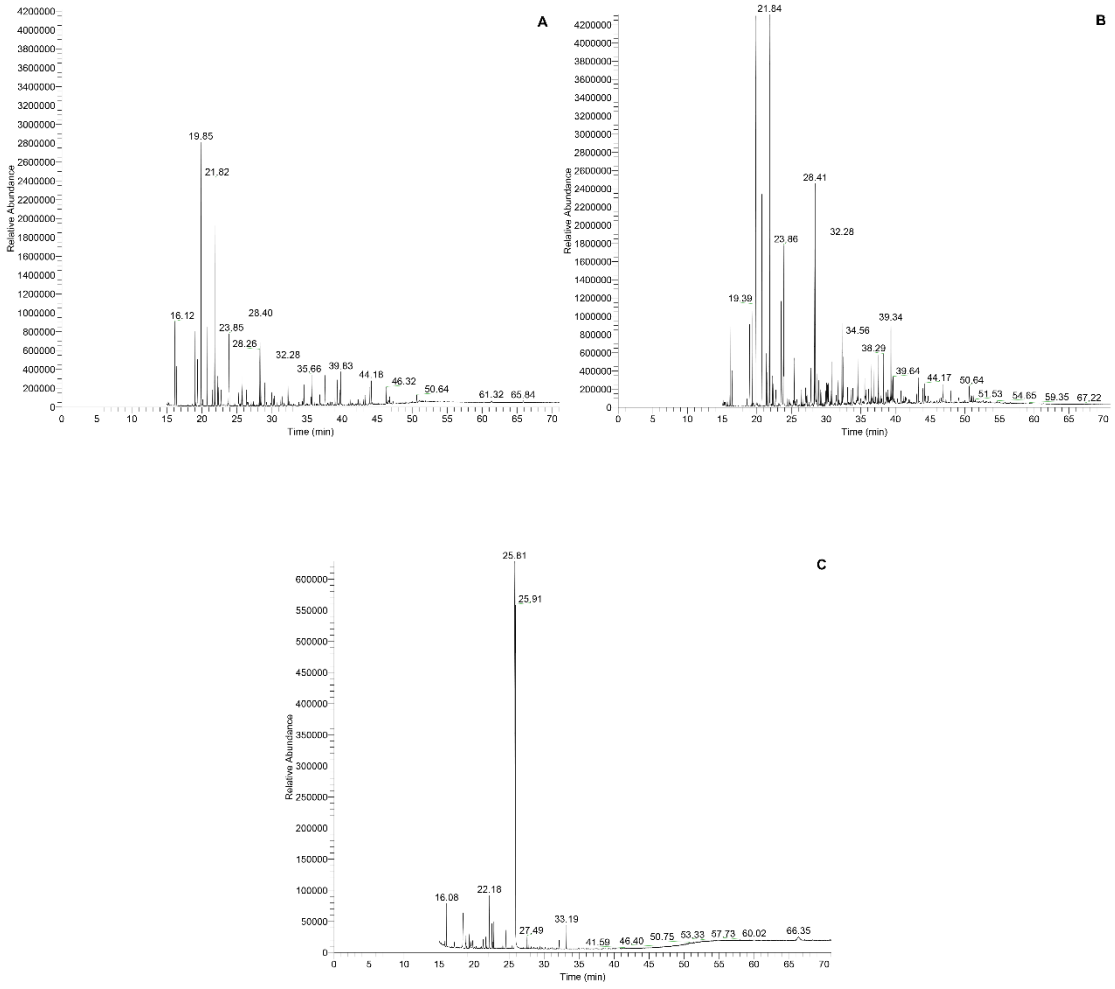

Figure 2-4 Chromatographs of TMS derivatives. A) uninoculated M9 minimal media 10% black liquor B) environmental microbial isolate grown for 450 hours in M9 minimal media supplemented with 10% black liquor under anaerobic conditions at 37 ˚C with shaking at 200 rpm. C) Trimethylsilyl derived guaiacol.

facultative anaerobe which can grow on 10% black liquor and use the lignocellulose extraction components as sole carbon sources for growth. This protocol could be used to determine the potential to degrade pulping waste by other microorganisms or microbial consortiums which have lignocellulolytic potential.

Approximately 1 L of black liquor was collected at a time; however, the amount collected should be determined by the needs of the experiments and the access one has to collect black liquor. While collecting black liquor, be sure to wear safety glasses and wear heat-resistant gloves to hold the collection bottle. In this protocol, the black liquor was neutralized to facilitate growth of the microbial environmental isolate used in this experiment; however, it may be that a different pH would enable optimum growth of other microorganisms. It might also be that other microorganisms are able to grow in a higher percentage of pulping waste. The dark color of this pulping waste interferes with optical density readings at high concentrations, and therefore, different methods should be used to determine cell growth (such as viable cell count or microscopy techniques).

The extraction process outlined here can be applied to other forms of biomass with variable particle sizes, but these changes may affect the yield. Minimal media agar studies were used here to provide qualitative information about the ability of this microbial environmental isolate to degrade the lignocellulose extraction components. This method was chosen for its simplicity. The minimal media agar studies could be supplemented with liquid minimal media growth studies to determine the specific growth rate on the lignocellulose extraction components. A comparison of the generation time would allow one to quantify carbon source preference. The results presented show that the microbial environmental

isolate can degrade agar, and minimal media growth curves can be used to distinguish the ability of the microbe to degrade agar from the ability to degrade the lignocellulose extract. Anaerobic growth conditions were used to analyze metabolic products because it was the view of the authors that fermentation might produce higher value by-products. Microbial growth cultures were transferred to a serum bottle during mid-log phase. The serum bottle was sealed under aerobic conditions which allowed the cells to continue growing aerobically until all the oxygen was depleted in the serum bottle. Since the microbial environmental isolate is a facultative anaerobe, we used this approach because the oxygen present in the serum bottle will be quickly used by the microorganism. However, if a different microorganism is being used that is strictly aerobic or anaerobic, the same growth conditions should be modified appropriately. This approach was chosen in an attempt to ensure that a greater amount of fermentation products were produced because of the high cell density characterized by mid log phase. The preparation of the spent culture media for GC analysis uses silylation to ensure stability of the metabolic products on the GC column used. Pyridine is essential for the silylation reaction. Incomplete silylation will result in instability of the compounds on the column. Incomplete silylation results in one major peak on the GC spectra that matches with trimethylsilyl derivitzing agent. If this occurs, add an additional 10 µl of pyridine and place the sample in an incubator at 60 °C with shaking for 15 minutes.

The experiments described are dependent upon access to black liquor. Black liquor samples used in this work were obtained from the Forest Biomaterials Department at North Carolina State University. The percentage of solids (see materials table) was determined by weight after drying in a 90 ˚C oven for 18 hours. The pH of black liquor ranges from 10-14;

neutralization was used to facilitate growth of microorganisms. Neutralization of black liquor results in the precipitation of lignin. Bacterial conversion of black liquor into value-added products would be the ideal application and thus requires that the bacteria used to degrade this pulping waste product could utilize black liquor without extensive modifications. Fermentation products such as ethanol, butanol and acetate are only some examples of compounds that could be produced from microbial degradation of black liquor, and there could be methods other than GC-MS to determine the products produced such as LC-MS or HPLC.

Unlike previous experiments analyzing the ability of microorganisms to grow on kraft lignin, the primary interest of this protocol is to determine if microorganisms can utilize pulping waste (Chandra et al. 2005; Chen et al. 2012; Bandoundas et al. 2011; Chandra et al. 2011). The extraction technique presented is advantageous as it offers a way to extract each of the constituent components from a lignocellulose source. This novel method uses $NaClO_2$ bleaching which offers increased purity over conventional extraction techniques. Lignin remaining bound to the cellulose and hemicellulose fibers gives a brownish color (Gellerdstedt 2009). Therefore, the increased whiteness of the $NaClO_2$ extraction method suggests increased purity (Figure 2-5). The centrifugation steps (2.19 and 2.29) can be completed in multiple days. To do so, remove the supernatant and store the pellet at room temperature.

Environmental and industrial wastes offer a ready supply of resources which could be converted into valuable products. Black liquor is an example of waste from the pulping process. Black liquor is currently used to create steam and energy in the recovery boiler;

however, the complex structure of lignocellulose provides sufficient chemical diversity to support conversion of its carbons into a variety of value-added products. The combination of techniques described is aimed at identifying and characterizing the microbial degradation of black liquor with the goal of identifying a microorganism that has the ability to produce valuable by-products from pulping waste.

Figure 2-5 Comparison of bleaching methods. A) Dried hollocellulose using $NaClO_2$ bleaching B) dried hollocellulose using NaOH bleaching. Comparison of color indicates increased delignification when $NaClO_2$ is used as a bleaching agent.

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We would like to thank Jim McMurray for providing the black liquor and Dr. David Tilotta and August Meng for their help with GC-MS. Support for Stephanie L. Mathews was provided by a USDA National Needs Fellowship (award number 2010-38420-20399).

Disclosures
The authors declare that they have no competing financial interests.

http://www.jove.com/video/51373/methods-for-facilitating-microbial-growth-on-pulp-mill-waste-streams

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CHAPTER 3

Isolation of *Paenibacillus glucanolyticus* from Pulp Mill Sources with Potential to Deconstruct Pulping Waste

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Abstract

Black liquor is a pulping waste generated by the kraft process that has potential for downstream bioconversion. A microorganism was isolated from a black liquor sample collected from the Department of Forest Biomaterials at North Carolina State University. The organism was identified as *Paenibacillus glucanolyticus* using 16S rRNA sequence analysis and was shown to be capable of growth on black liquor as the sole carbon source based on minimal media growth studies. Minimal media growth curves demonstrated that this facultative anaerobic microorganism can degrade black liquor as well as cellulose, hemicellulose, and lignin. Gas chromatography-mass spectrometry was used to identify products generated by *P. glucanolyticus* when it was grown anaerobically on black liquor. Fermentation products which could be converted into high-value chemicals such as succinic, propanoic, lactic, and malonic acids were detected.

KEYWORDS: Biodegradation (bacterial degradation); pulp mill waste; black liquor; cellulose; hemicellulose; lignin; GC-MS; *Paenibacillus glucanolyticus*

3.1 Introduction

The pulping of wood is a chemically intensive process that has been optimized over many years to create a system with minimal waste. However, some outputs of this process could be used to produce valuable product(s) (other than steam and heat). An example of an underutilized material is black liquor. It is generated from the kraft process which is responsible for 85% of world lignin production (Tejado et al., 2007). The kraft process (Figure 3-1) uses temperature (160-200 ˚C), pressure (120 psig), and the chemicals contained in white liquor (sodium hydroxide and sodium sulfide) to dissolve lignin from wood fibers (Biermann, 1996; Brannvall, 2009). Black liquor contains lignin, organic acids, and polysaccharide degradation by-products. Currently, black liquor is incinerated in the recovery boiler to produce steam for thermal energy and to recover chemicals for reuse in the pulping process. However, the amount of black liquor created by pulping can exceed the amount of black liquor that the recovery boiler can effectively process. Disposing of the black liquor as effluent negatively affects aquatic flora and fauna and thus is not an option. Isolation of microorganisms within the pulp mill that use black liquor for growth would be beneficial in terms of increasing chemical recovery and generation of value-added product(s) that would improve the overall life cycle analysis of the pulping system. The US Department of Energy (US DOE) published a list of chemicals that can be produced from biomass and converted into high-value chemicals. Among the top 30 are succinic, propanoic, lactic, and malonic acids which could be potentially produced by microbial fermentation (Werpy and Peterson, 2004). However, the recalcitrant nature of the lignocellulose, the chemical derivatives which constitute the majority of available organic carbon within black liquor, and the basic

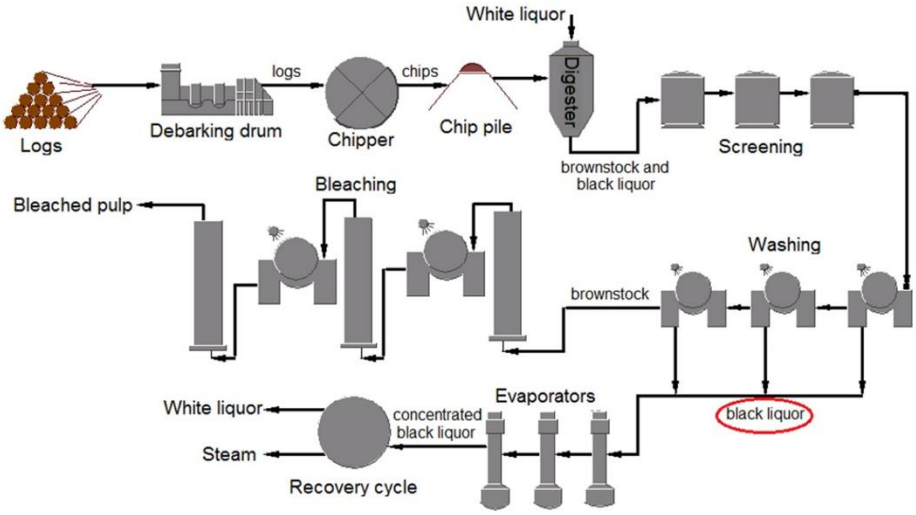

Figure 3-1 Flow diagram for a kraft pulp mill showing the process by which lignocellulosic biomass is processed into pulp. The pulping waste black liquor is produced as a result of the kraft process. The stage at which black liquor could be utilized for production of high-value production is indicated by the red oval.

pH of black liquor present challenges to microbial biodegradation of this material.

The degradation of black liquor may involve enzymes with the ability to degrade lignin, cellulose, and hemicellulose. Many microorganisms can degrade the polysaccharide components in black liquor, but not the lignin. Primarily, the complex and amorphous structure of lignin limits the biodegradation capacity of microorganisms. Lignin limits a microorganism's access to the carbon sources contained in the plant cell wall which is cellulose or hemicellulose. *Trametes elegans* is a lignin-degrading white-rot fungus that has been extensively studied that uses cellulose as its carbon source (Lara et al., 2003). Fungal lignin degradation has been studied for over 30 years, nonetheless there is no commercial biocatalytic process of lignin depolymerization (Pawlak, 2009). Bugg et al. (2011) attribute this to the challenges associated with fungal protein expression and genetic manipulation. Despite the structural complexity of lignin, some bacteria are capable of lignin degradation such as *Aneurinibacillus aneurinilyticus, Bacillus* sp, *Bacillus cereus* C10-1, *Critobacter* sp., *Nocardia, Novosphingobium* sp. B-7, *Paenibacillus* sp., *Pandoraea norimbergensis, Pseudomonas jessenii* PS06, *Pseudomonas putida, Rhodococcus jostii* RHA1, *Serratia marcescens, Sphingomonas paucimobilis* mt-2. *Streptomyces,* and *Viridosporus* T7A (Bandounas et al., 2011; Bugg et al., 2011; R. Chandra et al., 2011; Chen et al., 2012; Raj et al., 2007).

Bacillus glucanolyticus was first isolated from environmental soil samples by Alexander and Priest (1989). This gram positive, rod-shaped, facultative anaerobic bacterium is characterized by its terminal spore formation, motile colonies, and ability to degrade a variety of β-glucans (Alexander and Priest, 1989). *B. glucanolyticus* was shown to be capable

of hydrolyzing carboxymethyl cellulose (β, 1-4 linked glucose), curdlan (β, 1-3 linked glucose), pustulan (β, 1-6 linked glucose), and xylose (Alexander and Priest, 1989; Kanzawa et al., 1995). *Bacillus glucanolyticus* was renamed *Paenibacillus glucanolyticus* in 1997 by Shida et al. (1997), based on 16s rRNA gene similarity. There has been no subsequent work published on *Paenibacillus glucanolyticus*. However, bacteria within the genus *Paenibacillus* have been found to degrade black liquor components like cellulose and xylan (Ko et al., 2007).

The aims of these experiments were to 1) isolate a microorganism from the pulping waste black liquor, 2) identify the microorganism isolated, 3) characterize its growth on black liquor and black liquor constituents, and 4) identify fermentation products produced when it is grown on black liquor.

3.2 Methods

3.2.1 Materials

Chemicals

$NaClO_2$ was purchased from Sigma Aldrich (MO) and $CaCl_2$ from VWR (PA). NaOH was obtained from EMD Millipore (MA). Switchgrass was sourced from Cherry Research Farm (Goldsboro, NC). Filter paper (size 1) was purchased from Whatman (UK). Ampicillin, glucose, and isopropyl-β-D-thiogalactopyranoside (IPTG) were supplied by Fisher (MA). X-gal was purchased from Qiagen (CA) and xylose from Acros organics (NJ).

Solutions

1,4-dioxane (ACS grade), ethanol (microbiology grade), and H_2SO_4 (ACS grade) were purchased from Fisher (MA). Ethyl acetate, glacial acetic acid, and hydrochloric

acid were sourced from EMD Millipore (MA). Guaiacol, non-volatile acid standard mix, volatile acid standard mix, trans-caffeic acid, vanillic acid and gallic acid were obtained from Sigma Aldrich (MO) in certified reference material grade and used a standards for GC-MS analysis. N,O-Bis(trimethylsilyl)trifluoroacetamide 99:1% was purchased from Fluka (MO) and pyridine from Alfa Aesar (MA). Black liquor was collected from the NCSU Forest Biomaterials pulping labs.

Bacterial culture media

M9 minimal medium was composed of 0.04 M Na_2HPO_4, 0.02 M KH_2PO_4, 18 mM NH_4Cl, 8.6 mM NaCl, 27 µM $CaCl_2$, 1mM $MgSO_4$, 0.2% glucose according to Lech and Brent (1992). The medium was adjusted to pH 9 by the addition of 5 M NaOH. Luria-Bertani (LB) medium was prepared according to Lech and Brent (1992) and included 10 g tryptone, 5 g yeast extract, and 5 g NaCl (L^{-1} of distilled water).

Extraction and separation of switchgrass components

The extraction protocol was carried out as previously described (Mathews et al., 2013). Briefly, carbohydrate extraction was performed by sodium chlorite bleaching of switchgrass followed by filtration of lignin precipitant after which cellulose and hemicellulose were separated by base and acid precipitation. Cellulose was isolated through filtration while hemicellulose was isolated by centrifugation. Lignin was extracted from switchgrass through solubilization in a solution of NaOH and ethanol. Once separated from carbohydrates, lignin was precipitated at pH 2 and isolated by centrifugation.

3.2.2 Isolation of *P. glucanolyticus* from black liquor

A sample of black liquor from North Carolina State University Department of Forest Biomaterials was obtained. A 30 ml culture of LB media with 10% black liquor (v/v) was incubated at 37 °C for 10 days after which genomic DNA was isolated using the Qiagen Gentra Puregene Bacteria Kit (CA) and visualized by electrophoresis on a 1% agarose gel. Amplification of the 16s rRNA by polymerase chain reaction (PCR) was accomplished using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Baker et al., 2003). The PCR product was electrophoresed through a 1% agarose gel for visual inspection and then subsequently cloned into pPCRscript (Agilent Technologies LaJolla, CA) linearized by *Eco*RV restriction digestion (New England Biolabs, MA). *E. coli* XL-1 Blue (Novagen, EMD Biosciences, Germany) cells made competent by $CaCl_2$ were transformed with this construct and plated on LB agar plates supplemented with ampicillin (100 µg/ml), ITPG (400 µm) and X-gal (40 µg/ml) and incubated at 37 °C overnight. Plasmids were purified and sent for sequencing using 8F and 1492R primers. Sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) producing the best match: *Paenibacillus glucanolyticus* 98% coverage, 0.0 E-value, 99% identity.

3.2.3 Growth Studies

P. glucanolyticus was grown in LB media or M9 minimal media at 200 rpm at the specified temperature and pH. Glucose, xylose, cellulose, hemicellulose, and lignin were added as carbon sources at a concentration of 0.2% - 1% (w/v). Anaerobic cultures were transferred at late exponential phase to a sterile serum bottle sealed with a rubber stopper and

aluminum seal. An additional 0.2% carbon source was added during transfer. The serum bottle was sealed under aerobic conditions which allowed the cells to continue growing aerobically until all of the oxygen was depleted. This approach was used in an attempt to ensure efficient fermentation product generation that comes from having sufficient cell density at the onset of anaerobic growth.

Black liquor was collected from the kraft digester, allowed to cool, and subsequently filter sterilized using a 0.45 μm polyethersulfone (PES) membrane filter. Black liquor was added to the media at 10% (v/v). Growth was measured by optical density at 600 nm using a Biorad SmartSpec 3000 (CA). Samples of uninoculated media were used as a blank. Microbial growth was not detected in uninoculated samples of media supplemented with black liquor.

3.2.4 HPLC detection of ethanol

Uninoculated and inoculated cultures were analyzed for fermentation products by HPLC. Cultures were centrifuged at 9,500 rpm for 30 minutes and supernatant was filter sterilized through 0.22 μm PES membranes and analyzed using a Dionex UltiMate 3000 HPLC equipped with an Aminex HPX-87 column (Biorad, CA). The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.6 ml min^{-1}. The column temperature was maintained at 60 °C. The refractive index was recorded at 512 μRIU**.**

3.2.5 GC-MS analysis

Samples were prepared according to Raj et al. (2007). Uninoculated and inoculated cultures (50 ml) were centrifuged at 9,500 rpm for 30 minutes after 450 hours of growth, and the supernatant was acidified with concentrated HCl to pH 1-2. The supernatant was then

mixed with three volumes of ethyl acetate. The organic layer was collected and dewatered over anhydrous Na_2SO_4. It was then filtered through Whatman no. 1 filter paper and dried using a rotovap. Ethyl acetate extraction residues (3 mg) were dissolved in 100 µl of 1,4-dioxane and 10 µl pyridine. Samples were derivatized by the addition of 50 µl of BSTFA and incubated at 60°C with shaking for 15 minutes.

An aliquot of 1µl was injected into the ThermoFinigan trace gas chromatograph (GC) (Fisher, MA) interfaced with a Polaris Q mass spectrometer (MS) for separation by an Agilent HP-5 capillary column (60 m x 0.18mm internal diameter, 0.18 µm thickness). Helium was used as a carrier gas with a flow rate of 1 ml min^{-1}. The column was held at 50 °C for 5 minutes and increased to 280 °C by 5 °C min^{-1} and held for 20 minutes. The transfer line was maintained at 300°C. A solvent delay of 15 minutes was selected. Electron ionization mass spectra were recorded at 10-500 (*m/z*) at electron energy of 70 eV. Standard compounds were derivatized and chromatographed as above. Compounds were identified by comparing retention times of purchased standards or data in the NIST library.

3.3 Results and Discussion

3.3.1 Growth Characterization

Paenibacillus glucanolyticus optimal growth conditions

Paenibacillus glucanolyticus was previously isolated and described by Alexander and Priest (1989) as a facultative anaerobe characterized by long thin cells that form flat, smooth, opaque and motile colonies which produces terminal spores. In the current study, the *P. glucanolyticus* strain isolated from black liquor was evaluated for growth in response to pH (Figure 3-2A) and temperature (Figure 3-2B) and was found to have an

optimal growth pH of 9.0 and a temperature of 37 °C, respectively, when it was grown aerobically in LB. Table 3-1 lists the generation times for the conditions tested. Growth characteristics also provide additional information about the application of this bacterium to the pulping waste black liquor. Black liquor is characterized by an alkaline pH (10-14) (Mishra and Thakur, 2012), and these results suggest that black liquor should be diluted or acidified for optimal growth of *P. glucanolyticus.*

Growth requirements of *P. glucanolyticus*

Growth of *P. glucanolyticus* was analyzed in M9 minimal media with 10% black liquor to determine the media components necessary for microbial growth. Table 3-2 shows that *P. glucanolyticus* can grow in the presence of black liquor, use black liquor as a carbon source, and use black liquor as a fixed nitrogen source. Previous work has also demonstrated that other microorganisms can degrade black liquor such as *Serratia marcescens, Klebsiella pneumoniae, Citrobacter* sp., *Aeromonas formicans,* and *Trametes versicolor* when supplemented with carbon and nitrogen (R. Chandra et al., 2011; Font, et al., 2003; Gupta et al., 2001). Only Yang et al., (1995) showed bacterial degradation of the diluted pulping waste black liquor by *Bacillus* sp. Similarly, *P. glucanolyticus* requires no additional nutrients as it can grow on diluted black liquor alone (in deionized water).

Minimal media growth studies

Black liquor may contain a variety of carbon sources including lignin, cellulose, hemicellulose, and polysaccharide degradation products like glucose and xylose. Thus, growth of *P. glucanolyticus* was measured in M9 minimal media supplemented with a single carbon source for black liquor, glucose, xylose, cellulose, hemicellulose, and lignin.

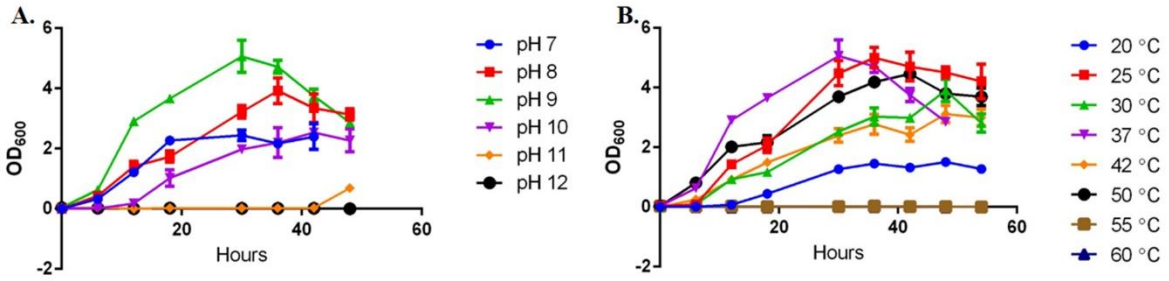

Figure 3-2 Growth curves of *P. glucanolyticus* in 30 ml LB media. A) Growth curve to dermine optimal pH for growth B) Growth curve to determine optimal temperature for growth under aerobic conditions with shaking at 200 rpm. n=3

Table 3-1

Generation time of *P. glucanolyticus* during log phase in response to pH and growth temperature.

Conditions	Generation Time (hours)
pH 7	13.2
pH 8	8.2
pH 9	5.7
pH 10	10.3
pH 11	11.23
pH 12	39.2
20˚C	16.7
25˚C	8.6
30˚C	7.15
37˚C	5.7
42˚C	9
50˚C	10.2
55˚C	67
60˚C	67

Table 3-2

Growth levels of *P. glucanolyticus* when cultured in various media.

Media	Hours of microbial growth	OD_{600}
M9 minimal media[a] pH 9 10% BL	144	+++[b]
M9 pH 10 10% BL without additional sugar source (glucose)	144	++
M9 pH 9 10% BL without additional fixed nitrogen source (NH_4Cl)	144	++
Molecular grade water 10% black liquor	264	+

[a]0.04 M Na_2HPO_4, 0.02 M KH_2PO_4, 18 mM NH_4Cl, 8.6 mM NaCl, 27 µM $CaCl_2$, 1mM $MgSO_4$, 0.2% glucose.
[b]+ OD_{600} < 0.2, ++ OD_{600} 0.2-0.5, +++ OD_{600} > 0.5.
BL= Black liquor

Figure 3-3A depicts aerobic growth; Figure 3-3B shows anaerobic growth in M9 minimal media with 0.2% carbon source. Figures 3-3C and 3-3D demonstrate the growth of *P. glucanolyticus* when cultured on 0.2-1% hemicellulose and lignin. *P. glucanolyticus* is capable of growing aerobically and anaerobically on black liquor, glucose, xylose, cellulose, hemicellulose, and lignin as sole carbon sources. While black liquor is the preferred carbon source under aerobic conditions, growth on glucose produced the highest optical density under anaerobic conditions. Aerobic growth on black liquor exhibited biphasic growth which suggests that this bacterium can use more than one carbon source present in this pulping waste. *P. glucanolyticus* was also capable of aerobic growth on cellulose, hemicellulose and

lignin but produced lower optical densities than when grown anaerobically. Increased amounts of hemicellulose and lignin resulted in increased optical density which suggests nutrient limitation was responsible for the low optical densities seen in Figures 3-3A and 3-3B. The ability of *P. glucanolyticus* to degrade the carbon sources tested here suggests that this bacterium has the ability to degrade the major components of lignocellulosic biomass.

P. glucanolyticus appears to produce enzymes capable of degrading the carbon sources used in this study (black liquor, glucose, xylose, cellulose, hemicellulose, and lignin) under both aerobic and anaerobic conditions as indicated in Figure 3-3. Another

Paenibacillus, P. curdlanolyticus, can degrade cellulose and hemicellulose (Pakdeedachakiat, et al., 2008; Pason et al., 2006). Enzymatic degradation of lignin occurs through the action of peroxidases and laccases. Microbial lignin degradation has been confirmed for a number of α-proteobacteria, γ-proteobacteria, and actinomycetes (Bugg et al., 2011; Mishra and Thakur, 2012).

3.3.2 Fermentation analysis

P. glucanolyticus culture medium was analyzed for fermentation products by HPLC to determine potential metabolic products after 400 hours of growth. Ethanol was produced (2.19 g L^{-1}) when *P. glucanolyticus* was grown under anaerobic conditions in LB media with 10% black liquor. The amount of ethanol produced in the presence of black liquor when cultured in LB media increased by 5% when compared to ethanol produced when grown in LB media alone. These results suggest that *P. glucanolyticus* is capable of producing ethanol in the presence of black liquor. In M9 minimal media with 10 % black liquor under anaerobic conditions, 0.439 g L^{-1} of ethanol was produced which suggests that *P. glucanolyticus* has the

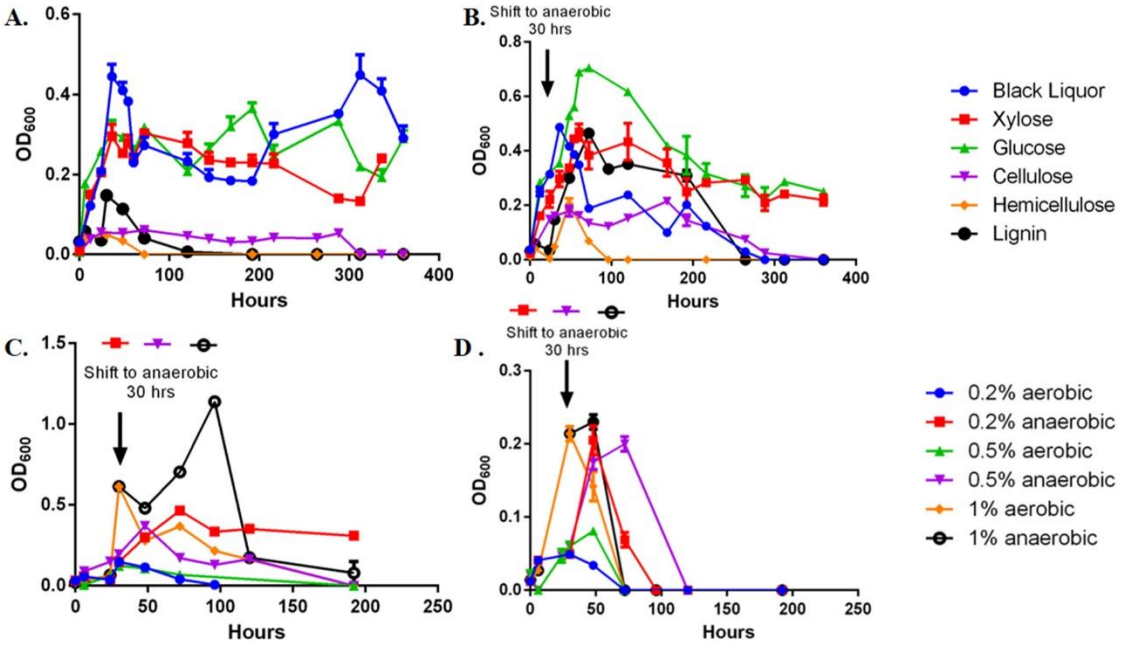

Figure 3-3 Growth of *P. glucanolyticus* on lignocellulosic carbon sources. *P. glucanolyticus* was grown in M9 minimal media, pH 9, 37 ºC supplemented with black liquor at 10% (v/v) or various carbon sources at 0.2% (w/v). A) Aerobic cultures, B) Anaerobic cultures, C) Growth of *P. glucanolyticus* on 0.2-1% hemicellulose, and D) Growth of *P. glucanolyticus* on 0.2-1% lignin. n=3

ability to anaerobically metabolize a carbon source contained within black liquor to produce ethanol.

The production of ethanol also suggests that *P. glucanolyticus* might be capable of other fermentation processes which could produce value-added by-products. Samples were also analyzed by GC-MS to determine if metabolic products of anaerobic growth on black liquor other than ethanol were produced. Table 3-3 summarizes the GC-MS results which confirm the presence of trimethylsilyl (TMS) organic acids and TMS lignin degradation products. Relative abundance of inoculated samples represents the average of three replicates. Succinic, propanoic, lactic, and malonic acids were products from the microbial fermentation of biomass that were identified by the US DOE as building blocks for high value byproducts (Werpy and Peterson, 2004). Succinic acid increased by 900%, propanoic acid increased by 500%, lactic acid increased by 400%, and malonic acid increased from 0 to

$4.0_E{}^4$ in the media supernatant when inoculated with *P. glucanolyticus*. Fermentation of black liquor by *P. glucanolyticus* also produced butyric acid which could be converted to the liquid fuel *n*-butanol. In addition to these value added products, vanillic acid and gallic acid were produced. Minimal media growth curves (3.1.3) demonstrated the ability of *P. glucanolyticus* to degrade lignin, and the presence of these compounds are indicative of the degradation of lignin present in black liquor. Previous work has also used GC-MS to identify metabolic products of kraft lignin degradation. Chandra et al. (2007), Raj et al. (2007), and Chen et al. 2012) demonstrated that other bacteria produced low molecular weight alcohols

Table 3-3

Compounds identified by GC-MS as TMS derivatives from *P. glucanolyticus* anaerobic growth cultures with 10% black liquor.

Retention Time (min)	Relative abundance					Identified Compounds
	Uninoculated Control		*P. Glucanolyticus* 10% Black liquor		% Change	
19.53	+	$6.0_E{}^4$	+	$2.0_E{}^5$	250% increase	Oxalic acid[a]
19.85	+	$2.0_E{}^5$	+	$1.0_E{}^6$	400% increase	DL-Lactic acid[a]
20.04	+	$2.0_E{}^4$	+	$4.0_E{}^4$	100% increase	Hexanoic acid[a]
20.71	+	$2.5_E{}^4$	+	$5.0_E{}^5$	1800% increase	Acetic acid[b]
21.82	+	$1.8_E{}^5$	+	$8.0_E{}^5$	350% increase	Butyric acid[b]
23.85	+	$7.0_E{}^4$	+	$4.0_E{}^5$	500% increase	Propanoic acid[b]
25.40	-	-	+	$4.0_E{}^4$		Malonic acid[a]
25.79	+	$5.0_E{}^4$	+	$4.0_E{}^4$	20% decrease	Guaiacol[a]
28.26	+	$2.5_E{}^4$	+	$3.0_E{}^5$	900% increase	Succinic acid[a]
39.32	+	$3.0_E{}^4$	+	$2.0_E{}^5$	600% increase	Vanillic acid[a]
42.28	-	-	+	$4.0_E{}^4$		Gallic acid[a]
46.37	+	$5.0_E{}^4$	+	$4.0_E{}^4$	20% decrease	*t*-Caffeic acid[a]

[a]Match based on purchased standard. [b]Match based on data from NIST library.

and lignin degradation products by analyzing culture media of *Bacillus* sp. ITRC S_8, *Paenibacillus* sp. (ITRC S_6, *Novosphingobium* sp. B-7, *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus*, and *Bacillus* sp. when grown on a different carbon source: kraft lignin. These studies showed that microorganisms can ferment kraft lignin to produce similar products to those identified in this study including propanoic acid (*Paenibacillus* sp., *Aneurinibacillus aneurinilyticus*, and *Bacillus* sp.), lactic acid (*Novosphingobium* sp. B-7), gallic acid (*Aneurinibacillus aneurinilyticus*) and, vanillic acid (*Novosphingobium* sp. B-7). This study is the first to identify bacterial fermentation products produced when grown on black liquor as the sole carbon source. Additionally, succinic, malonic and butyric acids have not been identified as bacterial metabolic products from growth on pulping byproducts.

Future experiments will analyze the products made by this microorganism when grown on the components of black liquor to determine the source of these value-added products. Future experiments will also attempt to use industrially relevant conditions (pH, temperature and scale of microbial cultures) to determine the amount of products that can be generated by *P. glucanolyticus*.

3.4 Conclusions

This study characterized the growth of *P. glucanolyticus* isolated from the pulping by-product black liquor. *P. glucanolyticus* grows optimally at pH 9 and 37℃ and can use black liquor as a carbon and fixed nitrogen source. *P. glucanolyticus* can also use glucose, xylose, cellulose, hemicellulose, and lignin as sole carbon sources under aerobic and anaerobic conditions. Finally, *P. glucanolyticus* can metabolize black liquor to produce value-added products including ethanol, succinic, propanoic, lactic, and malonic acids. These

results suggest that *P. glucanolyticus* could be used to convert the pulping waste black liquor into useful products such as chemicals or fuel.

The GC chromatograms shown in Supplementary Figure 3-1 display the differences between the TMS organic acids present in the uninoculated and inoculated samples while also demonstrating that additional unidentified compounds were present in the inoculated sample.

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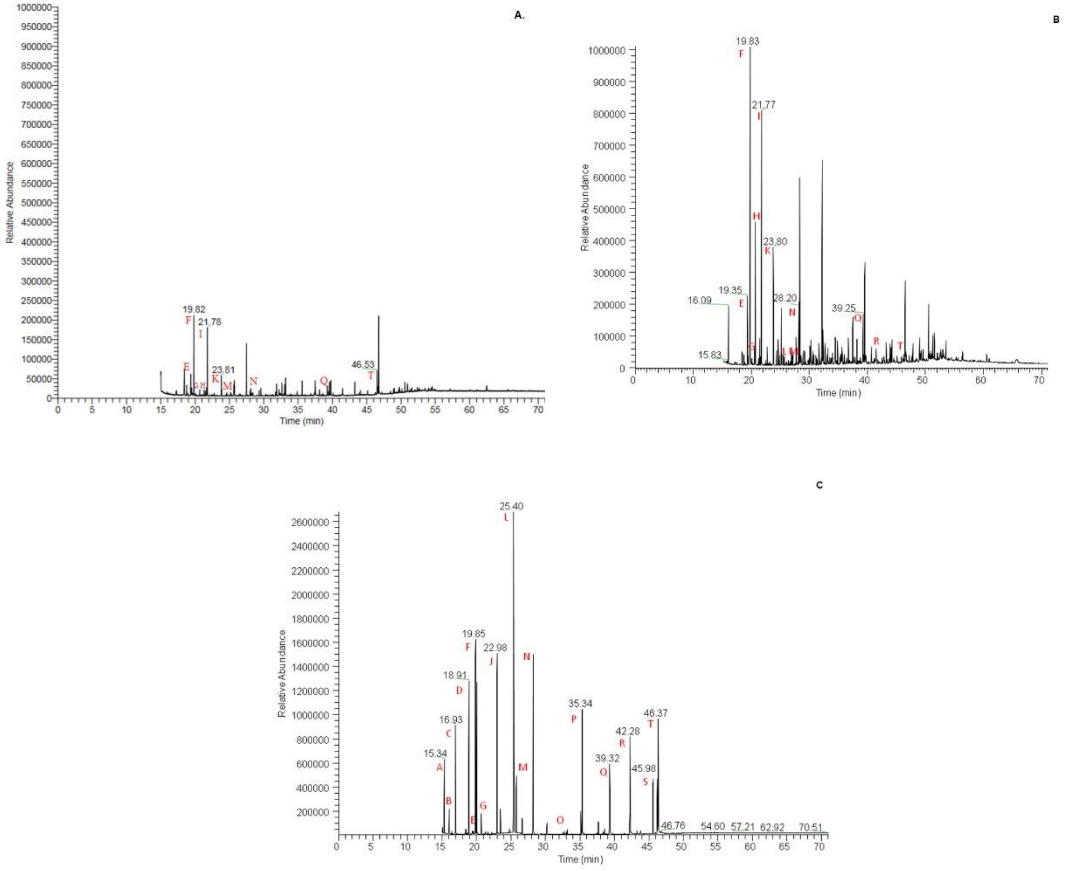

Supplementary Figure 3-1 Spectra of TMS derivatives. A) M9 minimal media 10% black liquor, B) M9 minimal media 10% black liquor inoculated with *P. glucanolyticus* after 450 hours of growth, C) Standards. Letters mark matching peaks and are listed with their corresponding compounds: A-Isobutyric acid, B-pyruvic acid, C-valeric acid, D-methylvaleric, E-oxalic acid, F-Lactic Acid, G-Hexanoic acid, H-Acetic Acid, I-Butryic acid, J-Heptanoic acid, K-Propanoic acid, L-Malonic acid, M-Guaiacol, N-Succinic acid, O-Fumaric acid, P-3,4-dimethoxy-benzaldehdye, Q-Vanillic acid, R-Gallic acid, S-t-4-hydroxy-3-methyl cinnamic acid, and T- *t*-Caffeic acid.

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CHAPTER 4

Degradation of Lignocellulose and Lignin by *Paenibacillus glucanolyticus*

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Abstract

Lignocellulose is an abundant renewable carbon source that has been used for fuel and chemical production. These are products of polysaccharide degradation, however, these processes do not use the remaining constituent of lignocellulose: lignin. Lignin is a recalcitrant amorphous aromatic compound. *Paenibacillus glucanolyticus* SLM1, a facultative anaerobe that grows optimally at pH 9, was isolated from pulp mill waste. Initial characterization suggested this bacterium may be able to degrade lignin. This work examines the ability of *P. glucanolyticus* SLM1 and the type strain *P. glucanolyticus* 5162 to degrade lignocellulose, lignin and, aromatic lignin-related compounds using growth studies, dye degradation assays, GC-MS and, GPC. These results show that both strains of *P. glucanolyticus* can degrade aromatic lignin-related compounds under both aerobic and anaerobic conditions as well as degrade polymeric lignin under anaerobic conditions. However, only *P. glucanolyticus* SLM1 can also degrade polymeric lignin under aerobic conditions.

Keywords: lignin degradation, lignocellulose degradation, *Paenibacillus glucanolyticus*

4.1 Introduction

Lignocellulose, a component of plant cell walls, is one of the most abundant renewable sources of carbon. Lignocellulose contains lignin, cellulose and hemicellulose, (Perez et al., 2002). It is utilized as a feedstock to produce liquid transportation fuels, paper, and organic chemicals. These industrial processes make use of the polysaccharides, cellulose and hemicellulose, in the plant cell wall which are composed of repeated sugar units linked by β,1-4 bonds. The lignocellulose conversion processes degrade the polysaccharides into their constituent sugars and then the desired end products through enzymatic or thermomechanical processes. These conversion processes typically produce lignin as a byproduct.

Lignin is an amorphous highly branched polymer present in vascular plants, which accounts for up to 30% of the dry weight of the plant biomass (Bugg, 2011b). In the plant cell wall, the function of lignin is to provide rigidity and resistance from compression. It is present in the cell wall bound to cellulose and hemicellulose (Crawford and Crawford, 1976; Perez et al., 2002). Unlike other polymers, lignin does not have a readily hydrolyzable bond at periodic intervals along a linear backbone. Lignin is formed when the aromatic amino acids phenylalanine and tyrosine are polymerized by plants through oxidative mechanisms to form the phenylpropane units known as monolignols (Lewis et al., 1998; Chakar and Ragauskas, 2004). There are three types of monolignols: coniferyl alcohol with an aryl-OCH3 group (known as guaiacyl), sinapyl alcohol with two aryl-OCH3 groups (syringyl), and p-coumaryl alcohol with no OCH_3 groups (p-hydroxyphenyl) (Crawford, 1981; Chakar and Ragauskas, 2004). The composition of lignin differs between plants with respect to the

monolignols present. Softwood lignin is predominantly composed of coniferyl alcohols (80%), while hardwoods are 56% coniferyl alcohols and 40% sinapyl alcohols. Grass lignin contains more p-coumaryl alcohol than softwoods and hardwoods (up to 10%) and equal amounts of coniferyl and sinapyl alcohols (Dhillon et al., 2012). These phenylpropane units are linked by β-aryl ether, di-aryl propane, biphenyl, diaryl ether, phenylcoumarane, spirodienone, and pinoresinol bonds. Lignin dimers are then polymerized to form the polymer. The structure of lignin has been described as a "seemingly random distribution of stable carbon-carbon and ether linkages between its monomeric units (Crawford, 1981)."

While, difficult to degrade due to its complex structure, lignin is non-toxic, versatile, inexpensive, renewable, and highly available either directly from plants or as byproducts from industrial lignocellulosic conversion processes. There are many industrially-relevant products that can be produced from lignin depolymerization including foam, rubber, films, plastics, and adhesives (Hatakeyama, 2002; Feldman, 2002). Vanillin is produced from lignin and is used as a food flavoring agent (Bandounas et al., 2011). Ferulic acid is also derived from lignin and can be employed as an anti-oxidant and food additive (Philbrook et al., 2013). The varied nature of the lignin structure also suggests that it could be used as a source of organic chemicals which would offer a renewable solution to the current processes that employ petroleum in the production of these chemicals.

Industrial lignin byproducts are also difficult to degrade and use in further conversion steps because of their structural heterogeneity (Dhillon et al., 2012). Industrial lignin sources are vastly modified as they are produced using heat, pressure and/or chemicals to separate

lignin from cellulose and hemicellulose (Crawford, 1981). While lignin is the second most abundant source of renewable carbon, and has structural potential to be used to produce fuels and chemicals, it is currently underutilized owing to its recalcitrance to conversion.

Some microorganisms (fungi and bacteria) produce enzymes that can degrade lignin (Vicuna et al., 2000; Bugg et al., 2011a). The most well studied lignin-degrading organisms are fungi. However, most lignin-degrading enzymes produced by these fungi are not active at pH and temperature extremes and in anaerobic or low oxygen conditions which characterize industrial lignocellulosic processes. Some bacteria have been shown to be capable of degrading aromatic compounds and polymeric lignin: *Aneurinibacillus aneurinilyticus, Bacillus* sp., *Bacillus cereus* C10-1, *Citrobacter* sp., *Nocardia, Novosphingobium* sp. B-7, *Paenibacillus* sp., *Pandoraea norimbergensis, Pseudomondas jessinii* PS06, *Pseudomonas putida, Rhodococcus jostii* RHA1, *Serratia marcescens, Sphingomonas paucimobilis, and Streptomyces viridosporus* T7A (Bandounas, et al., 2011; Bugg et al. 2011b; Chandra et al., 2011; Chen et al., 2012; Raj et al., 2007; Zimmerman, 1990). Bacteria are more easily genetically manipulated than fungi and some bacteria have increased stability in environmental conditions such as high or low pH, oxygen limitation, and high lignin concentration (Li et al., 2009). For these reasons, bacteria have great potential for a commercial lignin biocatalytic depolymerization process. Bacterial lignin degradation occurs through the activity of extracellular enzymes which are either secreted or attached to the outer membrane. There are two ways by which bacterial enzymes might degrade the lignin polymer: a) depolymerization of lignin which releases dimeric lignin fragments that can be transported into the cell and degraded or b) dearomatization of the intact polymer by ring

cleavage, which degrades the lignin network (Crawford 1981). *Sphingomonas paucimobilis* SYK6 is the bacterial lignin degrader which has been studied most extensively. This bacterium is able to cleave the most abundant linkage: β-aryl ether bonds, through the action of a glutathione-dependent β-etherase enzyme (Masai et al., 1989). Further oxidation produces vanillic acid which is demethylated to produce protocatechuic acid (Bugg et al., 2011a). Masai et al. (2007) has shown that *S. paucimobilis* SYK6 can degrade bi-phenyl, β-aryl ether, ferulate, vanillin, and syringaldehyde lignin structures. Bacteria known to degrade lignocellulose and lignin were described in a recent review by Mathews et al. (2015).

Bacillus glucanolyticus was first isolated from environmental soil samples by Alexander and Priest (1989). This gram positive, rod-shaped, facultative anaerobic bacterium is characterized by its terminal spore formation, motile colonies, and ability to degrade a variety of β-glucans (Alexander & Priest, 1989). *B. glucanolyticus* was shown to be capable of hydrolyzing carboxymethyl cellulose (β, 1-4 linked glucose), curdlan (β, 1-3 linked glucose), pustulan (β, 1-6 linked glucose), and xylose (Alexander and Priest, 1989; Kanzawa, et al., 1995).This strain was deposited into the DSMZ culture collection as strain 5162. *Bacillus glucanolyticus* was renamed *Paenibacillus glucanolyticus* 5162 in 1997 by Shida et al. based on 16 S rDNA gene similarity. A microorganism was isolated from the pulping waste black liquor, whose main component is lignin, and was identified by 16 S rDNA sequencing as *P. glucanolyticus* (Mathews et al. 2013). Characterization of this isolate of *P. glucanolyticus* revealed optimal growth at 37 ˚C and pH 9.0. Further growth experiments confirmed the ability of this *P. glucanolyticus* strain to hydrolyze cellulose and hemicellulose for growth and also demonstrated the ability to grow on lignin as the sole carbon source in

aerobic and anaerobic conditions (Mathews et al. 2014). These findings suggested that this isolate of *P. glucanolyticus* may produce enzymes which can break down cellulose, hemicellulose and, lignin.

The goal of this work was to further characterize the ability of this bacterium (*P. glucanolyticus* SLM1) and the type strain (5162) to degrade the components of lignocellulose. A variety of methods were used to elucidate lignocellulose depolymerization capabilities of *P. glucanolyticus.* Minimal media growth curves measured the ability of *P. glucanolyticus* to grow on polymeric lignin, fractionated lignin, and lignin like compounds, and Gas Chromatography Mass Spectrometry (GC-MS) was used to determine the metabolic products produced after growth on lignocellulose components. Gel Permeation Chromatography (GPC) was used to determine the effect of bacterial growth on chain length and molecular weight of lignin.

4.2 Methods

4.2.1 Bacterial Strains, Plasmids, and Enzymes. *P. glucanolyticus* SLM1 was isolated as described in Mathews et al. (2013). *P. glucanolyticus* 5162 was purchased from DSMZ (Germany).

4.2.2 Chemicals and Reagents. Biochoice lignin (BCL) was obtained from Domtar Inc. Lignin, cellulose and hemicellulose were extracted from switchgrass as in Mathews et al. (2013). Lignin model compounds: acetovanillone, anisoin, biphenyl, catechol, cinnamic acid, guiaciol vanillin, oxalic acid, malonic acid, and benzyaldehyde and dyes: Congo Red (CR), Xylidine Ponceau (XC), Bromocresol

Green (BG), Alizarin Yellow (AY), Brilliant Blue R (BB), Toluidene Blue O (TB) and Reactive Blue (RB), were obtained from Sigma Aldrich. Hexanes, acetone, acetyl anhydride, and tetrohydrofuran were purchased from Fisher Scientific. Woodflour was produced from birch (hardwood) species and pine (softwood) chips by milling to 0.2 mm particles (Lightsey et al. 1977).

4.2.3 Media and Growth Conditions. M9 minimal medium was used to measure growth of *P. glucanolyticus* and is composed of 0.04 M Na_2HPO_4, 0.02 M KH_2PO_4, 18 mM NH_4Cl, 8.6 mM NaCl, 27 µM $CaCl_2$, 1 mM $MgSO_4$ (Lech and Brent, 1992). Solid media was made using 15 g L^{-1} of agar. Dyes which mimic the structure of lignin were added to liquid cultures at 50 mg L^{-1} except for TB and RB which were added at 25 mg L^{-1} (Bandounas et al. 2011). These studies were conducted at 37℃, 200 rpm and pH 9 for *P. glucanolyticus* SLM1 and pH 7 for *P. glucanolyticus* 5162 according to optimal growth conditions as determined previously (Mathews et al. 2013 and unpublished). Bacterial growth was measured spectrophotometrically at λ = 600 nm using a Biorad SmartSpec 3000. Uninoculated medium samples were used as a blank.

4.2.4 Dye Decolorization Assays. Bacterial cultures (10 mL) were grown to mid log phase in LB or M9 minimal media, and Congo Red (CR), Xylidine ponceau (XC), Bromocresol Green (BG), Alizarin Yellow (AY), Brilliant Blue R (BB), Toluidene Blue O (TB) or, Reactive Blue (RB) were added in the concentrations indicated in section 2.3. Cultures were returned to incubation at 37℃ with shaking at 200 rpm. Decolorization was observed in liquid cultures spectrophotometrically

every 24 h for 5 days using the specific absorbance of each dye. Decolorization was measured as % of initial absorbance at λ max as follows AY (362 nm), CR (470 nm), BB and RB (595 nm), XC and BG (615 nm), Toluidene Blue O (635 nm). For solid assays, overnight cultures were streaked on LB and M9 agar plates and incubated at 37℃ for 10 days. Decolorization of solid media was visually inspected daily for bacterial growth and clearing zones after bacterial growth.

4.2.5 GC-MS. Samples were prepared according to Raj et al. (2007b). Uninoculated and inoculated cultures (50 ml) were centrifuged at 9,500 rpm for 30 min after 400 h of growth and the supernatant was acidified with concentrated HCl to pH 1-2. The supernatant was then mixed with three volumes of ethyl acetate. The organic layer was collected and dewatered over anhydrous Na_2SO_4. It was then filtered through Whatman no. 1 filter paper and dried using a rotovap. Ethyl acetate extraction residues (3 mg) were dissolved in 100 µl of 1,4-dioxane and 10 µl pyridine. Samples were derivatized by the addition of 50 µl of BSTFA and incubated at 60°C with shaking for 15 minutes. These samples were diluted 1:50 with 1,4-dioxane. Samples were run by the North Carolina State University Mass Spectrometry Facility. An aliquot of 1 ml was injected in the Agilent Technologies 5975 GC/MS (Santa Clara, CA) for separation by an Agilent HP-5 capillary column (30 m x .25 mm internal diameter, 0.25 µm thickness). Helium was used as a carrier gas with a flow rate of 1 ml min^{-1}. The column was held at 50 °C for 2.25 minutes and increased to 280 °C by 11.12 °C min^{-1} and held for 9 min. The transfer line was maintained at 300°C. A solvent delay of 6 min was

selected. Electron ionization mass spectra were recorded at 10-500 (*m/z*) at electron energy of 70 eV. Standard compounds were derivatized and chromatographed as above. Compounds were identified by comparing retention times of purchased standards or data in the NIST library.

4.2.6 Lignin Fractionation. Lignin fractionation was performed as described in Cui et al. (2014). BCL 100 g, was dissolved in 100 mL acetone for 6 h, and the solution was vacuum filtered using Whatman filter paper No. 2. The solid residue was rinsed with 200 mL acetone and then collected as fraction 1. The liquid fraction was brought up to 1 L with acetone and 250 mL of hexanes and stirred for 1 h. The supernatant was transferred to a new jar. The solids were washed with 20 mL hexanes and 80 mL acetone by mixing and the wash was placed into the new jar. The solids were collected as fraction 2. The supernatant was mixed with 750 mL hexanes for 1 h. The supernatant was poured off and the solid washed with a solution of 50 mL hexanes and 50 mL acetone. The solid was dried by evaporation and collected as fraction 3. The supernatant was then dried in the rotovap and redissolved in 200 mL acetone. After the addition of 800 mL hexanes, the solution was mixed for 1 h. The supernatant was discarded and the solid collected after rinsing with a solution containing 80 mL hexanes and 20 mL acetone. The solid was dried by evaporation and then collected as fraction 4.

4.2.7 Lignin Isolation. Inoculated and uninoculated M9 media with 0.2% BCL was incubated for 408 h. After bacterial culture growth, lignin was solubilized with 5 M NaOH, filter sterilized with a nylon 0.2 uM filter to remove any bacterial cells,

and lignin was precipitated with 12 N HCl and collected after centrifugation at 4700 rpm for 60 min. Conical tubes were placed into a 90℃ oven for 15 min to dry the lignin. The solid lignin powder was weighed or acetylated for GPC.

4.2.8 Lignin Acetylation. Lignin was dissolved in 500 µL of acetyl anhydride per 40 mg of lignin in a glass vial, and 500 µL of pyridine was added. The vials were covered in foil, and the reaction proceeded in the dark for 4 days. After which, the samples were resuspended in 10 mL of ethanol and evaporated using the rotovap. The samples were resuspended in 10 mL ethanol and evaporated until the smell of pyridine was no longer present. The samples were then dried under vacuum at 35℃ for 2 days.

4.2.9 Gel-Permeation Chromatography. Dried and acetylated lignin was added to tetrohydrofuran (THF) at 1 mg mL^{-1} and then filter sterilized using a 0.2 µm nylon filter. The molecular weight of the lignin sample was analyzed using a Waters GPC instrument. THF was used as the solvent at a flow rate of 0.7 mL min^{-1} at 35℃. The acetylated sample was loaded at 50 uL onto two styragel linear columns Styragel HR1 and Styragel HR5E (Waters, MA). The sample was run for 25 min with detection of absorbance at 254 and 280 nm. Polystyrene standards (820, 2330, 3680, 13200, 30000, 44000, 570000, 5185000 Da) were used for calibration.

4.3 Results

4.3.1 *Paenibacillus glucanolyticus* growth on lignocellulose and its components

Previous work showed that *P. glucanolyticus* SLM1 was capable of growth on black liquor which is produced from lignocellulose (Mathews et al. 2013). This work also demonstrated the ability of this isolate to grow on cellulose, hemicellulose and lignin as the sole carbon source under aerobic and anaerobic conditions. These findings led us to hypothesize that this bacterium may be capable of degrading the major components of lignocellulose. Table 4-1 compares the growth of *P. glucanolyticus* SLM1, isolated from black liquor, and *P. glucanolyticus* 5162, type strain, on black liquor, cellulose, hemicellulose, lignin isolated from switchgrass, and BCL. Table 4-1 shows that each strain is capable of growth on black liquor and the lignocellulose constituents tested. The fastest growth was exhibited by *P. glucanolyticus* SLM1 when grown in minimal media with black liquor or BCL as the sole carbon source under aerobic and anaerobic conditions with a generation time of 100 h. The slowest generation time (1400 h) was measured when *P. glucanolyticus* 5162 was grown on lignin under aerobic conditions.

Because these *P. glucanolyticus* strains are capable of growth on the constituents of lignocellulose, the ability of these strains to grow directly on lignocellulose was also determined. Milled wood from both softwood (pine) and hardwood (birch) species were used as the sole carbon source in M9 minimal media under aerobic and anaerobic conditions. An increase in optical density was observed for *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162 grown on softwood and hardwood woodflours under aerobic and anaerobic conditions (Figure 4-1).

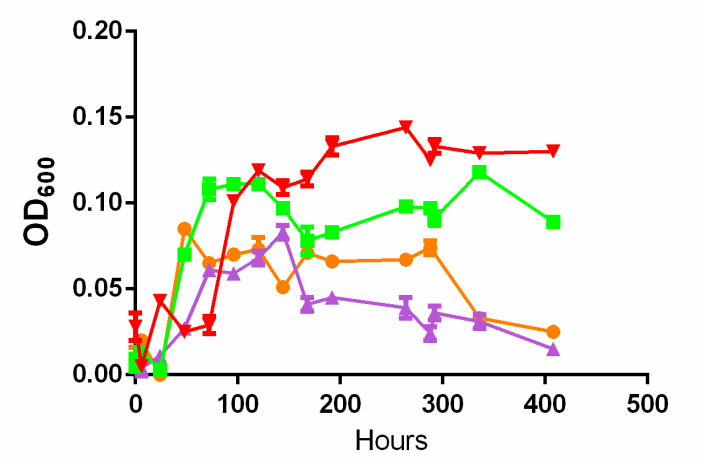
Table 4-1

Growth of *P. glucanolyticus* strains SLM1 and 5162 on lignocellulose components and lignin-like compounds as the sole carbon source in M9 minimal media.

Compound	**SLM1 growth**		**5162 growth**	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Black Liquor	++	++	+	++
Glucose	++	++	++	++
Xylose	++	++	++	+
Cellulose	++	+	++	+
Hemicellulose	+	++	++	++
Lignin	++	++	++	++
BioChoice Lignin	++	++	++	++
BCL Fraction 1	++	++	++	++
BCL Fraction 2	++	++	++	++
BCL Fraction 3	++	++	++	++
BCL Fraction 4	+	+	++	+
Acetovanillone	++	+	+	+
Anisoin	+	+	+	+
Catechol	++	+	++	+
t-Cinnamic Acid	-	-	-	-
Benzaldehyde	+	+	+	-
Guaiacol	+	+	+	+
Malonic Acid	+	+	+	-
Oxalic Acid	+	+	+	-
Vanillin	+	+	+	+

+ OD increases compared to uninoculated control
++ Generation ≤ 500 time hours

A.



B.

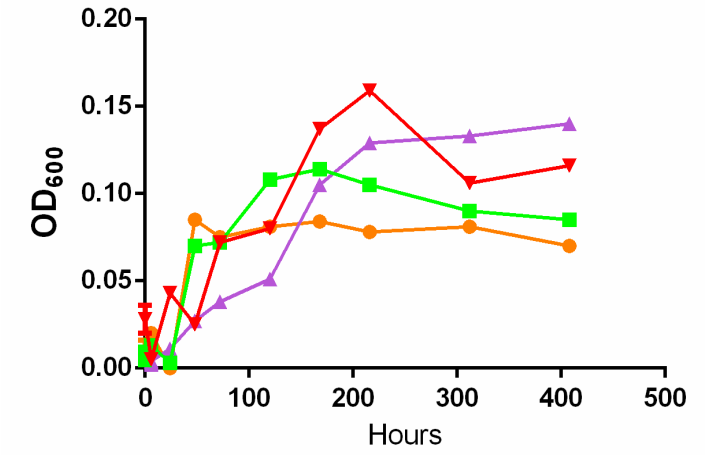


Figure 4-1 Minimal media growth studies of *P. glucanolyticus* SLM1 and 5162 cultured with woodflour under aerobic (A) and anaerobic conditions (B). Hardwood (birch, purple triangle and orange circle) and softwood (pine, red triangle and green square) milled to 0.2 mm particles were added to M9 minimal media pH 9 at 0.2% (w/v) and inoculated with 1% (v/v) of an ON culture of *P. glucanolyticus* and incubated for 408 h at 37℃ with shaking at 200 rpm. Anaerobic cultures were grown aerobically for 24 h and transferred to a serum bottle sealed with atmospheric oxygen. N = 4

4.3.2 Growth on lignin and lignin-related compounds.

Bacterial growth on lignin and lignin-related compounds was measured by optical density using a spectrophotometer. Lignin confers UV-visible light absorbance (Heitner et al., 2010) thus, uninoculated medium was used as blanks for all measurements. BioChoice lignin (BCL) was fractionated by hexanes and used as the sole carbon source for growth studies to determine the ability of *P. glucanolyticus* to grow on various sizes of the lignin polymer. Table 4-1 shows that both strains SLM1 and 5162 are capable of growth on BCL and fractionated BCL. Minimal-media growth studies were also conducted with lignin-monomers (acetovanillone, anisoin, benzaldehyde, catechol, cinnamic acid, guaiaciol and vanillin), and it was observed that both strains SLM1 and 5162 can grow on these lignin monomers with the exception of cinnamic acid (Table 4-1).

In addition to growth curves, screening for lignin degradation has been performed by Bandounas et al. (2011) using aromatic dyes with structural similarities to lignins. This method offers a simple screen with a color indication for the potential to degrade lignin-like aromatic monomers. The ability of *P. glucanolyticus* to degrade dyes was measured using solid and liquid assays. Colonies were present on rich and minimal media solid dye plates for each *P. glucanolyticus* strain. Decolorization was visualized on Congo Red, Bromocresol Green, and Toluidine Blue LB plates (Figure 4-2A). Minimal media plates were used to determine which dyes can be utilized as the sole carbon source but decolorization zones were difficult to visualize. Liquid dye decolorization assays were then performed to quantify dye degradation by the decrease in the λ_{max} of the dye used. Congo Red, Alizarin Yellow, Brilliant Blue and Reactive Blue were decolorized by both strains in rich media but little or

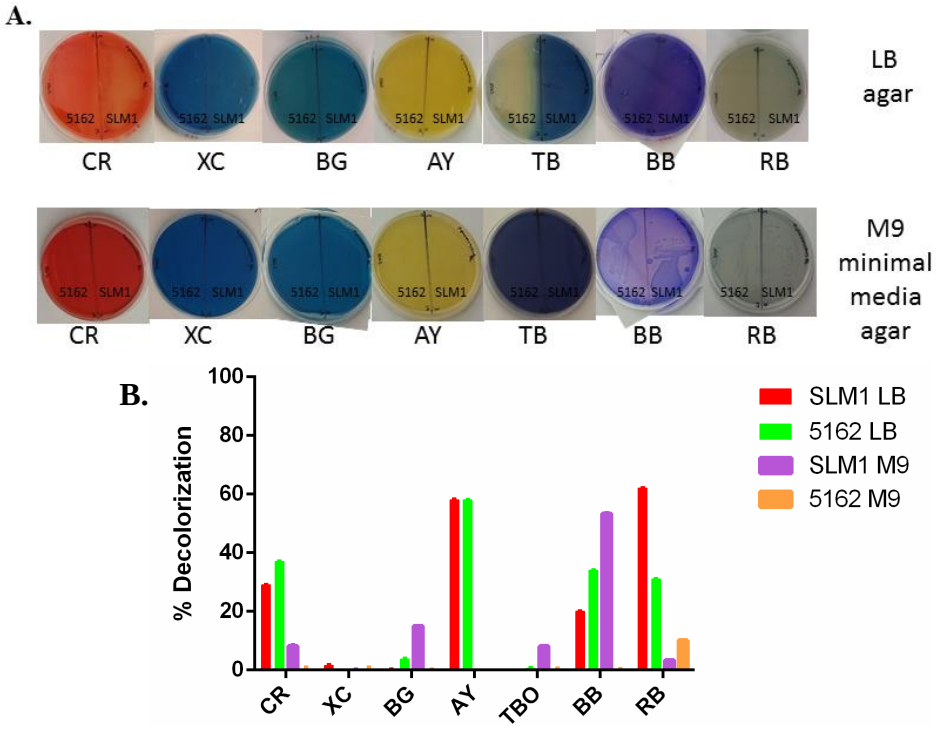


Figure 4-2 Dye decolorization assays. A. Image of decolorization zones produced by *Paenibacillus glucanolyticus* strains grow on LB and M9 agar plates with dye added at 50 mg L^{-1} for Congo Red (CR), Xylidine Ponceau (XC), Bromocresol Green (BG), Alizarin Yellow (AY), Brilliant Blue R (BB) or 25 mg L^{-1} Toluidene Blue O (TB) and Reactive Blue (RB) after incubation at 37 ℃ for for 5 days when grown on LB and 10 days when grown on M9. *P. glucanolyticus* SLM1 was inoculated on right side of the plates, *P. glu* 5162 on the left. Plates were washed with distilled water to visualize clearing zones produced as a result of dye degradation. B. Average percent decrease of λ_{max} of indicator dyes in liquid media. Liquid cultures were inoculated with *P. glucanolyticus* SLM1 or 5162 and in LB media at 37 ℃ until mid-log phase after which dye was added to liquid cultures at concentrations used in solid assays and incubation was continued for 5 days. Percent decolorization represents the average of 4 cultures, standard deviation is shown by the error bars. N = 4

no decolorization was measured in minimal media (Figure 4-2B). *P. glucanolyticus* SLM1 was able to decolorize several dyes in minimal media: Congo Red, Toluidine Blue O, Brilliant Blue and Reactive Blue, whereas, *P. glucanolyticus* 5162 was only able to decolorize Reactive Blue in minimal media.

4.3.3 Degradation of lignin and lignin-related compounds.

Previously we identified the presence of low molecular weight aromatics: vanillic and gallic acids, after growth of *P. glucanolyticus* SLM1 on black liquor as the sole carbon source (Mathews et al. 2013). Because the *P. glucanolyticus* SLM1 strain is more efficient at anaerobic growth on lignin than *P. glucanolyticus* 5162, the fermentation products produced by *P. glucanolyticus* SLM1 were determined when grown on the components of lignocellulose. *P. glucanolyticus* SLM1 produced oxalic, lactic, acetic, and propanoic acid when grown on plant cell wall carbohydrates cellulose and hemicellulose (Table 4-2). Malonic, vanillic and gallic acids were produced when grown on lignin as the sole carbon source. The production of these organic acids was also measured throughout bacterial growth (Supplemental Figure 4-1) showing that the increase in relative abundance occurs during bacterial growth.

While these results provide additional evidence of lignin degradation by *P. glucanolyticus,* the purity of lignin used in these growth studies may be questioned. To provide additional evidence of lignin degradation, gel permeation chromatography (GPC) was used to determine the weight of lignin before and after bacterial growth of both *P. glucanolyticus* strains. Since lignin is easily precipitated with acidification, lignin was collected by precipitation with HCl and the amount of lignin in inoculated and uninoculated

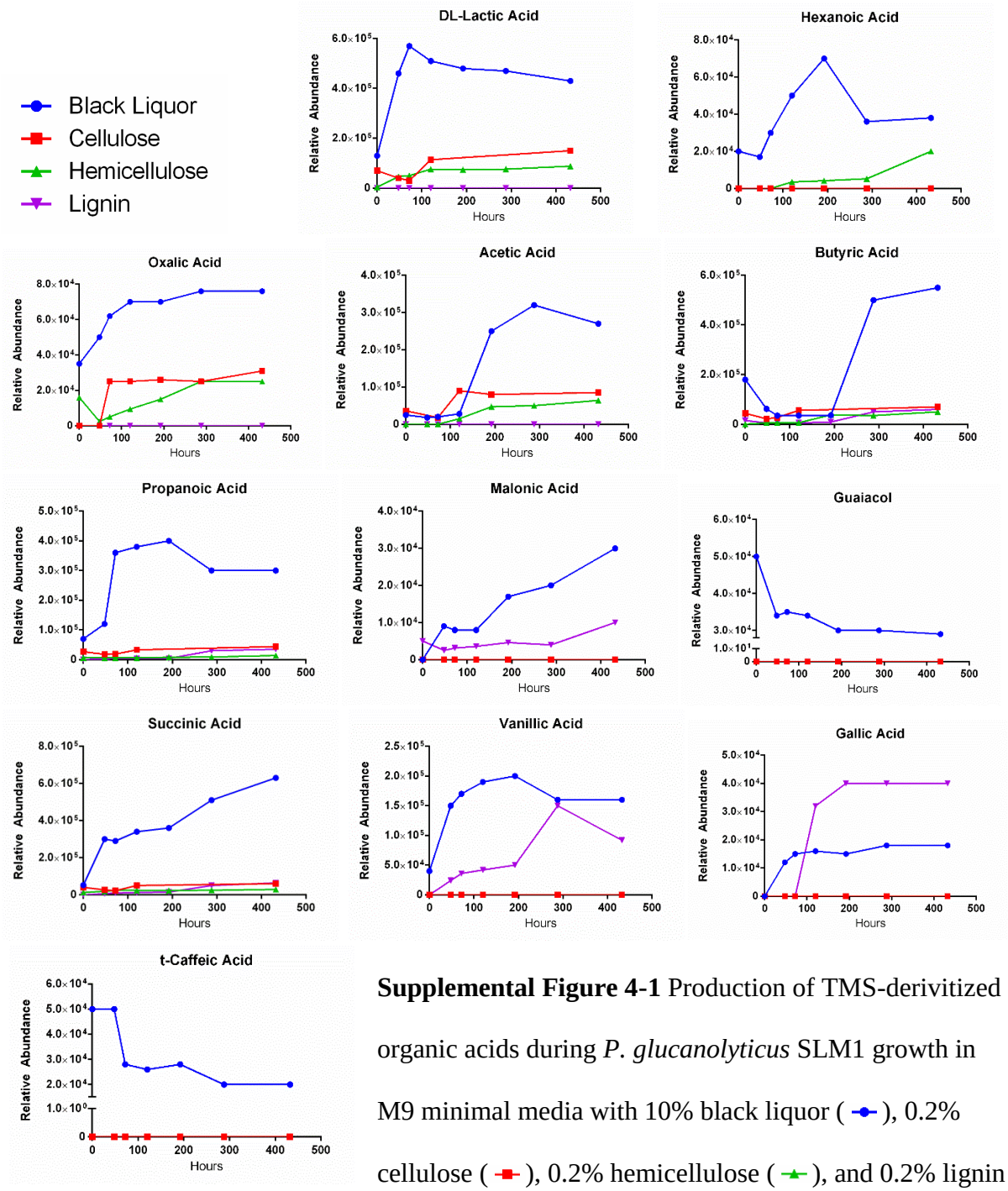
Table 4-2

Relative abundance of TMS organic acids identified by GC-MS in *Paenibacillus glucanolyticus* SLM1 culture supernatant after 400 h of growth in M9 minimal media with the specified carbon source.

Retention Time (min)	**Relative Abundance**										Identified Compound
	Uninoc. 0.2% Cell		*P. glu* 0.2% Cell		Uninoc. 0.2% Hemi		*P. glu* 0.2% Hemi		Uninoc. 0.2% Lignin		*P. glu* 0.2% Lignin		
7.46	-	-	+	3.2_E^6	-	-	+	1.8_E^6	-	-	-	-	Oxalic acid[a]
8.22	-	-	+	2.2_E^6	-	-	+	5.1_E^6	-	-	-	-	DL-Lactic acid[a]
8.26	-	-	-	-	-	-	+	1.3_E^7	-	-	-	-	Hexanoic acid[a]
8.40	-	-	+	5.0_E^6	+	1.5_E^6	+	5.6_E^6	-	-	-	-	Acetic acid[b]
9.11	-	-	+	4.5_E^6	-	-	+	3.4_E^6	+	2.1_E^6	+	6.8_E^6	Butyric acid[b]
9.23	-	-	+	6.5_E^6	-	-	+	7.8_E^6	-	-	+	4.9_E^6	Propanoic acid[b]
9.96	-	-	-	-	-	-	-	-	-	-	+	2.0_E^6	Malonic acid[a]
11.53	-	-	+	6.4_E^6	-	-	+	8.1_E^6	+	2.1_E^6	+	2.7_E^6	Succinic acid[a]
16.50	-	-	-	-	-	-	-	-	+	1.5_E^6	+	3.0_E^6	Vanillic acid[a]
16.73	-	-	-	-	-	-	-	-	-	-	+	2.4_E^6	Gallic acid[a]
18.11	-	-	-	-	-	-	-	-	+	5.7_E^6	+	2.5_E^6	Cinnamic acid[a]

[a]Match based on purchased standard. [b]Match based on data from NIST library.

P. glu (*Paenibacillus glucanolyticus* SLM1), Cell (cellulose), Hemi (hemicellulose)



Supplemental Figure 4-1 Production of TMS-derivitized organic acids during *P. glucanolyticus* SLM1 growth in M9 minimal media with 10% black liquor ( ), 0.2% cellulose ( ), 0.2% hemicellulose ( ), and 0.2% lignin ( ) under anaerobic conditions. Time 0 represents relative abundance of compounds present in uninoculated samples.

flasks was measured by weight after 400 h of growth. Lignin decreased after bacterial growth of both strains under aerobic and anaerobic conditions in M9 minimal media with 0.2% BCL (Table 4-3). GPC was also used to measure the molecular weight of lignin after bacterial growth. This technique separates lignin based on polymer size. Polymeric lignin, BCL, was fractionated and used as the sole carbon source for growth in M9 minimal media under aerobic and anaerobic conditions. The chromatographs in Figure 4-3 show increased retention time and abundance for lignin fractions after bacterial growth. Increased retention times for lignin fractions 2, 3, and 4 are most noticeable with peaks appearing after 30 min. The molecular weight and molecular average were calculated from these chromatographs (Table 4-3). Chain length and molecular weight decreased as a result of bacterial growth and indicate that lignin is depolymerized. *P. glucanolyticus* 5162 did not decrease in polymer size after aerobic growth on BCL.

Table 4-3

Lignin weight measurements from *P. glucanolyticus* SLM1 and 5162 cultures supplemented with 2 mg ml^{-1} BCL after 400 h of growth.

Sample	**lignin mg/mL**	**% Δ**	**Mn**	**% Δ**	**Mw**	**% Δ**
Uninoculated	16.7 ± 0.09		985		113660	
P. glu SLM1 aerobic	11.6 ± 0.07	-31%	770	-22%	2467	-98%
P. glu SLM1 anaerobic	12.9 ± 0.07	-28%	826	-16%	38820	-66%
P. glucanolyticus 5162 aerobic	15.6 ± 0.01	-7%	1031	+5%	150595	+32%
P. glucanolyticus 5162 anaerobic	12.7 ± 0.05	-24%	829	-16%	34763	-69%

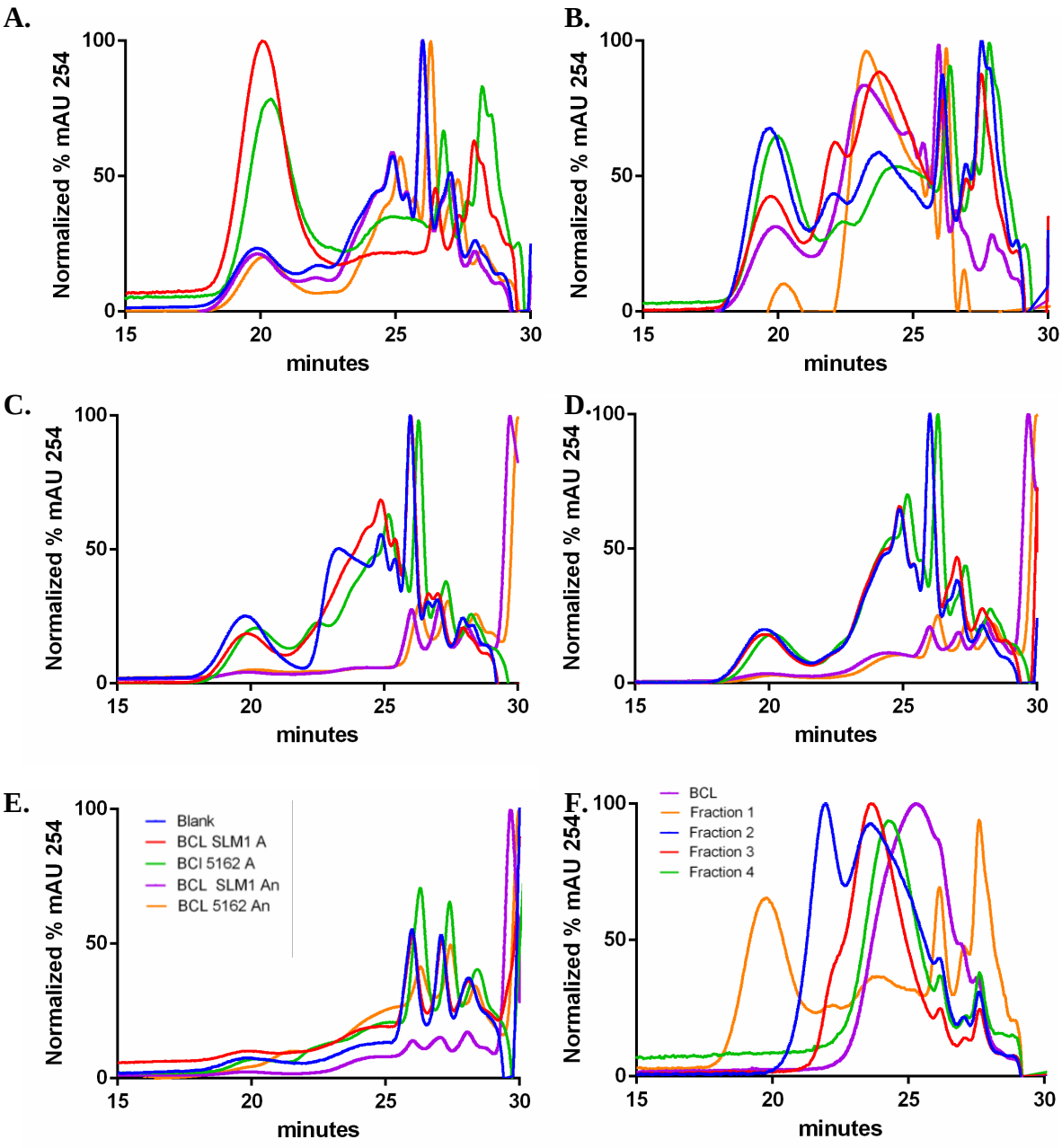


Figure 4-3 Molecular weight analysis of lignin by GPC. The effect of bacterial growth on the molecular weight of lignin was analyzed by gel-permeation chromatography. Absorbance was measured at 280 nm for four samples, and the average is shown here. Spectra are normalized to set highest value at 100% and lowest value at 0%. A. Normalized

Figure 4-3 [continued]

chromatographs of BCL after 408 h of bacterial growth. B-E. Normalized chromatographs of fractionated BCL. A-E. Blue lines represent BCL material without bacterial growth (Blank), red lines represent BCL material after aerobic growth of *P. glucanolyticus* SLM1, green line represents BCL material after aerobic *P. glucanolyticus* 5162 growth, purple line represents BCL material after anaerobic growth of *P. glucanolyticus* SLM1, and orange line represents BCL material after anaerobic growth of *P. glucanolyticus* 5162. F. Chromatogram of BCL and BCL fractions used as carbon sources for growth. Purple represents BCL, orange represents fraction 1, blue represents fraction 2, red represents fraction 3 and green represents faction 4.

4.4 Discussion

4.4.1 Growth on lignocellulose, lignin and lignin-related compounds

Growth curves were used to determine the ability of *P. glucanolyticus* SLM1 and 5162 to grow on lignocellulosic compounds as the sole carbon source. Both strains can grow on black liquor, cellulose, hemicellulose, switchgrass lignin, BCL, hardwood flour and, softwood flour. These bacteria can also grow on BCL that has been fractionated by polymer size. In these experiments, two types of lignin were used: lignin isolated from switchgrass and BCL. The method of isolation affects lignin structure and may also influence the ability of the bacteria to degrade that type of lignin (Crawford and Crawford 1976). Lignin isolated from switchgrass was used in previous research (Mathews et al. 2013), and BCL is industrially relevant as it is produced by a paper mill. These studies show that *P. glucanolyticus* can use BCL as the sole carbon source for growth and could therefore be used for the biological treatment of this industrial lignin to produce valuable byproducts.

Growth of *P. glucanolyticus* SLM1 and 5162 on lignocellulose and lignocellulosic components was slow with generation times at or over 100 hours. Previously characterized bacterial lignin degradation processes were also reported to be slow and to occur under oxidative conditions (Bungay et al., 2012). However, the basic growth studies presented in this study establish the ability of these two strains to use lignocellulose and lignocellulosic components for growth which imply that these bacteria can degrade these complex polymers. Further comparison between these *P. glucanolyticus* strains that are outside the scope of these experiments are being performed to determine the physiological and molecular differences between these *P. glucanolyticus* strains.

This present work is the first evidence that *P. glucanolyticus* 5162 can degrade black liquor and lignin. Two other bacteria, *Novospingobium* sp. B-7 and *P. glucanolyticus* SLM1, have been shown to degrade lignin as the sole carbon source; the ability of other bacteria to use these lignin sources in the presence of polysaccharides or sugars has been reviewed in Mathews et al. (2015) (Chen et al. 2012; Mathews et al. 2014).

The ability of these strains to grow on woodflours (Figure 1) under aerobic and anaerobic conditions, is the first documented evidence that bacteria, specifically *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162, can grow directly on milled wood. Lignocellulose degradation is not uncommon but this process is typically carried out by microbial communities which participate in the carbon cycle by breaking down humic matter. Fungi are the most well-known members of lignin degrading microbial communities and are able to oxidize polymeric lignin. These lignin-degrading enzymes are produced as a result of carbon limitation (Li et al. 2009). Fungi break down lignin to access cellulose and hemicellulose which are preferred carbon sources (Malherbe and Cloete 2003). Lignocellulose-composting microbial communities are responsible for degrading the components of lignocellulose, and this has been reviewed by Tuomela et al. (2000) and Sun and Cheng (2002).

Growth of *P. glucanolyticus* BL and 5162 on lignocellulose and lignin suggests that these bacteria can degrade lignin. However, lignin degradation may occur through the breakdown of lignin monomers as well as the degradation of polymeric lignin (Crawford 1981). Fungal lignin degradation pathways include the degradation of low molecular weight aromatics such as vanillyl, syringyl and coniferyl alcohols (Kawahara et al., 1980; Bugg et al., 2011a; Gonzalez et al. 1986). Thus, it may be that *P. glucanolyticus* can also break down

lignin monomers, so growth of *P. glucanolyticus* on lignin-monomers and lignin-like compounds were measured. These assays are advantageous because these substrates can be synthesized instead of being isolated from plant cell wall material which ensures the substrate purity and structure. These compounds are also more soluble than the polymeric lignin.

P. glucanolyticus strains SLM1 and 5162 are capable of growth on polymeric BCL and fractionated BCL. These strains can grow on the lignin monomers acetovanillone, anisoin, benzaldehyde, catechol, guaiaciol and vanillin. Strains SLM1 and 5162 could not grow on cinnamic acid. It may be that cinnamic acid or its degradation product(s) were toxic to *P. glucanolyticus.* These results suggest that *P. glucanolyticus* strains SLM1 and 5162 can degrade polymeric lignin and lignin-degradation products, which is an essential attribute for biological degradation and conversion of lignin and lignin-containing materials.

Dye degradation assays provide a colorimetric method by which we analyzed degradation of aromatic structures similar to lignin. Growth on LB plates with dye indicates that these strains can grow in the presence of the dyes tested. Some dyes are toxic to microorganisms; in fact Toluidine Blue is used to inactive several fungi (Donnelly et al. 2008). Our results indicated that *P. glucanolyticus* SLM2 and 5162 could grow on all of the different LB dye-containing plates. In addition, growth on dye-containing minimal media plates indicates that these bacteria can grow on the selected dyes as the sole carbon source. Decolorization was visualized on Congo Red, Bromocresol Green, and Toluidine Blue LB plates indicating that *P. glucanolyticus* can degrade these dyes. Dye degradation was also measured spectrophotometrically to quantify dye degradation. Both strains were able to decolorize dyes

when grown in rich media, but the level of decolorization was lower for the strains cultured in minimal media, for example Reactive Blue was 62% decolorized by strain SLM1 in rich media but only 3.4% decolorized in minimal media, suggesting these dyes are poor carbon sources. Strain SLM1 was able to degrade a greater number of dyes when grown in minimal media than strain 5162. The ability of SLM1 more efficiently decolorize dyes corroborates the findings in Section 3.1 that these two strains differ in their ability to degrade lignocellulose. Strain SLM1 appears to be more efficient at degrading lignin and lignin-related aromatic structures.

Reactive Blue and Toluidine Blue O are decolorized by enzymes similar to lignin peroxidases; therefore, these decolorization studies also indicate the presence of enzymes that depolymerize lignin in both strains. Dye-decolorization has been used to screen for lignin degradation capabilities in a number of studies. Harith et al. (2014) and Bandounas et al. (2011) demonstrated the ability of bacteria from the *Klebsiella* and *Enterobacter* genus, *Bacillus cereus*, and *Bacillus* sp. LD003 to decolorize Toluidine Blue. *Bacillus* sp. LD003 was also shown to grow on lignin as the sole carbon source.

4.4.2 Lignin degradation

Lignin degradation has been measured using several different techniques. ^{14}C labeled lignin can be used to measures the release of ^{14}C-CO_2 after bacterial growth but this method requires several weeks to prepare the labeled lignin substrate. GC-MS has been used to identify products of microbial growth on kraft lignin and black liquor (Raj et al. 2007; Chandra et al. 2011; Chen et al. 2012). The presence of low molecular weight aromatic compounds serves as evidence of lignin degradation. GC-MS was previously used to

characterize the ability of *P. glucanolyticus* SLM1 to degrade black liquor. In these experiments, *P. glucanolyticus* SLM1 was grown on cellulose, hemicellulose, and lignin as the sole carbons source. Anaerobic growth on these substrates produced several organic acids. Oxalic, lactic, acetic, and propanoic acids are mixed acid fermentation products. This metabolic pathway is present in other *Paenibacillus* species. Malonic, vanillic and gallic acids were only produced when grown on lignin as the sole carbon source confirming our conclusion from analysis of fermentation on black liquor, that these organic acids are produced as a result of lignin degradation. The production of organic acids was also measured throughout bacterial growth; the increase in abundance of these compounds provides additional evidence that the production of oxalic, lactic, acetic, propanoic, malonic, vanillic and gallic acids is not a result of spontaneous degradation during incubation with shaking.

Weight change analysis and GPC was also used to measure lignin degradation. Unlike GC-MS, this technique evaluates the effect of bacterial growth on the carbon source instead of the possible conversion into smaller compounds. Dry weight of the lignin decreased after bacterial growth suggesting that lignin is being degraded during bacterial growth (Table 3). The chromatographs shown in Figure 3 have noticeably larger peaks at increased retention times for the bacterial treated samples when compared to the untreated samples. The presence of these peaks indicates the production of smaller polymers of lignin as a result of bacterial growth. The quantified lignin degradation profiles as presented in Table 3 demonstrate the differences in degradation of BCL for *P. glucanolyticus* strains SLM1 and 5162under aerobic and anaerobic culturing conditions. While growth studies showed that *P.*

glucanolyticus 5162 can grow on lignin and lignin-related compounds under aerobic conditions, the GPC results suggest that this strain may not be able to degrade polymeric lignin as efficiently as SLM1 under aerobic conditions.

4.5 Conclusions

These experiments evaluated the ability of *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162 to grow on and degrade lignocellulose and lignin. Minimal media growth studies showed that *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162 can grow on hardwood flour, softwood flour, black liquor, cellulose, hemicellulose, lignin, lignin factions, acetovanillone, vanillin, catechol, oxalic acid, benzyaldehyde and malonic acid as the sole carbon source in M9 minimal media at pH 9, 37℃ under aerobic and anaerobic conditions. *P. glucanolyticus* SLM1 was also able to grow on and decolorize Congo Red, Bromocrenol Green, Toluidine Blue O, Brilliant Blue and Reactive Blue as the sole carbon source. *P. glucanolyticus* 5162 was only able to grow on and decolorize Reactive Blue as the sole carbon source. The ability of these strains to utilize lignin and lignin related structures suggest that these bacteria produce enzymes capable of degrading lignin. Additional methods were used to determine the effect of these strains on lignin after bacterial growth. Vanillic, malonic and gallic acids were present in the culture supernatant after bacterial growth of *P. glucanolyticus* with BCL lignin as the sole carbon source. These organic acids result from lignin degradation. The amount and weight average molecular weight of lignin were also measured after bacterial growth. The weight of lignin (BCL) decreased after 400 h of growth under aerobic and anaerobic conditions for both strain SLM1 and 5162. After growth on BCL, the lignin was also extracted, acetylated and separated by GPC showing that growth of

P. glucanolyticus SLM1 decreased the molecular weight after aerobic and anaerobic growth, while lignin weight average molecular weight only decreased after growth of *P. glucanolyticus* 5162 under anaerobic conditions. This study describes several experiments which show that *P. glucanolyticus* SLM1 can degrade polymeric lignin and aromatic lignin-related compounds under aerobic and anaerobic conditions. *P. glucanolyticus* 5162 can degrade aromatic lignin-related compounds under aerobic and anaerobic conditions but may not be able to degrade polymeric lignin under aerobic conditions. Future experiments will seek to determine additional physiological differences between these strains by direct comparisons of bacterial growth including optimal growth temperature, pH and generation time of lignocellulosic sources. Future experiments will also seek to optimize growth conditions for lignin and lignocellulose degradation for the biological treatment of lignocellulose or lignin waste streams produced by industries that utilize lignocellulose as the feedstock.

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CHAPTER 5

Genome Sequence and Characterization of Two Strains of *Paenibacillus glucanolyticus* That Degrade Lignocellulosic Biomass

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Abstract

In this study the physiology and genomic sequences of two strains of *Paenibacillus glucanolyticus* were compared. *P. glucanolyticus* 5162 was isolated from soil, while *P. glucanolyticus* SLM1 was isolated from a wood pulp mill process stream known as black liquor. Growth curves showed different pH preference with strain SLM1 growing optimally at pH 9 and strain 5162 at pH 7. Both strains can grow on black liquor and the components of lignocellulose including glucose, xylose, cellulose, hemicellulose and lignin. These minimal media growth curves suggest there are additional differences between the strains in their ability to degrade lignocellulosic components. We hypothesize that this is due to differences in the enzymes produced by each strain. Illumina MiSeq and PacBio technologies were used for genomic sequencing. These techniques did not provide sufficient coverage to close the genome but did allow us to identify putative enzymes involved in cellulose, hemicellulose and lignin degradation. Future experiments will involve additional sequencing to close the genome of both strains which will allow for genetic comparison of *P. glucanolyticus* 5162 and SLM1.

5.1 Introduction

Bacillus glucanolyticus was first isolated from environmental soil samples by Alexander and Priest (1989). This gram positive, rod-shaped, facultative anaerobic bacterium is characterized by its terminal spore formation, motile colonies, and ability to degrade a variety of β-glucans (Alexander & Priest, 1989). *B. glucanolyticus* was shown to be capable of hydrolyzing carboxymethyl cellulose (β, 1-4 linked glucose), curdlan (β, 1-3 linked glucose), pustulan (β, 1-6 linked glucose), and xylose (Alexander and Priest, 1989; Kanzawa,

et al., 1995). *Bacillus glucanolyticus* was renamed *Paenibacillus glucanolyticus* in 1997 by Shida et al. based on 16 S rDNA gene similarity. There has been no subsequent work published on *P. glucanolyticus*. A microorganism was isolated from waste black liquor generated during Kraft wood pulping, whose main organic component is lignin, and was identified by 16 S rDNA sequencing as *P. glucanolyticus* (Mathews et al., 2013). Characterization of this *P. glucanolyticus* strain revealed optimal growth at 37 ˚C and pH 9.0. Further growth experiments confirmed the ability of *P. glucanolyticus* to hydrolyze cellulose and hemicellulose and also demonstrated its ability to degrade lignin as the sole carbon source under aerobic and anaerobic conditions (Mathews et al., 2014). The ability to degrade lignin suggests that this microorganism contains genes which encode enzymes that are involved in lignin depolymerization. Elucidation of the lignin degradation pathway of *P. glucanolyticus* would provide additional information about genes and enzymes involved in bacterial lignin depolymerization, lignin degradation under basic conditions, and lignin degradation under anaerobic conditions.

This research will result in additional knowledge about novel lignin degradation pathways and could be used to produce industrial levels of lignin-degrading enzymes. There are currently only a limited number of studies focused on the genetic and enzymatic pathways by which lignin is degraded. This research seeks to determine how *P. glucanolyticus* can degrade lignin by identifying genes and characterizing the enzymes involved. This research will contribute to information about bacterial lignin degradation under novel conditions (anaerobic and basic pH) and may also contribute to the development of an industrial lignin degradation process, as *P. glucanolyticus* lignin degradation enzymes

could be produced and have activity at industrially relevant levels and conditions. A commercial lignin degradation process would serve to utilize waste lignin generated by industries that use lignocellulosic biomass. A commercial lignin degradation process would also enable the utilization of this renewable resource to produce valuable compounds and organic chemicals.

5.2 Methods

5.2.1 Bacterial Strains. *Paenibacillus glucanolyticus* SLM11 was isolated from pulping waste as described in Mathews et al. (2013). *Paenibacillus glucanolyticus* DSM 5162 was purchased from DSMZ (Germany).

5.2.2 Characterization of the isolates. Each strain was characterized for pH optima and degradation of lignocellulose components isolated as described in Mathews et al. (2013). Lignocellulose components were added to M9 minimal media at 0.2% (w/v) or 10% (v/v) for black liquor, and the cultures were inoculated with a 1% overnight inoculum of the *Paenibacillus* strain grown in LB.

5.2.3 Phylogenetic analysis. *Paenibacillus* and *Bacillus* 16S rDNA sequences were obtained from NCBI. *P. glucanolyticus* SLM1 16S rDNA sequence was obtained as described in Mathews et al. (2013). Multiple alignment of the sequences was performed using CLUSTAL on the MEGA 6 software (Tamura et al. 2013). The neighbor-joining tree boot strap consensus tree was inferred from 1000 replicates using the Jukes-Cantor method in Mega 6 (Tamura et al. 2013).

5.2.4 DNA isolation, sequencing, and genome assembly. Genomic DNA was extracted using the Qiagen Yeast/Bact. Kit. Library preparation and sequencing was performed by the Genomic Sciences Laboratory at North Carolina State University. Each strain was sequenced using Illumina MiSeq v3 (300x2 PE) chemistry and PacBio P6-C4 chemistry. The two strains were combined in one MiSeq library and generated over 26 million reads for each strain with an average sequence length of 285 bases. PacBio reads were sequence on individual SMRTcells producing 150 thousand reads with an average length of 2500 bp.

5.2.5 Secretion Prediction. The putative proteins were analyzed for secretion using PSORTb (http://www.psort.org/psortb/) because it has high precision (96.5%), allows multiple sequences to be analyzed at once, and differentiates between gram positive and gram negative bacteria (Gardy and Brinkman, 2006).

5.3 Results and Discussion

5.3.1 Phylogenetic analysis of the 16s rRNA genes. A phylogenetic tree was constructed from 16 S rDNA data collected by sequencing and 16 S rDNA sequences from the NCBI database (Figure 5-1). The neighbor joining tree showed that *P. glucanolyticus* SLM1 was more closely related to strain YNLB2-16-191104 than the type strain. Strain YNLB2-16-191104 was isolated in a clinical microbiology lab by Nagano et al. (2006). The bacterium has not been further characterized. However, the type strain is the most deeply rooted. Our hypothesis is that each strain of *Paenibacillus glucanolyticus* is adapted to its specific environment which results in genomic differences.

5.3.2 Bacterial growth and lignocellulose degradation. Bacterial growth at various pHs was measured for each strain. These comparisons revealed physiological differences between *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162. Strain SLM1 grows optimally

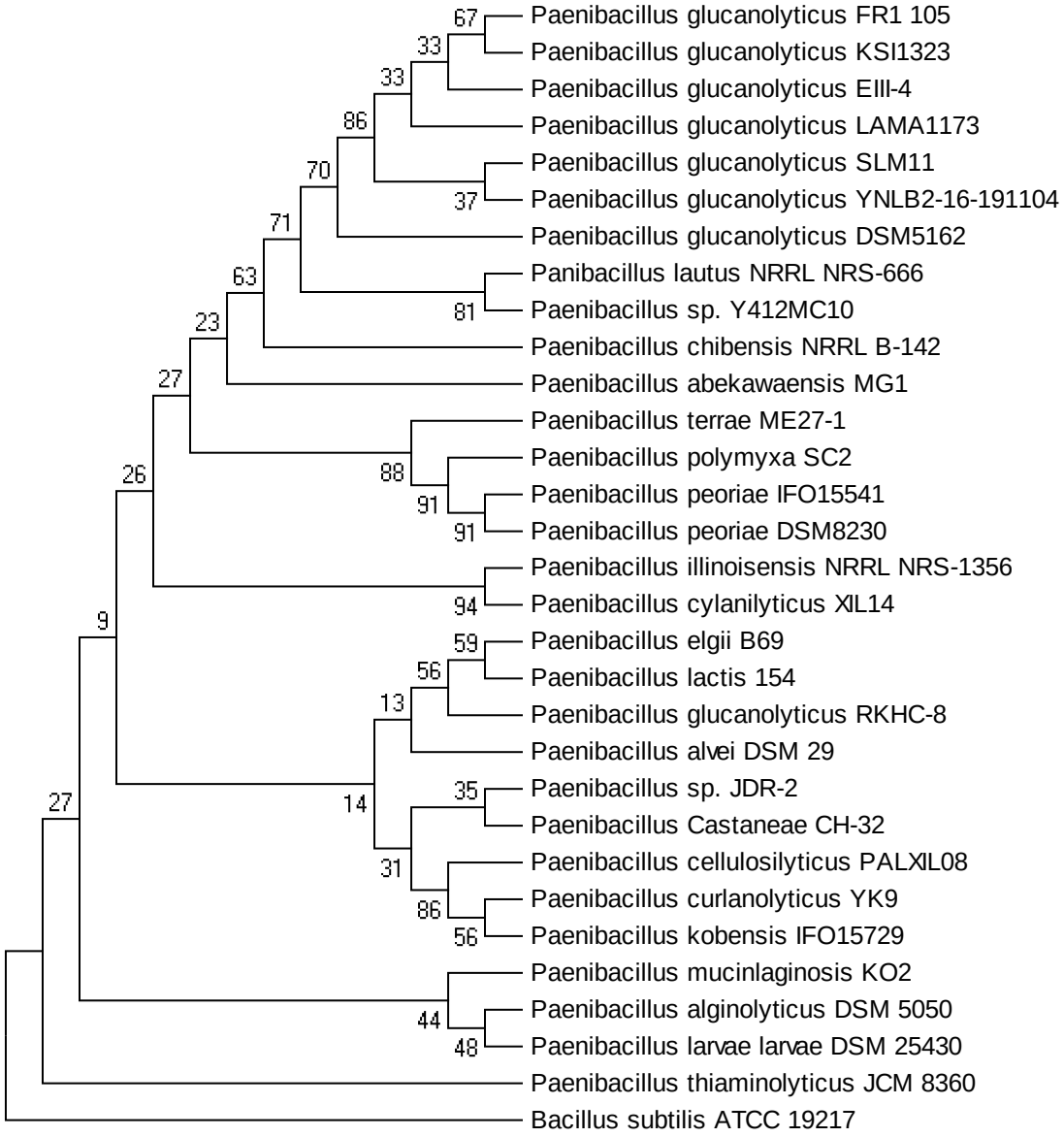

Figure 5-1 Phylogenetic tree of *Paenibacillus glucanolyticus* and related genera based on 16S rDNA sequence comparisons. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (value 1000) is shown next to the branches.

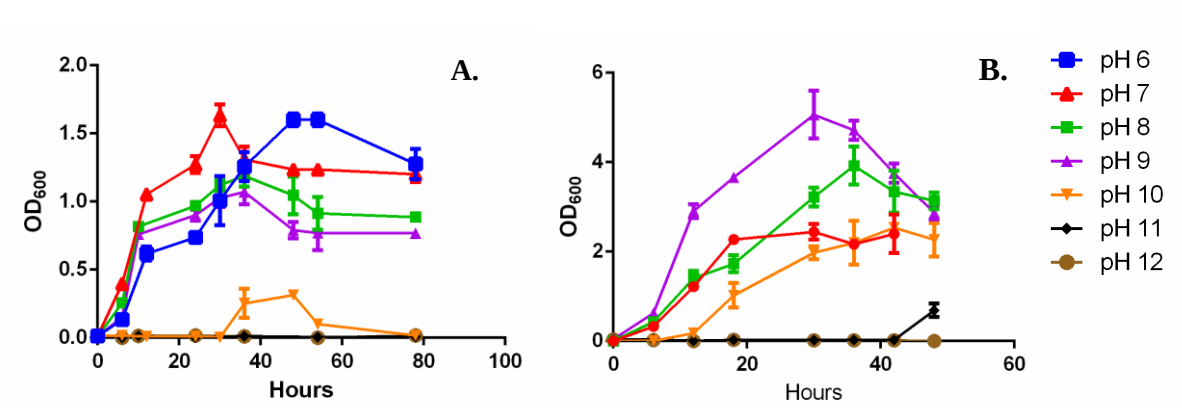

Figure 5-2 pH optimum of *P. glucanolyticus* 5162 (A) and *P. glucanolyticus* SLM1 isolate (B). The bacteria were cultured in LB media under aerobic conditions at 37℃ with shaking at 200 rpm. (n=4)

Table 5-1

Generation times of *P. glucanolyticus* 5162 at various pHs when grown in LB media

pH	Generation Time (hours)
6	32.8
7	16.8
8	68.5
9	80.7
10	31.25
11	625
12	625

Table 5-2 Generation times of *P. glucanolyticus* SLM1 at various pHs when grown in LB media.

pH	Generation Time (hours)
7	13.2
8	8.2
9	5.7
10	10.3
11	11.23
12	39.2

at pH 9 while the type strain grows optimally at pH 7 (Figure 5-2, Table 5-1, Table 5-2). Both strains can grow on black liquor under aerobic and anaerobic conditions (Figure 5-3). Both strains can also grow on black liquor components as the sole carbon source under aerobic and anaerobic conditions (Figure 5-4). *P. glucanolyticus* SLM1 grew faster and to a higher optical density on glucose, xylose, cellulose, hemicellulose and lignin under anaerobic conditions compared to strain 5162. These growth curves suggest that *P. glucanolyticus* SLM1 is better adapted to growth conditions which characterize black liquor (basic pH and using lignocellulosic components for growth) compared to 5162. Strain SLM1 can grow under more alkaline conditions and can grow more efficiently on lignocellulose components than strain 5162. Because, strain SLM1 was isolated from an alkaline environment containing cellulose, hemicellulose and lignin, these results were not surprising.

5.3.3 Sequencing. Assembly of the MiSeq and PacBio sequence information produced 21 contigs with a total of 6185025 bp for *P. glucanolyticus* 5162 and 39 contigs with a total of 6592552 bp for strain SLM1. The contigs were submitted to RAST and the putative proteins were analyzed for secretion signals. Enzymes involved in degradation of cellulose, hemicellulose or lignin must be located outside of the cell or on the outer membrane, as these polymers are too large to be transported inside of the cell and then degraded. Many extracellular and membrane localized enzymes involved in degrading these polymers have been characterized (Yang et al. 2011). Strain 5162 has 442 and SLM1 has 440 putative enzymes which have probability of secretion or localization to the membrane as determined by pSortB. Of these enzymes, several are involved in the transport of sugars, metal acquisition, electron transport, respiration and other necessary cellular functions. However, several enzymes were identified which could be responsible for degradation of lignocellulose components. These enzymes are listed in Table 5-3 and 5-4. Several canonical cellulose and hemicellulose degradation enzymes were identified by RAST. Cannonical lignin degradation enzymes, laccase, lignin peroxidase and manganese peroxidase were not identified by RAST with the exception of one enzyme produced by *P. glucanolyticus* SLM1, which has sequence similarity with a laccase enzyme. It may be that enzymes used in lignin degradation by these two strains are unique and are less homologous to fungal lignin enzymes that have been well studied.

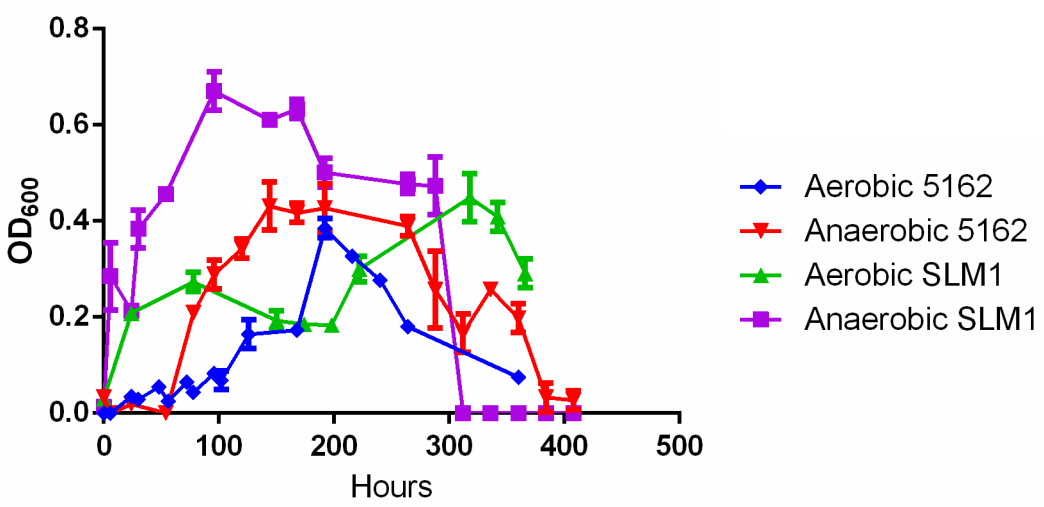

Figure 5-3 Comparison of growth on black liquor as the sole carbon source. *P. glucanolyticus* 5162 (red and blue) and *P. glucanolyticus* SLM1 were grown in M9 minimal media with 10% black liquor (filter-sterilized) as the sole carbon source. Minimal media was pH 7 for *P. glucanolyticus* 5162 and pH 9 for *P. glucanolyticus* SLM1. Both strains were cultured under aerobic (blue and green) and anaerobic conditions (red and purple) at 37˚C with shaking at 200 rpm.

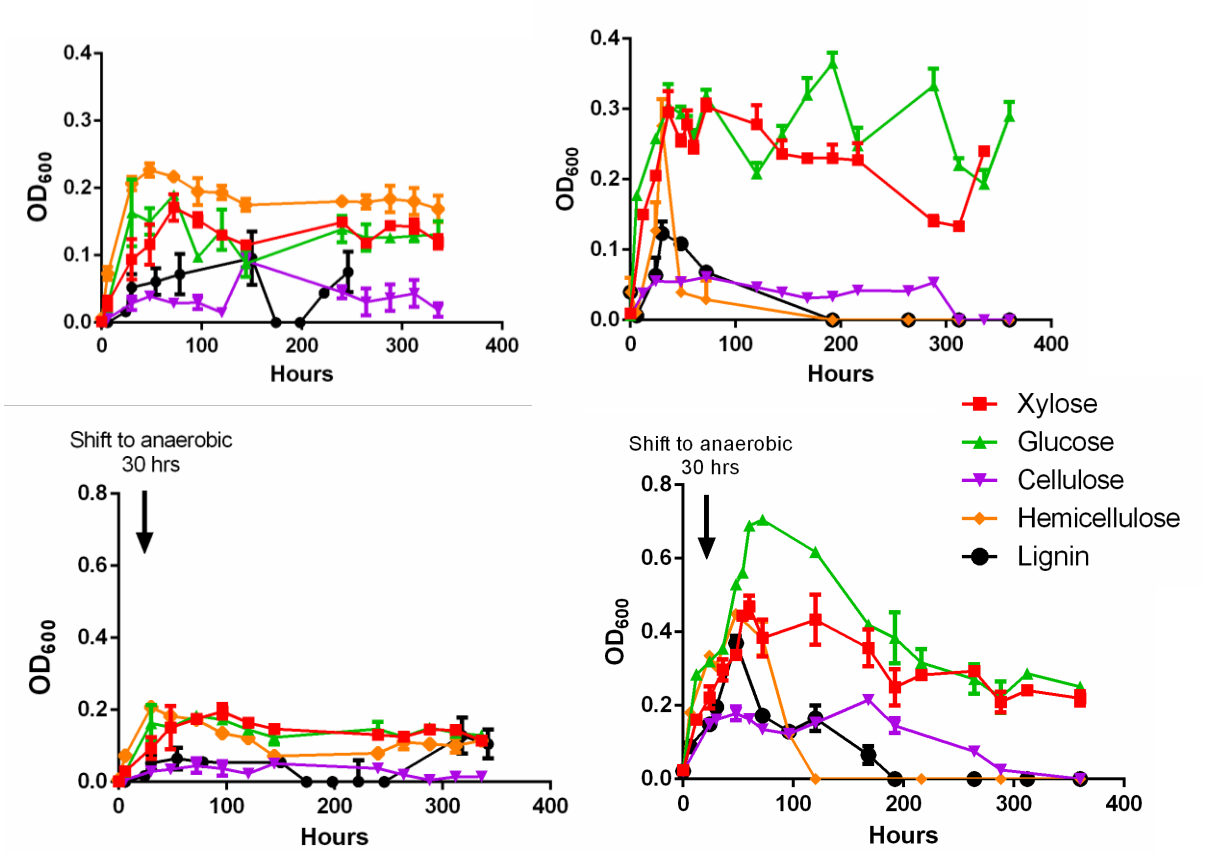

Figure 5-4 Comparison of growth in minimal media on lignocellulose substrates. Aerobic growth of *P. glucanolyticus* 5162 (A) and *P. glucanolyticus* SLM1 (B). Anaerobic growth of *P. glucanolyticus* 5162 (C) and *P. glucanolyticus* SLM1 (D). The bacteria were cultured in M9 minimal media with 0.2% carbon source at 37℃ with shaking at 200 rpm. (n=4)

Table 5-3 Putative **l**ignocellulose degrading genes of *P. glucanolyticus* 5162 identified by sequence similarity.

Location	**Length**	**Predicted function**
Hemicellulose		
Contig_10_73780_72428	1352	Endo-1,4-beta-xylanase A precursor (EC 3.2.1.8)
Contig_10_92260_95157	2897	Endo-1,4-beta-xylanase A precursor (EC 3.2.1.8)
Contig_10_399188_400138	950	Arabinan endo-1,5-alpha-L-arabinosidase (EC 3.2.1.99)
Contig_10_1442432_1441035	1397	Alpha-L-arabinofuranosidase II precursor (EC 3.2.1.55)
Contig_10_1411361_1412344	983	Xylosidase/arabinosidase
Contig_10_1697187_1700309	3122	Alpha-mannosidase (EC 3.2.1.24)
Contig_10_1704810_1707545	2735	Alpha-mannosidase (EC 3.2.1.24)
Cellulose		
Contig_10_147728_145896	1832	Glycosyl hydrolase, family 30
Contig_10_1216574_1217758	1184	COG1306 predicted glycoside hydrolase
Contig_30_409521_413390	3862	secreted glycosyl hydrolase
Contig_30_755406_756461	1055	glycosyl hydrolase 53
Contig_32_381651_379939	1712	Endoglucanase B precursor (EC 3.2.1.4) (Endo-1,4-beta-glucanase B) (Cellulase B) (EG-B)
Contig_34_181320_179764	1556	COG1649 predicted glycoside hydrolase
Contig_32_210017_209088	929	Glycoside hydrolase, family 10:Glycoside hydrolase, family 5
Contig_10_1707683_1709599	1916	Glycosyl hydrolase-related protein
Contig_30_286988_286299	689	Beta-glucosidase (EC 3.2.1.21)
Contig_30_287590_287018	572	Beta-glucosidase (EC 3.2.1.21)
Contig_30_361940_364234	229	Beta-glucosidase (EC 3.2.1.21)
Contig_30_378740_378036	704	putative glycosyl hydrolase, family 43
Contig_30_378934_378809	125	putative glycosyl hydrolase, family 43
Contig_30_380606_378960	1646	glycoside hydrolase, family 37
Contig_30_404121_406250	2129	Beta-glucosidase (EC 3.2.1.21)
Contig_30_460746_462365	1619	endoglucanase
Contig_31_145424_147052	1628	Cellulase(EC:3.2.1.4)
Polysaccharides		
Contig_10_329479_331803	2324	Chitinase (EC 3.2.1.14)
Contig_10_1597338_1596568	770	Endo-beta-1,3-1,4 glucanase (Licheninase) (EC 3.2.1.73)
Contig_30_448318_449541	1223	Chitinase (EC 3.2.1.14)
Contig_32_213326_212043	1283	Chitinase (EC 3.2.1.14)
Lignin		
Contig_30_364418_365500	1082	copper amine oxidase domain protein
Contig_30_568803_567643	1160	copper amine oxidase-like protein

Table 5-4 Putative **l**ignocellulose degrading genes of *P. glucanolyticus* SLM1 identified by sequence similarity.

Location	**Length**	**Predicted function**
Hemicellulose		
Contig_1_5902_4667	1235	Endo-1,4-beta-xylanase A precursor (EC 3.2.1.8)
Contig_11_7143_11129	3986	Endo-1,4-beta-xylanase A precursor (EC 3.2.1.8)
Contig_42_412820_411870	950	Arabinan endo-1,5-alpha-L-arabinosidase (EC 3.2.1.99)
Contig_43_72823_71471	1352	Endo-1,4-beta-xylanase A precursor (EC 3.2.1.8)
Contig_45_448918_447521	1397	Alpha-L-arabinofuranosidase II precursor (EC 3.2.1.55)
Cellulose		
Contig_18_108493_110205	1712	Endoglucanase B precursor (EC 3.2.1.4) (Endo-1,4-beta-glucanase B) (Cellulase B) (EG-B)
Contig_18_282014_282913	899	Glycoside hydrolase, family 10:Glycoside hydrolase, family 5
Contig_28_118647_122516	3869	secreted glycosyl hydrolase
Contig_28_455113_456168	1055	glycosyl hydrolase 53
Contig_44_223274_221718	1556	COG1649 predicted glycoside hydrolase
Contig_45_221432_222616	1184	COG1306 predicted glycoside hydrolase
Polysaccharides		
Contig_18_278675_279958	1283	Chitinase (EC 3.2.1.14)
Contig_18_382934_381918	1016	Chitinase (EC 3.2.1.14)
Contig_28_157447_158670	1223	Chitinase (EC 3.2.1.14)
Contig_45_603575_602805	770	Endo-beta-1,3-1,4 glucanase (Licheninase) (EC 3.2.1.73)
Lignin		
Contig_40_34973_33270	1703	Multicopper oxidase, sequence similarity to Cu-dependent laccases in Coprinus Cinereus At 1.68 A Resolution
Contig_11_22105_18464	3641	hypothetical protein, Metabolism of aromatic compounds
Contig_28_73539_74621	1082	copper amine oxidase domain protein
Contig_28_268218_267058	1160	copper amine oxidase-like protein
Contig_28_496578_497840	1262	copper amine oxidase domain protein
Contig_11_137864_137103	761	Protein of unknown function DUF541, sequence similarity to oxidative Stress Defense
Contig_43_189298_188621	677	putative oxygenase subunit
Contig_16_119004_120467	1463	Glycolate dehydrogenase (EC 1.1.99.14), subunit GlcD, homology to fungal vanillyl-alcohol oxidase
Contig_45_197565_198092	527	Thioredoxin, can regulate lignin polymerization in plants

Table 5-4 (continued)

Contig_28_55040_53916	1124	Copper amine oxidase
Contig_28_131341_132375	1034	Copper amine oxidase
Contig_11_22105_18464	3641	Hypothetical enzyme, possible involved in metabolism of aromatic compounds
Contig_42_167075_167866	1007	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)
Contig_42_445979_446776	1166	oxidoreductase, short chain dehydrogenase/reductase family

5.4 Conclusions

These results confirm that these two strains of *P. glucanolyticus* have genetic and physiological differences. Strain SLM1 appears to be well-suited to grow in the presence of lignocellulose and degrade its components. Further sequencing will be performed to close the genome. This will allow for genetic comparison of these two strains and a better understanding of the enzymes produced which may be involved in lignocellulose degradation. Future work will be performed to characterize the enzymes produced by these two strains responsible for lignin degradation.

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CHAPTER 6

Conclusions

Bacterial Biodegradation and Bioconversion of Industrial Lignocellulosic Streams

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6.1 Introduction

This research began with the challenge to isolate a microorganism from black liquor, what was once previously thought to be an inhospitable environment for microbial growth. Black liquor has been described in each chapter of this dissertation. It is a paper mill waste produced from the kraft pulping process. The wood is treated with high temperature, high pressure, sodium hydroxide and sodium sulfide. Many living organisms cannot survive high temperatures which occur during kraft digestion. The results of the kraft process is the solid pulp (primarily cellulose) which is used to make number of products including paper, and the liquid black liquor (primarily lignin, hemicellulose and waste pulping chemicals). Black liquor is characterized by a high pH and the presence of lignin. Lignin is considered toxic to many microorganisms because of its aromatic structure. Degradation of lignin results in the cleavage of phenolic groups present in lignin which are also toxic to many organisms. Phenol is toxic due to its hydrophobicity which causes loss of membrane integrity. Degradation can also produce phenoxy radicals formed by donation of an electron, and this reactive oxygen species can damage DNA, and proteins in cells.

6.2 Signficant Study Results

Black liquor was collected from the pulping lab at North Carolina State University. Jim McMurray conducted the kraft pulping and provided access to the black liquor in the pulping labs. Isolation of microorganisms directly from black liquor was attempted initially without much success. Genomic DNA isolation from the black liquor storage tank failed. However, we were able to use the black liquor as an inoculum into rich media and culture a

microorganism. This work is outlined in Chapter 2. A strain of *Paenibacillus glucanolyticus* named strain SLM1 was isolated from black liquor.

Once isolated, we determined this microbe was of industrial importance. Black liquor is an underutilized resource. Pulp and paper mills burn the black liquor in the recovery boiler to produce energy and steam, and recover chemicals used in the kraft process. We hypothesized that this bacterium may have interesting properties because of its ability to grow on the black liquor. Characterization of this bacterium and its ability to use black liquor and black liquor components for growth were described in Chapter 3. *Paenibacillus glucanolyticus* SLM1 isolated from black liquor can grow on black liquor, cellulose, hemicellulose, and lignin as the sole carbon source under aerobic and anaerobic conditions and this bacterium grows optimally at pH 9. In addition to interesting growth abilities, gas chromatography mass spectrometry was used to determine if this organism could be producing any valuable metabolic products from growth on black liquor. *P. glucanolyticus* was able to produce butyric, lactic, succinic, and propanoic acids when grown on black liquor as the sole carbon source under anaerobic conditions. These compounds are valuable chemical building blocks which are used to produce a variety of commodities including film, foam, rubber and fuel. These results suggested that *P. glucanolyticus* SLM1 may be of industrial importance as it has the potential to degrade black liquor and transform it into valuable products.

These results presented many questions concerning the unique ability of *P. glucanolyticus* SLM1 to grow on and degrade lignocellulose components. One of the most unique findings was growth of *P. glucanolyticus* SLM1 on lignin as the sole carbon source

under anaerobic conditions. In this study, *P. glucanolyticus* was grown on lignin as the sole carbon source under aerobic conditions and was transferred to a serum bottle after 30 hours. This growth scheme was used to determine the effect of bacterial growth on lignin under anaerobic conditions at a higher optical density. Some bacteria can degrade lignin, and a few bacterial consortia can degrade lignin under anaerobic conditions, *but no single bacterium has ever been shown to degrade lignin under anaerobic conditions alone*. Chapter 4 presents our findings on the ability of *P. glucanolyticus* SLM1 to degrade lignocellulose, lignin and lignin-like compounds. These results confirm our hypothesis that this isolate can degrade lignin under aerobic and anaerobic conditions. This study also characterized the ability of the type strain, *P. glucanolyticus* 5162, to grow on and degrade lignin. It was found that it too was able to degrade lignin but to a lesser extent than the black liquor isolate. The ability of these bacteria to grow on wood was also tested. Both strains were shown to grow on hardwood and softwood flour as the sole carbon source. These data suggest that the strains produce enzymes capable of degrading cellulose, hemicellulose and lignin.

Physiological differences between the two strains of *P. glucanolyticus* (SLM1 and 5162) were presented in Chapter 4, and we suspected that the ability to degrade lignin and lignin-like compounds may not be the only difference between the strains. Chapter 5 includes the strain comparison in which we characterized optimal temperature, pH, and their ability to grow on black liquor and the components of black liquor.

Growth of these two strains of *P. glucanolyticus* on black liquor and lignocellulosic components suggest that these strains produce enzymes capable of degrading these components. These enzymes could have great industrial significance because they could be

used in enzymatic treatment of lignocellulosic material. Alternatively the bacteria could be engineered by increasing the production of these enzymes for biological treatment of lignocellulosic material. The genomic DNA from each strain was isolated and sequenced using MiSeq and PacBio technology. The results of sequencing are outlined in Chapter 5. While we have not been able to close the genome for each strain, preliminary sequence information was used to identify potential lignocellulose degrading enzymes present in each strain.

The ability of *P. glucanolyticus* strains to grown on and degrade lignocellulose is of great industrial significance. Several industries use only the polysaccharides or sugars contained within plant cell walls to produce valuable compounds, however this organism is also capable of degrading lignin.

6.3 Applications

The industrial importance of these strains and their ability to produce value added products was not forgotten. We are currently validating methods for electroporation of *P. glucanolyticus* to engineer both strains for the production of fuels and chemicals.
This dissertation presents several novel findings:

1) Isolation of a new bacterium, *Paenibacillus glucanolyticus* SLM1, from black liquor
2) Demonstration of the ability of *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162 to grow on black liquor as the sole carbon source.

3) Demonstration of the ability of *P. glucanolyticus* SLM1 and *P. glucanolyticus* 5162 to grow on hardwood flour, softwood flour, cellulose, hemicellulose, lignin and, BioChoice lignin as the sole carbon source.
4) Demonstration of the ability of *P. glucanolyticus* SLM1 to degrade lignin under aerobic and anaerobic conditions.
5) Demonstration of the ability of *P. glucanolyticus* 5162 to degrade lignin under aerobic conditions.

6.4 Future Work

The ultimate goal of this research is the use of *P. glucanolyticus* for biological treatment of lignocellulosic material for the production of valuable products. This process allows for the complete utilization of a renewable resource while also using less energy or chemicals than thermomechanical or chemical treatment of lignocellulose. Additional research is required to meet the end goal:

1. Complete genomic sequencing. This research is currently being completed. New libraries have been created of each strain and are being sequenced using PacBio technology. This data should provide us with enough genome coverage to produce a closed genome. The complete genomic sequence of these strains will provide us with information about the enzymes responsible for the degradation of lignocellulose and the production of valuable by products.
2. Engineering *P. glucanolyticus* for production of limited high value byproducts. While able to produce several promising metabolic products, industrialization will require either an energy intensive separation process or a simple distillation process to

separate a limited number of the highest value products. We believe that the latter scheme would be most efficient in terms of cost of the process and value of the products.

3. Optimizing growth of *P. glucanolyticus* for industrial use. These results have shown that this bacterium can produce valuable byproducts with limited additional input. However, industrial use of this bacterium may require faster growth. Optimal growth conditions should be determined at industrial scale. Conditions that should be tested include oxygen levels required for optimal growth, amount and type of carbon source(s) and, presence of additional bacterial strains capable of degrading lignocellulosic components.
4. Recombinant expression and characterization of lignocellulose-degrading enzymes produced by *Paenibacillus glucanolyticus*. Enzymes produced by this bacterium may also be useful for other industrial processes such as the treatment of wastewater, enzymatic pretreatment of lignocellulose for biofuel production and paper making.