Accepted Manuscript

Degradation of Lignocellulose and Lignin by *Paenibacillus glucanolyticus*

Stephanie L. Mathews, Amy M. Grunden, Joel Pawlak

PII: S0964-8305(16)30046-4

DOI: [10.1016/j.ibiod.2016.02.012](https://doi.org/10.1016/j.ibiod.2016.02.012)

Reference: INBI 3879

To appear in: *International Biodeterioration & Biodegradation*

Received Date: 29 July 2015

Revised Date: 8 February 2016

Accepted Date: 20 February 2016

Please cite this article as: Mathews, S.L., Grunden, A.M., Pawlak, J., Degradation of Lignocellulose and Lignin by *Paenibacillus glucanolyticus*, *International Biodeterioration & Biodegradation* (2016), doi: 10.1016/j.ibiod.2016.02.012.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Degradation of Lignocellulose and Lignin by *Paenibacillus glucanolyticus*

Mathews, Stephanie L.^{AB}, Grunden, Amy M.^B, and Pawlak, Joel^{A*}

^ADepartment of Forest Biomaterials
Biltmore Hall, Campus Box 8005
North Carolina State University
Raleigh, NC 27695 USA

^BDepartment of Plant and Microbial Biology
4550A Thomas Hall Box 7612
North Carolina State University
Raleigh, NC 27695 USA

*Corresponding Author

Email Address:

Stephanie L. Mathews
stephanie_mathews@ncsu.edu

Amy M. Grunden
amy_grunden@ncsu.edu

Joel Pawlak
jipawlak@ncsu.edu
1-919-513-0511

Abstract

Lignocellulose is an abundant renewable carbon source that has been used for fuel and chemical production. Lignocellulose refers to the plant cell wall and is composed of cellulose, hemicellulose, and 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 showed that this bacterium could degrade cellulose and hemicellulose and also suggested that it 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. Our results show that both strains of *P. glucanolyticus* can degrade aromatic lignin-related compounds under aerobic and anaerobic conditions. These strains can also 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*, bioproducts

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, which are composed of repeated sugar units linked by β ,1-4 bonds. The lignocellulose conversion processes occurs by the breakdown of polysaccharides into their

constituent sugars through chemical, 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. Lignin is formed when the aromatic amino acids phenylalanine and tyrosine are polymerized by plants to form the phenylpropane units known as monolignols (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 OCH₃ 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).”

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 the chemical structure to potentially be used to produce fuels and chemicals, it is currently underutilized owing to its recalcitrance to conversion.

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 depolymerized lignin including foam, rubber, films, plastics, adhesives, and food flavoring agents (vanillin and ferulic acid) (Hatakeyama, 2002; Bandounas et al., 2011; Philbrook et al., 2013). Thus, 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.

Some microorganisms (fungi and bacteria) produce enzymes that can degrade lignin (Vicuna et al., 2000; Bugg et al., 2011a). Fungi can grow on lignocellulose as the sole carbon source, degrading lignin to access the preferred energy source: cellulose. 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 (Mathews et al. 2015). No bacterium has been shown to be capable of growth on lignocellulose as the sole carbon source. 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. Oxidation of lignin by bacteria has been shown to produce vanillic acid and protocatechuic acid (Bugg et al., 2011a). Other bacteria can degrade lignin monomers such as bi-phenyl, β -aryl ether, ferulate, vanillin, and syringaldehyde lignin structures (Masai et al. 2007).

Measurement of lignocellulose and/or lignin degradation has been performed by analyzing growth on lignin and lignin-like compounds or the metabolites produced when grown on lignin or lignin-rich materials as the sole carbon source (Bandounas et al. 2011; Chandra et al. 2011; Mathews et al. 2013). These methods are not ideal as lignin used for these growth studies may differ in structure and contain polysaccharide residues as the result of lignin extraction from plant materials. Lignin-like compounds, such as dyes, have been used because they are synthesized and therefore have a known structure without polysaccharide residue contamination. These dyes can be incorporated into colorimetric screens to evaluate the potential to degrade lignin-like aromatic monomers. Others have also measured lignin-degrading enzyme activity by these bacteria (Chandra et al. 2011; Chen et al. 2012; Raj et al. 2007) which confirms that ability of enzymes produced by the bacteria to act on a particular lignin-like substrate, but not the lignin polymer itself. An alternative method for analyzing the degradation of the lignin polymer is to analyze the molecular weight of the lignin substrate before and after bacterial growth. Lignin polymer size may be measured using high performance liquid chromatography (HPLC) and is described by Cui et al. (2014).

The bacterial species described here, *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). *Bacillus 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.

From the pulping waste black liquor, the main component of which is lignin, a microorganism was isolated 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 of this *P. glucanolyticus* strain 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 objective of this work was to further characterize the ability of this bacterium (*P. glucanolyticus* SLM1) as well as an additional bacterium, *P. glucanolyticus* 5162 (the type strain), to degrade lignocellulose and the components of lignocellulose using a variety of methods to measure lignin degradation. In addition to analyzing bacterial-lignin degradation by growth studies on lignin, and lignin related compounds, and metabolite production, this work also uses a novel approach to characterize lignin degradation by measuring the molecular weight of lignin after bacterial-lignin degradation.

2. Materials and Methods

2.1 Bacterial strains, plasmids, and enzymes

P. glucanolyticus SLM1 was isolated as described in Mathews et al. (2013). *Paenibacillus glucanolyticus* 5162 was purchased from DSMZ (Germany).

2.2 Chemicals and reagents

Biochoice lignin (BCL) was obtained from Domtar Inc. Lignin, cellulose, and hemicellulose were extracted from switchgrass as described in Mathews et al. (2013). Lignin model compounds: acetovanillone, anisoin, biphenyl, catechol, cinnamic acid, guaiacol vanillin, oxalic acid, malonic acid, and benzaldehyde 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 tetrahydrofuran were purchased from Fisher Scientific. Wood flour was produced from birch (hardwood) species and pine (softwood) chips by milling to 0.2 mm particles (Lightsey et al. 1977).

2.3 Media and growth conditions

M9 minimal medium was used to measure growth of *P. glucanolyticus* and is composed of 0.04 M Na₂HPO₄, 0.02 M KH₂PO₄, 18 mM NH₄Cl, 8.6 mM NaCl, 27 µM CaCl₂, 1 mM MgSO₄ (Lech and Brent, 1992). Solid media were made using 15 g L⁻¹ of agar. Dyes that 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 °C, 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). Bacterial growth was measured spectrophotometrically at λ = 600 nm using a Biorad SmartSpec 3000. Uninoculated medium samples were used as a blank.

2.4 Dye decolorization assay

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 as described in Bandounas et al. (2011). Cultures were returned to incubation at 37 °C 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 percent 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 °C for 10 days. Decolorization of solid media was visually inspected daily for bacterial growth and clearing zones after bacterial growth.

2.5 Gas Chromatography-Mass Spectroscopy (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 Na₂SO₄. 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 μ l of 1,4-dioxane and 10 μ l pyridine. Samples were derivatized by the addition of 50 μ l 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 μ m 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.

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 dissolved 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.

2.7 Lignin isolation

Inoculated and uninoculated M9 media with 0.2% BCL were 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 °C oven for 15 min to dry the lignin. The solid lignin powder was weighed or acetylated for GPC.

2.8 Lignin Acetylation

Lignin was derivatized for size separation by gel-permeation chromatography as described by Cui et al. (2014). 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 suspended in 10 mL of ethanol and evaporated using the rotovap. The samples were suspended in 10 mL ethanol and evaporated until the smell of pyridine was no longer present. The samples were then dried under vacuum at 35 °C for 2 days.

2.9 Gel-Permeation Chromatography (GPC)

Gel permeation chromatography was performed as described by Cui et al. (2014). Dried and acetylated lignin was added to tetrahydrofuran (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 °C. The acetylated sample was loaded at 50 µL 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.

3. Results

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. In this study, we compared 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 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 wood flours under aerobic and anaerobic conditions (Fig. 1).

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 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, guaiacol 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 1).

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. 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 $\lambda_{\max}$ of the dye used. Congo Red, Alizarin Yellow, Brilliant Blue and Reactive Blue were decolorized by both strains in rich media but little or no decolorization was measured in minimal media (Fig. 2). *Paenibacillus 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.

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.

Paenibacillus glucanolyticus SLM1 produced oxalic, lactic, acetic, and propanoic acid when grown on plant cell wall carbohydrates cellulose and hemicellulose (Table 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 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 flasks was measured by weight after 400 h of growth. Lignin mass decreased after bacterial growth of both strains under aerobic and anaerobic conditions in M9 minimal media with 0.2% BCL (Table 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 Fig. 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 3). Chain length and molecular weight decreased as a result of bacterial growth and indicate that lignin is depolymerized. *Paenibacillus glucanolyticus* 5162 did not decrease lignin polymer size after aerobic growth on BCL.

4. Discussion

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 currently produced at an industrial scale. These studies show that *P. glucanolyticus* can use BCL as the sole carbon source for growth and could therefore also 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 trials 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 wood flours (Fig. 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 lignocellulose.

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 was 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, guaiacol 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 inactivate 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 to 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.

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.

Lignin degradation has been measured using several different techniques. ^{14}C labeled lignin can be used to measure the release of ^{14}C -carbon dioxide 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 carbon 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 were also used to measure lignin degradation. Dry weight of the lignin decreased after bacterial growth suggesting that lignin is being degraded during bacterial growth (Table 3). GPC provides further proof of lignin degradation. The chromatographs shown in Fig. 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 both the ability of these strains to degrade lignin and the differences in the ability to degrade BCL for *P. glucanolyticus* strains SLM1 and 5162 under 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.

5. Conclusions

These experiments presented in this study 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 fractions, acetovanillone, vanillin, catechol, oxalic acid, benzaldehyde and malonic acid as the sole carbon source in M9 minimal media at pH 9, 37 °C under aerobic and anaerobic conditions. *Paenibacillus glucanolyticus* SLM1 was also able to grow on and decolorize Congo Red, Bromocresol Green, Toluidine Blue O, Brilliant Blue and Reactive Blue as the sole carbon

source. *Paenibacillus glucanolyticus* 5162 was only able to grow on and decolorize Reactive Blue as the sole carbon source. 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. 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 both aerobic and anaerobic growth, while for *P. glucanolyticus* 5162 the lignin average molecular weight only decreased after growth 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. *Paenibacillus 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.

Acknowledgements

The authors would like to acknowledge the United State Department of Agriculture Award No. 2010-38420-20399 for supplying graduate student funding for this project. They would also like to acknowledge Domtar Inc. for the generous donation of materials as well as financial support.

References

- Alexander B, Priest FG.** 1989. *Bacillus glucanolyticus*, a new species that degrades a variety of B-glucans. *Int. J. Syst. Bact.* **39**(2):112-115.
- Bandounas L, Wiercks NJP, de Winde JH, Ruijsenaars HJ.** 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *BMC Biotechnol.* **11**(94).
- Bugg TDH, Ahmad M, Hardiman EM, Rahmanpour R.** 2011a. Pathways for degradation of lignin in bacteria and fungi. *Nat. Prod. Rep.* **28**:1883-1896.
- Bugg TDH, Ahmad M, Hardiman EM, Singh R.** 2011b. The emerging role for bacteria in lignin degradation and bio-product formation. *Curr. Opin. Biotechnol.* **22**:394-400.
- Bungay H.** 2012. Production opportunities for biomass refining. *Enzyme Microb. Technol.* **14**:501-507.
- Chakar FS, Ragauskas AJ.** 2004. Review of current future softwood kraft lignin process chemistry. *Ind. Crops, and Prod.* **20**:121-141.
- Chandra R, Abhishek A, Sankhwar M.** 2011. Bacterial decolorization and detoxification of black liquor from rayon grade pulp manufacturing paper industry and detection of their metabolic products. *Bioresour. Technol.* **102**:6429-6436.

- 454 **Chen Y, Chai L, Tang C, Yang Z, Zheng Y, Shim Y, Zhang H.** 2012. Kraft lignin
455 biodegradation by *novosphingobium* sp. B-7 and analysis of the degradation
456 process. *Bioresour. Technol.* **123**:682-685.
- 457 **Crawford RL.** 1981. Lignin biodegradation and transformation. John Wiley and Sons, New
458 York, NY.
- 459 **Crawford DL, Crawford RL.** 1976. Microbial degradation of lignocellulose: The lignin
460 component. *Appl. Environ. Microbiol.* **31**(5):714-717.
- 461 **Cui C, Sun R, Argyropoulos DS.** 2014. Fractional precipitation of softwood kraft lignin:
462 isolation of fractions common to a variety of lignins. *ACS Sustainable Chem. Eng.* **2**:959-
463 968.
- 464 **Dhillon GS, Fssara F, Brar SK, Verma M.** 2012. Biotechnological applications of lignin, p
465 1-34. *In* R. J. Patterson, RJ (ed), *Lignin: Properties and application in biotechnology and*
466 *bioenergy.* Nova Science Publishers, New York, NY.
- 467 **Donnelly RF, McCarron PA, Tunney MM.** 2008. Antifungal photodynamic therapy.
468 *Microbiol. Research* **163**(1):1-12.
- 469 **Gonzalez B, Merino A, Almedia M, Vicuna R.** 1986. Comparative growth of natural
470 bacterial isolates on various lignin-related compounds. *Appl. Environ. Microbiol.*
471 **52**(6):1428-1432.
- 472 **Harith ZT, Ibrahim NR, Yusoff N.** 2014. Isolation and identification of locally isolated
473 lignin degrading bacteria. *J. Sustain. Sci. Manag.* **9**(2):114-118.
- 474 **Hatakeyama H.** 2002. Polyurethanes containing lignin. *In* Hu TQ (ed), *Chemical*
475 *modifications, properties, and usage of lignin.* Kluwer Academic Press and Plenum
476 Publishers, New York, NY.

- Heitner C, Dimmel DR, Schmidt JA.** 2010. Lignin and lignans: advances in chemistry. CRC Press, Boca Raton, FL.
- Kanzawa Y, Harada A, Takeuchi M, Yokota A, Harada T.** 1995. *Bacillus curdlanolyticus* sp. nov. and *bacillus kobensis* sp. nov., which hydrolyze resistant curdlan. Int. J. Syst. Bacteriol. **43**(3):515-521.
- Kawahara M.** 1980. Metabolism of lignin-related compounds by bacteria. In Kirk TK, Higuchi T, Chang H. (eds), Lignin biodegradation: microbiology, chemistry and potential applications. CRC Press Inc, Boca Raton, FL.
- Lech K, Brent R.** 1992. *Escherichia coli*, plasmids and bacteriophages. In Ausubel FM, Brent R, Kingston RE, Moore DD, Sediman JG, Smith JA, Struhl K (eds), Short protocols in molecular biology. John Wiley and Sons INC, New York.
- Li J, Yuan H, Yang J.** 2009. Bacteria and lignin degradation. Front. Biol. China. **4**(1):29-38.
- Lightsey GR, Short PH, Sinha VKK.** Low cost polyolefin composites containing pulp mill wood residue. Polymer Engin. Sci. **17**(5):305-309.
- Masai E, Katayama Y, Fukuda M.** 2007. Genetic and biochemical investigations on bacterial catabolic pathways for lignin-derived aromatic compounds. Biosci. Biotechnol. Biochem. **71**(1):1-15.
- Mathews SL, Ayoub AS, Pawlak J, Grunden AM.** 2013. Methods for facilitating microbial growth on pulp mill waste streams and characterization of the biodegradation potential of cultured microbes. J. of Vis. Exp. 82.
- Mathews SL, Pawlak J, Grunden AM.** 2014. Isolation of *Paenibacillus glucanolyticus* from pulp mill sources with the potential to deconstruct pulping waste. Bioresour. Technol. **164**:100-105.

- 500 **Mathews SL, Pawlak J, Grunden AM.** 2015. Bacterial biodegradation and bioconversion
501 of industrial lignocellulosic streams. *Appl. Microbiol. Biotechnol.* 99:2939-2954.
- 502 **Perez J, Munox-Dorado J, de la Rubia T. Martinez J.** 2002. Biodegradation and
503 biological treatments of cellulose-hemicellulose and lignin: an overview. *Int. Microbiol.*
504 **5**:53-63.
- 505 **Philbrook A, Alissandratos A, Easton CJ.** 2013. Biochemical processes for generating
506 fuels and commodity chemicals from lignocellulosic biomass. *In* Petre M(ed), *Environmental*
507 *biotechnology-New approaches and prospective applications.* InTech.
- 508 **Raj A, Reddy MMK, Chandra R.** 2007. Identification of low molecular weight aromatic
509 compounds by gas chromatography-mass spectrometry (GC-MS) from kraft lignin
510 degradation by three *bacillus* sp. *Int. Biodeter. Biodegrad.* **59**:292-296.
- 511 **Shida O, Hiroaki T, Kiyoshi K, Lawrence NK, Kazuo K.** 1997. Transfer of *Bacillus*
512 *aligonolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*,
513 *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the Genus *Paenibacillus* and Emended
514 description of the Genus *Paenibacillus*. *Int. J. Syst. Bacteriol.* **47**(2):289.
- 515 **Vicuna R.** 2000. Ligninolysis. *Molec. Biotechnol.* **14**:173-176.
- 516

Figure Captions

Figure 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 °C 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

Figure 2. Dye decolorization assays. Decolorization produced by *Paenibacillus glucanolyticus* strains grown in LB or M9 minimal media at a concentration of 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) was measured by the average percent decrease of λ_{max} of the indicator dyes. Liquid cultures were inoculated with *P. glucanolyticus* SLM1 or 5162 and incubated at 37 °C until mid-log phase after which dye was added to liquid cultures, 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

Figure 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 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. Chromatograph 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 fraction 4.

Tables

Table 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 time $\leq$ 500 hours

Table 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		<i>P. glu</i> 0.2% Cell		Uninoc. 0.2% Hemi		<i>P. glu</i> 0.2% Hemi		Uninoc. 0.2% Lignin		<i>P. glu</i> 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

^aMatch based on purchased standard. ^bMatch based on data from NIST library.

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

Table 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 ⁻¹	% Δ	Mn	% Δ	Mw	% Δ
Uninoculated	16.7 ± 0.09		985		113660	
<i>P. glu</i> SLM1 aerobic	11.6 ± 0.07	-31%	770	-22%	2467	-98%
<i>P. glu</i> SLM1 anaerobic	12.9 ± 0.07	-28%	826	-16%	38820	-66%
<i>P. glucanolyticus</i> 5162 aerobic	15.6 ± 0.01	-7%	1031	+5%	150595	+32%
<i>P. glucanolyticus</i> 5162 anaerobic	12.7 ± 0.05	-24%	829	-16%	34763	-69%

Highlights

- *P. glucanolyticus* SLM1 and 5162 can grow on lignin as the sole carbon source.
- These strains can grow on hardwood and softwood flour as the sole carbon source.
- Both strains can reduce the molecular weight and chain length of BioChoice lignin.
- *P. glucanolyticus* 5162 can degrade lignin only under anaerobic conditions.
- *P. glucanolyticus* SLM1 can degrade lignin under aerobic and anaerobic conditions.

Abbreviations

BCL – BioChoice Lignin

CR – Congo Red

XC - Xylidine Ponceau

BG - Bromocresol Green

AY - Alizarin Yellow

BB - Brilliant Blue R

TB - Toluidene Blue O

RB - Reactive Blue