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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. 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.

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 occur 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 et al., 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-OCH_3 group (known as guaiacyl), sinapyl alcohol with two aryl-OCH_3 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)."

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,

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.

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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, 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 and 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. (1997) 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). *P. 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, 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. 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 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 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 $\lambda = 600$ nm using a Biorad SmartSpec 3000. Uninoculated medium samples were used as a