ABSTRACT

CUEBAS IRIZARRY, MARA FRANCHESKA. Characterization of Bacterial Isolates from Carpenter Bees for Biodegradation of Lignocellulose Compounds (Under the direction of Dr. Amy M. Grunden).

Complex polymers represent a challenge for environmental pollution as well as an opportunity for microbial catalyzed conversion to generate valorized chemicals. For example, black liquor, a by-product from paper milling, contains lignocellulose components; however, it is typically burned to generate steam energy for the paper pulping process. Here, we investigate the lignin and hemicellulose-derived compound biodegradation potential of bacterial strains isolated from carpenter bees when they are grown on black liquor or lignin. The isolates were identified as *Streptomyces spp.* by 16S rRNA and whole genome sequencing. Growth was assessed for the isolates cultured in minimal media with the lignocellulose constituents (cellulose, xylan, and lignin) or black liquor (pulping waste) added as the only source of carbon. Filter paper deconstruction, dye decolorization assays, and % lignin reduction assays were used to determine the cellulose, hemicellulose, and lignin degradation potential of the isolates, respectively. *Streptomyces spp.* strains 2-6 and 2-10 were able to decolorize azo, trimethylmethane, and thiazine dyes up to 60, 100, and 18%, respectively, after one week incubation without the addition of a reaction mediator. Cellulose deconstruction experiments showed degradation of up to 30% of the filter paper within 10 days. Growth on lignin revealed that *Streptomyces sp.* strain 2-6 could degrade up to 24% of the lignin mass within 30 days. Whole genome analysis of *Streptomyces sp.* 2-6 was employed to identify potential lignin and lignocellulose metabolism genes and associated pathway which are comparable with previously characterize lignin-degrading *Streptomyces* spp. The potential of lignin deconstruction by *Streptomyces sp.* 2-6 was evaluated using Gel-Permeation Chromatography, and GC-MS was used to identify products

generated when *Streptomyces sp.* 2-6 was culture on lignin and black liquor. This work demonstrated that *Streptomyces sp.* 2-6 can use low molecular weight lignin as a sole carbon source and that it promotes de-polymerization of high molecular weight lignin and produces organic acids with industrial relevance. Findings suggest the exploration of carpenter bee microbiota for the biodegradation and bioconversion of lignocellulosic derivatives.

Characterization of Bacterial Isolates from Carpenter Bees for Biodegradation of Lignocellulose Compounds

by
Mara F. Cuebas-Irizarry

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in partial fulfillment of the
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APPROVED BY:

_____	_____
Dr. Amy M. Grunden Committee Chair	Dr. José M. Bruno-Bárcena
_____	_____
Dr. Paul T. Hamilton	Dr. Joel J. Pawlak

DEDICATION

To the girl who tried so many times to make paper from scratch following instructions Discovery Kids and now is trying to bioconvert paper waste; the same girl that enjoyed puzzles, and now was able to put a dissertation together.

To misma.

BIOGRAPHY

Mara was born in the southwest coast of Puerto Rico in the beautiful town of San Germán, and raised between Cabo Rojo, and Lajas. She attended the Academia San Luis until 11th grade and then graduated from high school at Escuela Leonides Morales Rodríguez in Lajas. She started under the Chemical Engineering degree at the University of Puerto Rico – Mayagüez. During sophomore year, she changed her major to Industrial Microbiology. During that time, she did undergraduate research on several topics in environmental microbiology with mentoring from Dr. Martha López-Moreno, Dr. Luis Ríos-Hernández, and Dr. Rafael Montalvo-Rodríguez. She also participated during two consecutive summers in the George Washington Carver Internship Program in Iowa State University and was mentored by Dr. Diane F. Birt (2012), and Dr. Amy L. Toth (2013).

For graduate school, she was admitted to the University of Puerto Rico – Mayagüez, to pursue a Master in Sciences in Microbiology, where she graduated in 2017. There she taught four different laboratory courses and mentored 15 undergraduate research trainees. After that, she was admitted to the Microbiology Graduate program in the Department of Plant and Microbial Biology at North Carolina State University. During her PhD program, she taught BIT core technologies, participated in the Preparing for professoriate program, worked as a Student trainee at the Environmental Protection Agency, served in different board leadership roles for the Latin American Student Association of NC State where she lead the first online research symposium, and she worked on the development of an outreach program for students from the English as a Second Language classes at Garner Middle School.

Her favorite hobby is planning the next trip.

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CHAPTER 1

Dissertation overview

The concerns about global climate change have led to increased interest in developing alternatives to fossil fuels to meet energy demands. Initiatives such as the United Nations' Paris Agreement focus on fostering efforts to keep global mean temperature increase below 1.5ºC. These efforts include substantial reductions in green gas emissions coming from the combustion of fossil fuels (*The Paris Agreement | UNFCCC*, 2015). In addition, due to the lifespan of fossil fuels, energy companies and chemical production industries are looking for the development of alternative energy sources.

For this challenge, lignocellulosic biomass represents an opportunity as a renewable and sustainable source for the generation of biofuels and bioproducts. Lignocellulose is biofuels' most abundant renewable feedstock (Sharma, Xu, and Qin, 2019). However, its components: cellulose, hemicellulose, and lignin, are hard to break down due to structural complexity. One pound of lignin is equivalent to two pounds of sugar (reducing equivalents). Understanding lignin deconstruction will benefit biofuel industries because lignin reduces the efficiency of cellulose bioconversion used for ethanol production. The U.S. Department of Energy aims to use lignocellulosic biomass to produce ~36 billion gallons of renewable transportation biofuels by 2022 ('Bioenergy Technologies Office Multi-Year Program Plan, March 2016', (2016)). Now, this increase in biofuel production will decrease the dependence on fossil fuels and reduce greenhouse emissions.

Studies in the Grunden laboratory have demonstrated lignin degradation by *Paenibacillus glucanolyticus*, a gram-positive bacterium isolated from pulp milling streams (Mathews et al. 2014; Mathews et al. 2016). To date, most lignin degradation and depolymerization research has

involved fungal species. However, fungi can be challenging to work with as a biotransformation chassis because of slow specific growth rates and the lack of facile genetic systems. Therefore, expanding bacterial research on lignin degradation will increase the enzymatic options for depolymerization and have whole-cell biotransformation systems.

Contaminated soils from landfills and industrial runoff have become favored targets for isolating microbes for polymeric degradation applications (Rodríguez-Fonseca *et al.*, 2021). Microbes from contaminated soils are undergoing selective pressure in landfills that can enable isolates to metabolize waste polymers as a sole carbon source (Saxena and Bharagava 2020).

Symbiotic relationships could also be a source of lignin degradation. For example, insects can be an interesting source of microbes with the potential to degrade lignin. Wood-feeding insects such as termites have been the subjects of microflora study for years (Watanabe et al. 2003; Geib et al. 2008; Gales et al. 2018; Subta et al. 2020). However, insects exposed to lignocellulosic environments, such as carpenter bees, are underexplored for this application. A recent publication reported *Xanthomonas* sp. HY-71 from carpenter bees for plastic degradation (Kim et al. 2022). This serve as an indication that the microbiota from carpenter bees can be a potential source of microbial degradation or bionconversion factories.

In this dissertation, I have characterized bacterial isolates from carpenter bees with the ability to degrade lignocellulosic compounds. In addition, I have identified and reported the products generated from their metabolism of lignin and lignocellulosic compounds, and predicted which pathways could be involved in pulp mill bioconversion and lignin decomposition based on whole genome analyses.

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CHAPTER 2

Streptomyces spp. as biocatalyst sources in pulp and paper and textile industries:

Biodegradation, Bioconversion, and Valorization of Waste

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Summary

Complex polymers represent a challenge for remediating environmental pollution and an opportunity for microbial catalyzed conversion to generate valorized chemicals. Members of the genus *Streptomyces* are of interest because of their potential use in biotechnological applications. Their versatility makes them excellent sources of biocatalysts for environmentally responsible bioconversion, as they have a broad substrate range and are active over a wide range of pH and temperature. Most *Streptomyces* studies have focused on the isolation of strains, recombinant work, and enzyme characterization for evaluating their potential for biotechnological application. This review discusses reports on *Streptomyces*-based technologies for use in the textile and pulp milling industry and describes the challenges and recent advances aimed at achieving improved biodegradation methods featuring these microbial catalysts.

Key Points

- *Streptomyces enzymes for use in dye decolorization and lignocellulosic biodegradation*
- *Biotechnological processes for textile and pulp and paper waste treatment*
- *Challenges and advances for textile and pulp and paper effluent treatment*

Keywords: Lignocellulose, Lignin, Dye decolorization, *Streptomyces* spp., Lignin depolymerization, Bioremediation

2.1 Introduction

In recent years, the contribution of wastewater to environmental pollution has been of global concern. The presence of bioactive organic chemicals in water effluents is particularly problematic. Industries such as textile and paper production plants release synthetic dyes and lignin, respectively, as part of their wastewater (Andersson *et al.*, 2021) which could have further negative implications for the environment (Yadav and Chandra, 2018; Al-Tohamy *et al.*, 2022; Parmar *et al.*, 2022). For example, in paper production, the kraft pulping process is used, which consists of digesting wood under alkaline conditions, followed by several steps of washing and screening to separate brownstock and black liquor (Mathews *et al.*, 2015), a process that generates paper mill sludge wastewater. The effluents become a collection of fibers, dissolved organic solids, salts, chlorinated compounds, heavy metals, and a variety of lignocellulose biomass compounds (Brown *et al.*, 2021), ultimately resulting in both liquid and solid wastes that are of environmental concern (Chandra *et al.*, 2017). They could induce toxicity and endocrine damage if released into the environment (Yadav and Chandra, 2018; Kumar and Chandra, 2020), including negatively impacting aquatic life if introduced into waterways (Singh and Chandra, 2019).

Potential pollutants from these effluents are regulated by environmental agencies in different jurisdictions around the world. For example, the United States Environmental Protection Agency provides a list of pollutants and guidelines for discharge and best management practices to manage effluents and waste streams (US EPA, 2016). In the European Union, there is a “Circular Economy Action Plan for a greener and more competitive Europe” that provides a framework for the prevention of waste, monitoring of toxic substances, and the improvement of management practices of secondary raw materials and local waste (European

Commission, 2020). However, different countries around the world have varied standards and regulations governing the release of paper and textile industry effluents (Central Pollution Control Board (CPCB), 2000; Meriläinen and Oikari, 2008; Zinabu *et al.*, 2018).

Current treatment approaches for the paper industry effluent have been summarized and are separated into three-step treatments: physicochemical as the primary treatment (sedimentation; flotation, and filtration; oxidation, and ozonation); biological as secondary treatment, which is divided into aerobic (activated sludge systems and aerated lagoons) and anaerobic; and emerging approaches as tertiary treatments (membrane filtration, adsorption and activated carbon, membrane bioreactors) (Adarsh Kumar *et al.*, 2021). Several investigations, reviews and book chapters have described microbial degradation and decolorization of pulp and paper effluents as promising environmentally friendly technologies that need to be developed and further investigated (Singh *et al.*, 2008; Adarsh Kumar *et al.*, 2021; Amit Kumar *et al.*, 2021; Kumar *et al.*, 2022).

Effluents from industrial textile operations can contain a range of synthetic dyes mixed with other contaminants at various concentrations (Yaseen and Scholz, 2019); (Forgacs *et al.*, 2004). Researchers have reported dye contamination levels of textile and printing effluents ranging from 2-50% (w/v) (Díez-Méndez *et al.*, 2019); (Tan *et al.*, 2000) and that 15-30% of the dyestuff used in a dye/printing operations may be released to the environment (Bechtold and Turcanu, 2004), while others present information about the types of common dyes that are found in the wastewater effluent (Petrinić *et al.*, 2015). In addition to the textile industry, synthetic dyes are used in pharmaceutical, food, cosmetics, paper, leather, and carpet manufacturing (John Sundar *et al.*, 2011; Pérez-Ibarbia *et al.*, 2016; Saxena *et al.*, 2017; Guerra *et al.*, 2018; Castro *et al.*, 2021). Improper disposal of these dyes represents health risks because of toxic products that

biodegradation could produce (Parmar *et al.*, 2022). The increase in fade-resistant fabric production creates a problem because it uses stable dyes that are more resistant to traditional chemical and biological bioremediation methods (Carney Almroth *et al.*, 2021; Al-Tohamy *et al.*, 2022). Currently, decolorization and degradation methods are expensive and sometimes generate toxic compounds that are difficult to degrade (Parmar *et al.*, 2022). Therefore, developing low-cost, effective bioremediation methods for these color-fast dyes is needed. **Figure 2-1** summarizes possible chemical fates of textile and paper pulping effluents based on interactions with enzymes, which can lead to downstream consequences in the environment.

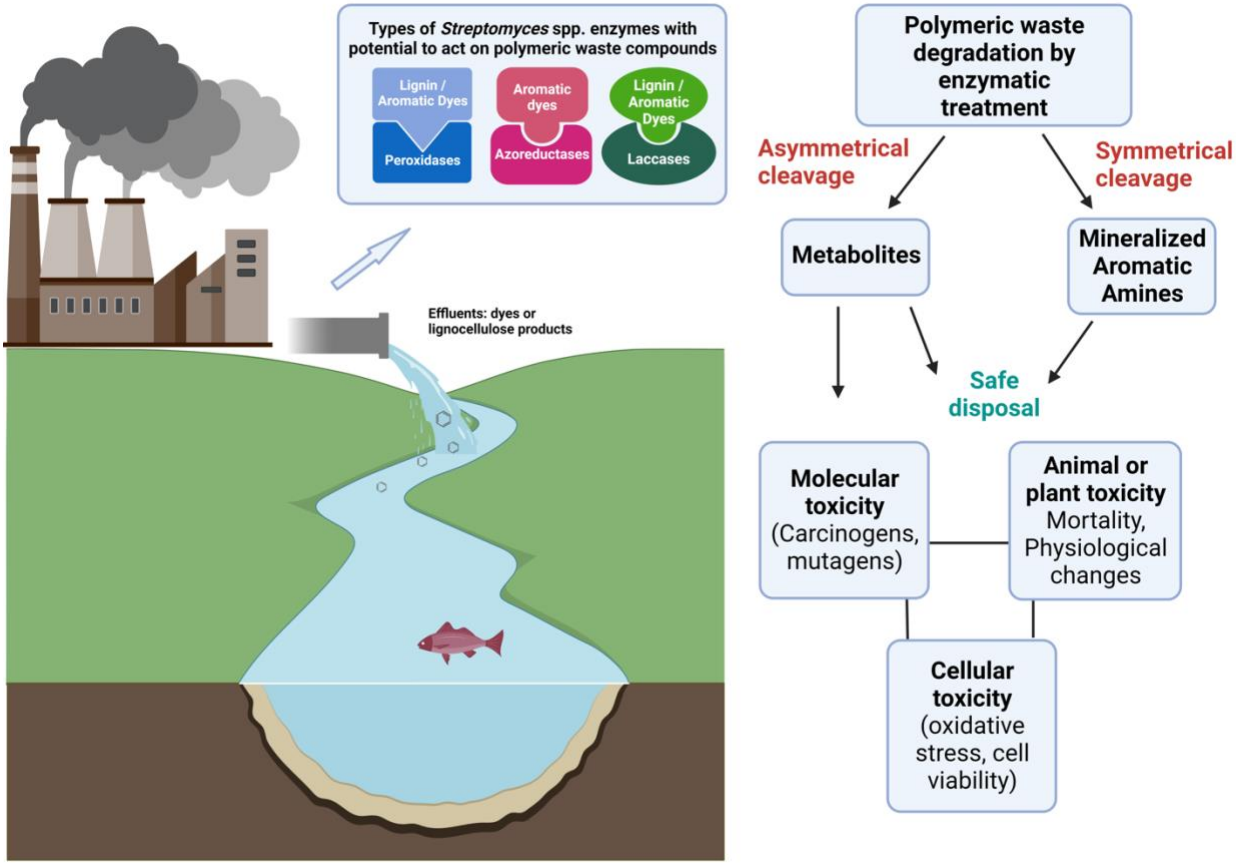


Figure 2-1 Enzyme types from *Streptomyces* spp. with the ability to degrade pollutants, as well as possible degradation byproducts formed and potential effects in the biosphere. Created with BioRender.com

Lignocellulose is the most globally abundant organic renewable source and can be used as a feedstock to produce sustainable bioproducts (i.e., fuels, chemicals, and molecules) (Riyadi *et al.*, 2020). Lignocellulose is a complex biopolymer composed of cellulose, hemicellulose, and lignin. Lignin, the second most abundant component in lignocellulose after cellulose (Xiao *et al.*, 2020), provides rigidity and robustness to the plant cell wall, notably protecting against pathogens and oxidative stress (Achyuthan *et al.*, 2010; Mathews *et al.*, 2015). However, because of its amorphous structure, lignin can be difficult to break down (Kumar and Chandra, 2020). The removal of lignin is necessary for efficient bioconversion of the hemicellulose and cellulose components of lignocellulose into sugars for fermentation applications (Zhang *et al.*, 2016; Yu *et al.*, 2018) as well as for wood pulp processing for paper production (Wang *et al.*, 2013). Once removed, lignin is often burned to use as steam energy (low-cost fuel to power paper mills) instead of being reused (Ko *et al.*, 2016). However, the valorization of lignin for its beneficial properties such as biodegradation, antioxidant activity, thermostability, high carbon content, and stiffness has been recently reviewed (Rinaldi *et al.*, 2016) as has the bacterial conversion routes for the valorization of lignin (Liu *et al.*, 2022). Challenges remain in the processing of lignin for complete valorization through its bioconversion to bio-based products (e.g. commodity chemicals like plastics, resins, fibers, bioplastics, resins, fibers, and phenols, as well as biofuels) (Sethupathy *et al.*, 2022). Existing pretreatment methods for lignin degradation may negatively affect downstream stages in energy bioconversion from cellulose biomass and can generate toxic waste products (Palmqvist, 2000; Palmqvist and Hahn-Hägerdal, 2000; Klinke *et al.*, 2004). Generation of harmful pollutants is a potential risk to the environment and human health (Yang *et al.*, 2017; Benslama *et al.*, 2021). Therefore, treatment options are needed to increase the efficiency of lignin degradation and bioconversion. Using microbial enzymes for

lignin and dye degradation is promising for enhancing available treatment options. The purpose of this review is to highlight the ability of multiuse enzymes from a specific bacterial genus, *Streptomyces* spp., to bioremediate textile and pulp-milling waste streams.

2.2 Taxonomy and life cycle of *Streptomyces*

The genus *Streptomyces*, phylum Actinobacteriota (Panda *et al.*, 2022), class Actinobacteria are Gram-positive, aerobic bacteria with high G+C content (69-78 mol %) that form an extensive mycelium (Kämpfer, 2006; Barbuto Ferraiuolo *et al.*, 2021). *Streptomyces* are ubiquitous in a variety of ecological niches but are commonly found in soil and are ecologically significant due to their role in decomposing cellulose (Takasuka *et al.*, 2013). The genus is arguably most known for secondary metabolite production (Kinkel *et al.*, 2014; Lee *et al.*, 2014; Dávila Costa *et al.*, 2020; Goel *et al.*, 2021, 2022; Alam *et al.*, 2022). *Streptomyces* are well studied as sources for antibiotic production in the pharmaceutical industry (Chevrette *et al.*, 2019). Recent advances in the production of drugs have been reviewed and highlighted as microbial cell factories (Barbuto Ferraiuolo *et al.*, 2021). These microbes contain multiple biosynthetic gene clusters (BGCs) that are sources of bioactive compounds with biomedical and agricultural applications (Ward and Allenby, 2018; Nicault *et al.*, 2021). However, comparably much less discovery has occurred to date focused on developing *Streptomyces*-based technologies for biodegradation applications.

Physiologically, *Streptomyces* spp. have a life cycle divided into two phases (vegetative and reproductive) that can vary depending on whether the culture is being cultivated on solid or in liquid media (Lajtai-Szabó *et al.*, 2022). When grown on solid-based media, the spores germinate into the hyphae which produces the vegetative mycelium. The mycelia grow deeply into the solid media, and some proportion of the mycelia could be present during the whole life

cycle (Flärdh and Buttner, 2009). Later, programmed cell death starts a degradation process where the hyphae become multinucleated (Manteca *et al.*, 2005). At this stage, the secondary metabolites are generated, and the aerial mycelia appear. This process is followed by a second programmed cell death event where spore formation occurs (Manteca *et al.*, 2008). If suitable conditions exist to promote germination of the spores, the cycle starts again (Barbuto Ferraiuolo *et al.*, 2021). When *Streptomyces* are cultivated in broth cultures, spore germination and mycelium development are similar to growth on solid media; however, differences in morphological characteristics like the propensity for clumping, pellet formation, or having disperse mycelia can result (Lajtai-Szabó *et al.*, 2022).

2.3 *Streptomyces* spp. enzymes with utility for lignin polymer modification and dye decolorization and degradation applications

Most lignin degradation studies focus on fungal enzymes, suggesting that fungal lignin metabolism is more efficient for lignin degradation than in bacteria (Liu *et al.*, 2022). Fungal species that have been reported to possess lignin depolymerization properties are white-rot and brown-rot members, where lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), dye-decolorizing peroxidases (DyP), and laccases (Lac) have been identified and characterized as the key enzymes for this process (Barrasa *et al.*, 1995; Soden *et al.*, 2002; Salvachúa *et al.*, 2013; Salame *et al.*, 2014; de Eugenio *et al.*, 2021). However, the slow growth of fungi makes the process more challenging. Extracellular activity from fungi with the ability to bioremediate dye and lignocellulosic waste has been demonstrated and extensively studied (Fu and Viraraghavan, 2001; Ajaz *et al.*, 2020; del Cerro *et al.*, 2021; Kita *et al.*, 2022). Challenges in improving the expression and activity of fungal enzymes have opened opportunities to investigate the possible use of oxidase-producing prokaryotic organisms for dye bioremediation

(Ajaz *et al.*, 2020). Compared to fungi, bacterial strains are easier to maintain in bioreactor systems. Also, maintaining fungal growth for extended periods (e.g. ≥ 7 days) is unfavorable for high decolorization rates (Banat *et al.*, 1996; Chang *et al.*, 2004). Bacterial-based dye bioremediation applications are preferred because prokaryotes are often faster growers and can generate more biomass that can ultimately support higher functional enzyme titers (Kuhad *et al.*, 2012). In addition, bacterial enzymes can exhibit better stability over a range of temperatures, which is important for in situ bioremediation applications (Wang *et al.*, 2022).

Bacterial lignin degradation has not yet been explored to the degree that fungal processes have been studied, but studies have reported lignin-degrading bacteria in the following classes: Actinobacteria, Alphaproteobacteria, Bacilli and Gammaproteobacteria (Ball *et al.*, 1989; Bugg *et al.*, 2011; DeAngelis *et al.*, 2011, 2013; Manter *et al.*, 2011; Chen *et al.*, 2013; Tian *et al.*, 2014; S. L. Mathews *et al.*, 2016; Jiang *et al.*, 2022). Genera belonging to these classes are best known for their degradation capabilities because it has been shown through metagenomic analyses and biochemical characterization that several of them possess highly conserved versions of well-characterized lignin-degradation enzymes (Tian *et al.*, 2014). The study of enzymatic breakdown of lignin and lignocellulose has fortuitously also resulted in the identification of enzymes capable of decolorizing various chromophores such as those found in industrial dye waste streams (Wang *et al.*, 2018). It is been reported that lignocellulose solubilizing microbes were also capable of the decolorization of triphenylmethane and Poly R-478 dyes (Ball *et al.*, 1989; Vasdev and Kuhad, 1994; Abou-Dobara and Omar, 2015). The decolorization process typically occurs through the oxidation of the chromophore portions of dyes or other colored compounds by enzymes such as laccases, azoreductases, and peroxidases (Chen *et al.*, 2003; Satheesh Babu *et al.*, 2015; John *et al.*, 2020).

Several research studies have shown the presence and potential of enzymes from *Streptomyces* spp. for lignocellulose degradation (Takasuka *et al.*, 2013; Feng *et al.*, 2015; Ventorino *et al.*, 2016; Blánquez *et al.*, 2017, 2022; Pinheiro *et al.*, 2017; Cecchini *et al.*, 2018; Riyadi *et al.*, 2020; Wadler *et al.*, 2022), and it has been shown that these microbes produce dye-decolorizing, detergent-stable peroxidases that could replace sodium perborate for distaining synthetic textile dyes (Rekik *et al.*, 2015). The presence and/or detected activity of these enzymes in *Streptomyces* spp. supports the idea that this group should be further investigated as a source of enzymes for lignocellulolytic-degrading and decolorization applications.

2.4 Applications of *Streptomyces* spp. in textile and pulp and paper industries

For textile and pulp industries, microbial enzymes have proven valuable to treat water effluents or waste byproducts (Rajoria and Roy, 2022). Because of these potential capabilities, researchers can use different screening methods to discover the presence of relevant enzymes from a variety of environmental samples (Parmar *et al.*, 2022). For example, a dye decolorization screening method was initially used to identify laccases and other enzymes that can break down aromatic compounds (Glenn and Gold, 1983; Pasti and Crawford, 1991; Ollikka *et al.*, 1993). Dye decolorization screens can employ a solid culture media format wherein the breakdown of the dye results in a distinguishable halo surrounding the microbial colony producing the enzyme(s) of interest, or the microbe can be culture in a liquid medium to which a dye has been added. Color intensity is then quantified using a spectrophotometer (Shah *et al.*, 2021). The dyes used in the screens (**Table 2-1**) are complex in chemical composition and are used for screening methods for these enzymes because they mimic the structural complexity of lignocellulose compounds and can therefore indicate aromatic-degrading activity (Tian *et al.*, 2014; Mathews *et al.*, 2016). These type of plate assays are described in recent reviews as part of the isolation

methods for lignin-modifying enzymes secreted by microorganisms (Kameshwar and Qin, 2017; Kaur *et al.*, 2022).

Table 2-1 Dyes of environmental concern in the textile industry and examples of *Streptomyces* spp. that have been involved in the treatment of these dyes.

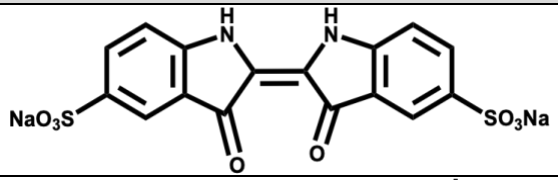
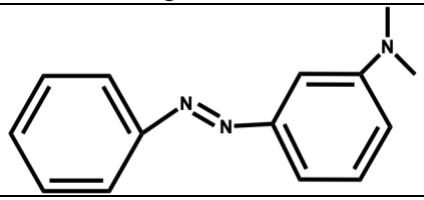
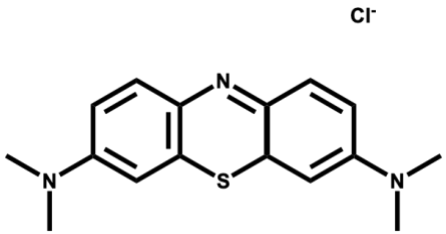
Dye Type	Commercial name	Material dye is applied to	Structure	Strains involved in decontamination	References
Acid	Acid blue 74 (Indigo Carmine)	Leather, Wool		*S. coelicolor*	(Dubé, Shareck, Hurtubise, Daneault, *et al.*, 2008)
Azo	Azo Red	Silk, Cotton, Nylon		*S.* sp. S27	(Dong *et al.*, 2019)
Basic	Methylene Blue	Ink		*S. tuirus*	(Mechouche *et al.*, 2022)

Table 2-1. (Continued)

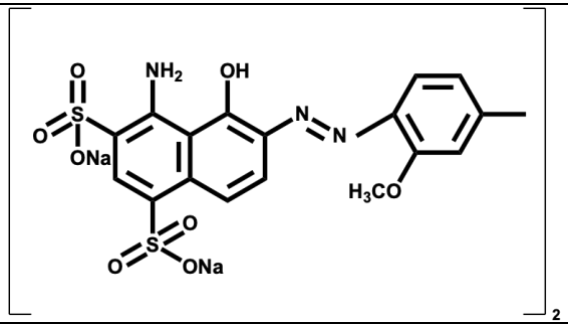
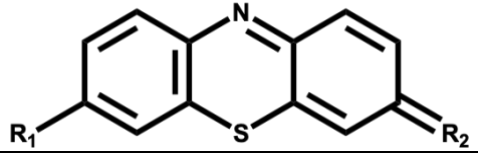
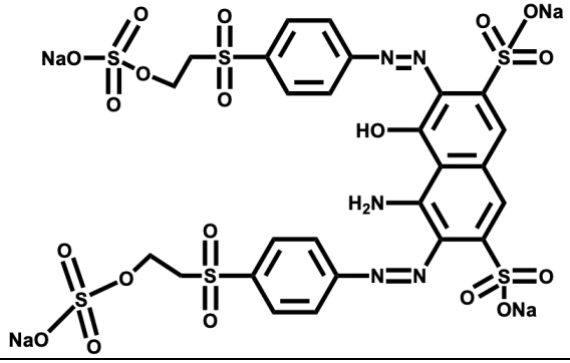
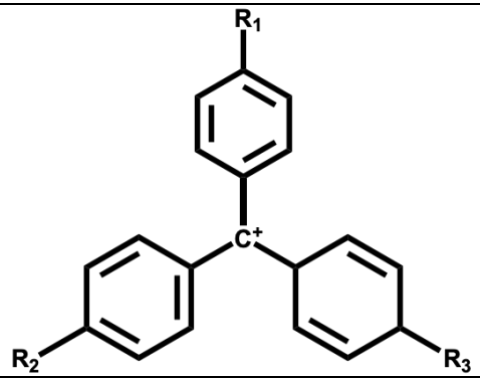
Direct	Direct Sky Blue 6b	Leather, Cotton, Wool, Ink		*S. coelicolor*	(Dubé, Shareck, Hurtubise, Beauregard, *et al.*, 2008)
Sulfur	Thiazine	Cotton, Paper, Silk		*S. ambofaciens*	(Díez-Méndez *et al.*, 2019)
Reactive	Reactive Black 5	Wool, Silk, Nylon		*S. ipomoneae* CECT 3341	(Blánquez *et al.*, 2019)

Table 2-1. (Continued)

Triphenyl methane	Malaquite green, Crystal Violet, Cotton Blue, Methyl Violet	Wool, Silk, Nylon, Cotton		*S.* sp. MN262194	(Adenan *et al.*, 2022)

Development of applications using *Streptomyces* to biotransform recalcitrant materials is an area of significant interest. Better understanding of the metabolism of lignocellulosic materials by *Streptomyces* spp. has recently opened a door for the valorization of microbial-generated products from food waste. As example in a recent study by Schalchi *et al.* *Streptomyces* spp. was used to produce antifungals and biopigments from potato solid waste (Schalchli *et al.*, 2021). Additionally, in the food waste valorization field, an immobilized polygalacturonase from *S. halstedii* ATCC 10897 was used in a bioreactor for the degradation of pear and cucumber residues increasing the sugar content up to 15.33 and 9.35 mg/mL, respectively (Ramírez-Tapias *et al.*, 2018). Another application for lignocellulosic bioconversion was developed for the generation of branched-chain fatty acids for lipid-based biofuel applications where *S. lividans* bioconverted sunflower stalks and rape straw residues into triacyglycerols with a yield of 19-44% conversion (Dulermo *et al.*, 2016). Therefore, exploring enzymes from *Streptomyces* spp. for potential biotechnological applications such as those discussed here represents an opportunity for both industries to treat polymeric waste material before discharge in both textile and pulp and paper industries. Additional reports and details about the application of *Streptomyces* spp. in these industries are presented in **Table 2-2**.

Table 2-2 Examples of the applied technologies using *Streptomyces* spp. in pulp, paper, and textile industries

Strain	Application	Industry	Key points	Reference
S. cellulosae	Bioaugmentation	Textile industry	-*S. cellulosae* was tested as part of the consortia that accelerated a textile waste compost process by reducing organic matter from 72 weeks to 12 weeks	(Biyada *et al.*, 2022)
S. rutgersensis UTMC 2445	Biobleaching	Pulp and Paper	-Lignocellulosic soil isolates as sources of enzymes in bleaching applications using xylanases -7% increase in brightness at 30°C, pH 5-7 compared to untreated control -55% of final brightness after biotreatment	(Hamedi *et al.*, 2020)
S. griseorubens LH-3	Biobleaching	Pulp and Paper	-Purified and characterized endo-xylanase increased the brightness of eucalyptus kraft pulp by 14.5% and reduced the kappa number by 24.5%	(Wu *et al.*, 2018)
S. olivaceus (MSU3)	Biobleaching	Pulp and Paper	-Endo-β-1,4 xylanase yielded the reduction of sugars of sugars 110 mg/g, the reduction of kappa number of 14.69 *k* and the degree of brightness of 43.62% ISO using sugarcane bagasse pulp. -Better fibrillation and porosity of pulp than the zinc oxide-mediated bleaching	(Kumar *et al.*, 2020)

Table 2-2. (Continued)

S. sp. S27	Bioscouring	Textile	-Alkali stable pectate lyase (pH 10) exhibited high activity (>70%) at pH 12.0 showed similar results in reducing the viscosity of polygaracturonic acid compared to a commercial complex (49% vs 49.7%) while when combined with other enzymes was able to be comparable in bioscouring jute fabric (22.39% vs. 22.99%)	(Yuan *et al.*, 2012)
S. sp. NP2, *S.* sp. NP4	Bio-colorants	Textile	-Isolates were able to generate diffusible deep blue and deep red pigments. -Dyes were tested in polyamide and acrylic fibers with darker colors, polyester and triacetate fibers to a lower dark color and cotton and cellulosic fibers were weakly colored. -Crude bacterial pigments showed similar characteristics of ionic and disperse dyes due to the polypyrrolic prodigiosin-like structures that are found in these synthetic dyes.	(Kramar *et al.*, 2014)

Table 2-2. (Continued)

S. cyaneus	Biopulping	Pulp and Paper	-The pretreatment of wheat straw in Solid State Fermentation improved the strength the optical and mechanical properties -Tear index and burst index were improved (15%, and 19% respectively)	(Berrocal *et al.*, 2004)
S. drozdowiczii	Stonewashing and biopolishing	Textile	-Cellulase activity worked up to 87% in the presence of commercial detergents, and was able to reduce the weight of cotton fabric comparable to commercial treatments (0.75g vs 1.12g from an initial 1.5g)	(Grigorevski de Lima *et al.*, 2005)
S. sviceus	Bioremediation of reactive azo dyes	Textile	-Optimal decolorization of Congo red-21 without the release of toxic molecules.	(Chakravarthi *et al.*, 2020)
S. sp. APL3	Degradation of synthetic polyesters	Textile	-*Streptomyces* isolated from compost was able to degrade Polylactic acid, poly-L-lactide, polycaprolactone, poly-(butylene succinate), and polybutylene succinate-*co*-adipate.	(Somyoonsap *et al.*, 2018)
S. sp. UAH 15, 23, 30, 51.	Decolorization of paper mill effluent	Pulp and Paper	-*Streptomyces* isolates were able to decolorize paper mill effluent after pulping wheat straw alkali treatment by 60-65%. Two specific strains (UAH 30, UAH 51) were able to also reduce the molecular weights in high and medium fractions of the effluent.	(Hernández *et al.*, 1994)

2.5. Oxidative lignin-degrading enzymes from *Streptomyces* spp. for use in waste detoxification

Microorganisms can be used for the detoxification (degradation, decolorization) of industrial waste streams. **Figure 2-1** summarizes several interactions between the chemical structure of the polymer and environmental factors that can lead to downstream consequences in the environment. Microbial enzymes have been shown to degrade synthetic dyes into uncolored compounds or mineralize them in the environment (Saratale *et al.*, 2011; dos Santos Bazanella *et al.*, 2013; Khan *et al.*, 2013). The loss of color does not necessarily mean or guarantee that complete mineralization has occurred (Franca *et al.*, 2015; Albahnasawi *et al.*, 2020). One possible fate is the production of amines or intermediates that need to be bioconverted by other microorganisms or methods (Figure 1) (Chang *et al.*, 2004). To remove these amines, chemical, physical, and biological methods have been used. Examples include the use of activated carbon adsorption, membrane separation, steam-distillation, bacterial oxidation, chemical oxidation, electrochemical techniques, and irradiation (Klibanov and Morris, 1981; Reynolds *et al.*, 2016; Bhat and Gogate, 2021). Biological methods have been receiving attention because of their low cost as an alternative to chemical and physical removal techniques which are expensive and require time-consuming analyses (Rathi *et al.*, 2021). However, research in this area is considered to be in the early stages, and the primary goal for implementation is to achieve high degradation rates to avoid transfer of pollutants from the facility to the environment (Rathi *et al.*, 2021).

The ideal scenario is to have microbes with high catalytic versatility to degrade complex mixtures of dyes and that can tolerate harsh conditions such as exposure to detergents, surfactants, and metals. Therefore, microbes capable of growing or surviving over a wide range

of pH, temperatures, and salinity can be helpful for these applications (Anjaneyulu *et al.,* 2005). A summary of enzymes characterized from *Streptomyces* spp. exhibiting lignocellulosic activity and dye decolorization properties are shown in Table 3.

One of the key enzymes known to be involved in lignin metabolisms is lignin peroxidase (EC 1.11.1.14; LiP) which was first isolated from the fungus *Phanerochaete chrysosporium*, and it was shown that its heme cofactor is required for enzymatic oxidation of aromatic rings (Tien and Kirk, 1984). Because this type of enzyme needs peroxide for activating catalysis, it is called a peroxidase. A peroxidase isoform (P3) from *S. viridosporus* T7A was characterized and overproduced in the presence of Acid-Precipitable Polymeric Lignin (APPL) and was demonstrated to have the ability to oxidize lignin and phenolic compounds, and because of this, it was classified as a lignin peroxidase called "Actinomycetes lignin-peroxidase" (ALiP-P3) (Ramachandra *et al.*, 1988; Spiker *et al.*, 1992). This AliP-P3 could catalyze the oxidation of 2,4-dichlorophenol in the presence of hydrogen peroxide, which suggested its usefulness for degradation of xenobiotics (Yee and Thomas K. Wood, 1997).

Unfortunately, no protein or gene sequence of the lignin peroxidase was deposited which makes difficult to compare to other studies for further enzyme characterization (de Gonzalo *et al.*, 2016) describing the characterization of this enzyme (Ramachandra *et al.*, 1988; Wang *et al.*, 1990; Thomas and Crawford, 1998). One of the characterization studies demonstrated the recombinant expression of the enzyme has been studied by using *Pichia pastoris* as the expression system since (Thomas and Crawford, 1998). In the same publication, the researchers reported the coexpression of an endoglucanase gene, suggesting that the lignocellulosic system of *S. viridosporus* was chromosomally clustered (Thomas and Crawford, 1998).

Streptomyces spp. such as *S. coelicolor* A3 may also have a role in lignin modification since it has been shown to use grass lignocellulose as a growth substrate and for forming APPL (Majumdar *et al.*, 2014a). In light of the importance of LiP enzymes for lignin deconstruction, lignin peroxidase-like activity has been screened for in other *Streptomyces* sp., for example, in *Streptomyces* sp. S6 (Riyadi *et al.*, 2020). However, no homologs or annotated lignin peroxidases have been found in *Streptomyces* that appear to be similar to fungal lignin peroxidases (de Gonzalo *et al.*, 2016; Riyadi *et al.*, 2020). Moreover, homologs of ligninolytic peroxidases have not been extensively studied in bacteria, and no homologs have been found in lignin-degrading bacteria using gene sequencing predictions or proteomes (Davis *et al.*, 2013; de Gonzalo *et al.*, 2016). Reviews about these enzymes called to attention the need of bioprospecting for lignin peroxidases in bacteria because little is known about them (Lambertz *et al.*, 2016; Falade *et al.*, 2017). Recently high LiP activity (1132-2899 U/L) was detected in *Vibrio* strains which could serve as research models for bacterial LiP (Li *et al.*, 2023).

The genome of *Streptomyces* sp. S6 contains high homologous peroxidases belonging to other family: DyP-type peroxidases (DyP), however, low activity was observed (Riyadi *et al.*, 2020). Interestingly, in the genome of *S. viridosporous* T7A, an annotated gene encoding for a putative Tat-secreted DyP (Davis *et al.*, 2013) was hypothesized to be the enzyme described before been deposited (de Gonzalo *et al.*, 2016).These dye peroxidases (DyP, EC 1.11.1.19) or dye-decolorizing peroxidases were initially characterized for decolorizing industrial dyes. However, these enzymes act on various substrates, including types of lignin. They differ from typical peroxidases in its substrate preference for anthraquinone dyes and its high peroxidase activity compared to a variety of other organic compounds (Mechouche *et al.*, 2022). Available peroxidases, also utilized for dye decolorization, are heme-containing proteins that require

hydrogen peroxide (H_2O_2) or organic hydroperoxides (R-OOH) to oxidize reducing substrates. The ability to oxidize various substrates makes them applicable for multiple biological processes such dye decolorization, and oxidation of small phenolic compounds including molecules that mimic lignin (Chen and Li, 2016). Peroxidase-based technology systems are catalytically active at acidic pH from 4-6, a limitation for its incorporation as biocatalysts in detergent formulations (Rekik *et al.*, 2015). The current status regarding the role of bacterial DyP-type peroxidases in lignin degradation is still under discussion and was recent reviewed (Sugano and Yoshida, 2021). Interestingly, DyP-type peroxidase expression has been shown to play a role in life cycle control in *Streptomyces* (Sugano and Yoshida, 2021), and it was demonstrated to participate in switching between vegetative mycelium and aerial hyphae in *S. lividans* (Chaplin *et al.*, 2015).

Another important oxidative enzyme involved in lignin modification and dye decolorization are laccases (EC 1.10.3.2), which were first discovered in the sap of the Japanese lacquer tree (Yoshida, 1883; Hoegger *et al.*, 2006), and are a type of blue polyphenol oxidase belonging to the family of blue multicopper oxidases. These enzymes are encoded in the genomes of fungi, bacteria, and animals. A recent review has highlighted information about the mechanism, structure, enzymatic assays, genomic distribution, biotechnological properties, and genetic engineering tools including a mention of their use in the pulp, paper, wood, dye/textile industries (Kaur *et al.*, 2022). Laccases from *Trametes versicolor*, a white-rot fungi, have been demonstrated to exhibit higher activity (20 times greater) compared to *Streptomyces* laccases (Margot *et al.*, 2013). However, *Streptomyces* laccases have shown improved stability over a broader range of temperature, alkaline conditions, and salinity, which is helpful for applications in pulp and textile industries for biobleaching and decolorization, respectively (Singh *et al.*, 2011).

Several species of *Streptomyces* are known to express laccases that have been biochemically characterized (Fernandes, Silveira, *et al.*, 2014). Some examples include a laccase from *S. griseus* that has an optimal pH and temperature of 6.5 and 40°C, and was shown to oxidize *N,N*-dimethyl-*p*-phenylenediamine sulfate (Endo *et al.*, 2003). *S. cyaneus* produces a laccase with an optimal pH and temperature of 4.5 and 70°C and reported activity with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) and 2,6-dimethoxyphenol (DMP) (Arias *et al.*, 2003). The *S. ipomoea* laccase has optimal pH of 8 and temperature of 60°C when acting on phenolic substrates (Molina-Guijarro and Pérez, 2009), and *S. sviceus* laccase has optimal pH of 9 temperature of 60°C for reactions with DMP and guaiacol (Gunne and Urlacher, 2012). Reports dealing with laccases associated with lignocellulose, dye deconstruction, degradation, or decolorization can be found in **Table 2-3**.

Polyphenol oxidases (PPO) are members of the multicopper oxidases that share similar catalytic properties to laccases (Janusz *et al.*, 2020). These enzymes require oxygen to catalyze the oxidation of mono- and di-phenols (Sharma and Kuhad, 2009). Interestingly, these enzymes contribute to the melanin pigment formation in bacterial cells (McMahon *et al.*, 2007; Wang *et al.*, 2019). The production of melanin by these enzymes has been observed in *Streptomyces* and was correlated with the production of laccase (Claus and Decker, 2006). The activity in bacterial cells and spores suggests a protective role against environmental stress factors such as UV radiation, reactive oxygen species (ROS), and toxic heavy metals (Faccio *et al.*, 2012). In addition, extracellular PPOs could have a role in the polymerization and detoxification of plant phenolic compounds in soil environments (Janusz *et al.*, 2020).

Table 2-3 Strains of *Streptomyces* spp. associated with lignocellulose and dye decolorization

Strain	Lignin Substrate	Dye Substrate	Enzyme	NCBI Accession numbers of characterized enzymes	Enzyme characterization method	Reference
S. flavorivens	Lignified sclereids					(Sutherland *et al.*, 1979)
S. badius ATCC 39117	Labeled lignin	Aromatic dyes	Laccase			(McCarthy, 1987)
S. viridosporus T7A	Kraft lignin, synthetic lignin, 2,4-diclorophenol (wood preservatives) Wheat Straw	Aromatic dyes	Laccase, H_2O_2 peroxidase AliP3		Characterization from crude extract	(Yee and T.K. Wood, 1997; Zerbini *et al.*, 1999; Zeng *et al.*, 2013)
S.cyaneus CECT 3335	Kraft pulp biobleaching	Evans Blue dye, Amido Black 10B, Reactive Black 5	Laccase	HQ857207	(Ece *et al.*, 2017)	(Arias *et al.*, 2003; Moya *et al.*, 2010; Popović *et al.*, 2021)
S. psammonitcus		Acid orange, Methyl orange, Bismarck brown	Lignin peroxidase, manganese peroxidase, Laccase		Characterization from crude extract	(Niladevi and Prema, 2005; Niladevi *et al.*, 2008)

Table 2-3. (Continued)

S. coelicolor		Direct Sky Blue, Acid blue 74, Reactive black 5	Laccase	CAB45586	(Dubé, Shareck, Hurtubise, Beauregard, *et al.*, 2008)	(Dubé, Shareck, Hurtubise, Beauregard, *et al.*, 2008)
S. coelicolor A3 (2)	Grass lignocellulose	Brilliant Blue G, and Trypan Blue	Laccase	CAB45586.1	(Machczynski *et al.*, 2004)	(Majumdar *et al.*, 2014a; Yadav and Chandra, 2018)
S. lividans TK24	Kraft lignin		AliP3	reported as not available (de Gonzalo *et al.*, 2016)	Wang *et al.*, 1990	(Wang *et al.*, 1990)
S. sp. SB086			Laccase		Characterization from crude extract	(Fernandes, da Silveira, *et al.*, 2014)
S. griseorubens JSD-1	Lignin, Rice straw, Xylan	Indigo carmine				(Feng *et al.*, 2015)
S. griseosporeus SN9			Lignin peroxidase (LiP-SN)		Characterization from crude extract	(Rekik *et al.*, 2015)
S. schrestomycetus S20		Malachite Green				(Vignesh et al., 2020)

Table 2-3. (Continued)

S. ipomoea CECT 3341	Wheat straw	Orange II, Reactive Black 5, Indigo Carmine	Laccase, Laccase-mediator system	DQ832180.1	(Molina-Guijarro and Pérez, 2009)	(Molina-Guijarro and Pérez, 2009; Blánquez *et al.*, 2017, 2019)
S. fulvissimus CKS7		Crystal Violet				(Buntić *et al.*, 2016)
S. sviceus SN3		Congo Red	Laccase	SSEG_02446 from CM000951.1	(Gunne and Urlacher, 2012)	(Chakravarthi, Bhoodevi *et al.*, 2021)
S. sviceus KN3		Congo Red, Navy Blue, Textile azo dye effluent		SSEG_02446 from CM000951.1	(Gunne and Urlacher, 2012)	(Chakravarthi *et al.*, 2017)
S. albus ATCC 3005	Eucalyptus Kraft pulp, Chlorophenols compounds		Laccase, Lignin peroxidase		Characterization in crude extract	(Antonopoulos *et al.*, 2001a; Antonopoulos *et al.*, 2001b)
S. coeliflavus CS-29		Blue dye 21, and red dye 34	Laccase, Peroxidase, Manganese peroxidase			(Mon *et al.*, 2022)

Table 2-3. (Continued)

S. bacillaris		Malachite Green, Crystal Violet, Cotton Blue, Methyl Violet			(Adenan *et al.*, 2022)
S. sp. C1		Indigo carmine and diamond black PV	Laccase mediator system	Characterization in crude extract	(Lu *et al.*, 2013)
S. thermocarboxydus	Alkali lignin		MnP-like, Laccase	Assay detected	(Tan *et al.*, 2022)
S. coelicolor,S. griseorubens, S.avermitilis	Wheat bran		Peroxidase in mono and co-cultures with fungi		

Azoreductases are another type of industrially relevant enzymes that have been reported to be expressed in *Streptomyces* sp. in addition to laccases and peroxidases that have described earlier. Azoreductases (EC. 1.7.1.6) can be defined as oxidoreductases which are mostly known for the degradation of azo dyes by reducing the azo linkage (-N=N-), for example, the reactive dye structures presented in **Table 2-1**. These enzymes are categorized into groups according to their cofactor preference (NADH or NADPH) as their electron donor (Dong *et al.*, 2019). A limiting factor in using these enzymes for wastewater treatment is their cofactor requirement as well as their need for redox mediators to facilitate the transfer of electrons from NAD(P)H to the colored substrate (Mahmood *et al.*, 2016; Verma *et al.*, 2019). However, some research studies have shown that this problem can be overcome by adding coenzymes or integrated enzymatic systems. This was the case for the discovery of a novel azoreductase "AzoRed2" from *Streptomyces* sp. S27 which was used in combination with a glucose-1-dehydrogenase from *Bacillus subtilis* to achieve 99% completion of the degradation of the azo dye within 120 min (Dong *et al.*, 2019). An alternative study involving *S. coelicolor* showed the importance of the azoreductase as the main biocatalyst of decolorization, while the presence of an active DyP-type peroxidase and laccase played a role in achieving the mineralization of methylene blue in 72 h at 97.5% decolorization (Preethi and Pathy, 2020). Therefore, azoreductases from *Streptomyces* spp. can serve as additional potential bioremediation catalyst, but they need to be more systematically explored for their use in wastewater treatment technologies.

2.6 Biotechnological approaches using *Streptomyces* spp. for decontamination in textile and pulp and paper industries

Bacterial decontamination in general is a promising environment-friendly and cost-effective alternative to physiochemical methods that are commonly used (Parmar *et al.*, 2022). The evaluation of these systems requires the analyses of degradation products or metabolites that are produced during the decontamination process. Suitable methods for these analyses can include analytical techniques such as UV-vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), gas chromatography-mass spectrometry (GC/MS), high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), among others to gain understanding as to the degree of waste compound degradation and compound fate that can be achieved using the microbial catalysts (Saratale *et al.*, 2011). The current approaches for potential decontamination are discussed in the following sections.

2.6.1 *Streptomyces* Whole cell technologies

A whole-cell system screening process is helpful to determine if the microbe can grow and/or metabolize a specific substrate under specific conditions required for degradation (Joutey *et al.*, 2013). In *Streptomyces*, whole-cell applications have been developed involving native planktonic whole-cell biocatalysts, immobilized systems, and recombinant whole-cell systems (Salama *et al.*, 2022). Even if the whole-cell approach might not represent an advantage over using enzymes as catalysts in terms of efficiency, it is often more cost-effective because protein isolation and purification steps can be avoided (de Carvalho, 2016).

Examples of application of this whole-cell approach for *Streptomyces* bioconversion include the degradation of organophosphorous compounds, which are found in pesticides, using *S. phaeochromogenes* (Santillan *et al.*, 2020). An alternative study demonstrated that

Streptomyces sp. MTCC 7546 could convert acrylonitrile into acrylic acid using both immobilized and planktonic cells (Nigam *et al.*, 2009). The biotransformation of nitriles is important because its toxicity has been associated with cancer, respiratory, and neuronal disorders (Ramteke *et al.*, 2013). Recombinant whole cells of *Streptomyces* overexpressing proteins have been employed to avoid problems that can arise with the use of non-native systems (e.g. the potential for the formation of aggregated recombinant enzyme inclusions when using *E.coli* expression systems) (Salama *et al.*, 2022). As an example, *S. lividans* has been extensively studied since it is readily genetically accessible and serves as a robust host for recombinant protein expression (Sevillano *et al.*, 2016). In addition, other *Streptomyces* spp. have been evaluated (Hwang *et al.*, 2021) to determine whether different genetic modification approaches could be used to efficiently produce secondary metabolites, biosynthetic gene clusters (BGC), and recombinant as described in **Table 2-4**.

Table 2-3 Genomic tools for cloning, assembly, and modifying secondary metabolites and recombinant proteins. (Updated and modified from Hwang et al., 2021)

Genomic tools	Heterologous host (*Streptomyces* spp.)	References
Genomic Libraries	*S. ambofaciens* BES2074, *S. albus* J1074, *S. coelicolor* M1152, *S. lividans* TK21, *S. coelicolor* strains	(Alexander *et al.*, 2010; Li *et al.*, 2017; Tan *et al.*, 2017; D'Agostino and Gulder, 2018; Liu *et al.*, 2018; Tu *et al.*, 2018)
Artificial Chromosome Vectors (pSBAC)	*S. lividans* TK21, *S. coelicolor* M145, *S. lividans* K4-114	(Liu *et al.*, 2009; Nah *et al.*, 2015; Luo *et al.*, 2016; Pyeon *et al.*, 2017)
Homologous recombination: Linear-to-circular (LCHR), and Linear-to-linear (LLHR)	*S. coelicolor* M1146, *S coelicolor* M1152/M1154, *S. venezuelae* WVR2006	(Jiang *et al.*, 2013; Yin *et al.*, 2015, 2016; Qian *et al.*, 2020)

Table 2-4. (Continued)

Homologous recombination: pSA3239 vector system	*S. lavendulae* subsp. *lavenduale* CCM 3239	(Novakova *et al.*, 2022)
In vivo homologous recombination: Exonuclease Combined with RecET recombination (ExoCET)	*S. coelicolor* A3 (2)	(Wang *et al.*, 2018)
In vivo homologous recombination: Transformation-Associated Recombination (TAR)	*S. lividans* TK23, *S. coelicolor* M1146, *S. albus* J1074, *S. albus* TK24, *S. coelicolor* M145	(Yamanaka *et al.*, 2014; Bonet *et al.*, 2015; Tang *et al.*, 2015; Bilyk *et al.*, 2016; Novakova *et al.*, 2018)
In vivo assembly: DNA Assembler	*S. lividans* 66, *S. lividans*	(Luo *et al.*, 2013; Shao and Zhao, 2013; Shao *et al.*, 2013)
In vivo assembly: Gibson Assembly	*S. coelicolor* M1146, M1152, M1154	(Zhou *et al.*, 2015; Linares-Otoya *et al.*, 2017; Vior *et al.*, 2018)
Direct Pathway Cloning (DiPAC)	*S. coelicolor* M1152/M1154	(D'Agostino and Gulder, 2018; Greunke *et al.*, 2018)
Cas9- Assisted Targeting of Chromosome segments (CATCH) combined with Gibson Assembly	*S. albus* J1074, *S. avermitilis* MA4680	(Jiang *et al.*, 2015; Tang *et al.*, 2018; Tao *et al.*, 2019)

To date, several *Streptomyces* spp. have been identified as sources of enzymes that could be used in dye and lignin degradation either as whole cells or as sources of recombinant enzymes (Kaur *et al.*, 2022). Vanillin bioconversion to vanillic acid was performed by whole cell suspensions of *S. viridosporus*, nearly achieving 96% of purity yield (Pometto and Crawford, 1983). A recombinantly laccase from *S. coelicolor* was expressed in *S. lividans* generating a titer of 350 mg/L with demonstrated activity over a pH range of 4.0-9.0, thermostability up to 70°C,

and the ability to decolorize indigo dye with mediator (Dubé, Shareck, Hurtubise, Daneault, *et al.*, 2008).

2.6.2 Enzyme mediator systems for *Streptomyces* applications

The lignin degradation process can be improved by the presence of small aromatic molecules called "mediators". In enzymes such as laccases, these mediators can act as electron carriers between the substrate and the enzyme to modulate redox potential of the reaction and expand the capacity of the laccase to oxidize structures (Roth and Spiess, 2015). The use of mediators for lignin depolymerization is important since it has been reported that without mediators, laccases can only polymerize lignin from small compounds instead of operating in the depolymerization direction (Longe *et al.*, 2018). Furthermore, the polymerization of lignin by a *Streptomyces* laccase has been observed (Majumdar *et al.*, 2014b), and it has been determined that higher reduction of laccases by mediators favors depolymerization, and a lower reduction of laccases in the absence of mediators favors the repolymerization (Chan *et al.*, 2020).

An example of laccase-mediator system in *Streptomyces* is shown in *Streptomyces* sp. C1, the small laccase SilA decolorized indigo carmine and diamond black using syringaldehyde as a redox mediator achieving a decolorization of 83.7% and 56.4%, respectively, suggesting that this microbial laccase should be explored for treatment of textile effluents (Lu *et al.*, 2013). This is a particularly promising application not only because of *Streptomyces* sp. C1 SilA's decolorization properties, but also because its stability under high pH (up to 10) and temperatures (40-50°C) (Lu *et al.*, 2013). Several years later, the first example of the use of SilA in textile dye decolorization application was published, demonstrating that SilA from *S. ipomoea* CECT 3341 can improve the degradation of textile dyes by up to 60-fold and 20-fold, respectively. To achieve this values a laccase mediator system along with acetosyringone and

methyl syringate was used (Blánquez *et al.*, 2019). In addition, the same small laccase SilA was recombinantly expressed in *E.coli* and shown to achieve high decolorization levels (more than 80% indigo carmine and malachite green at different pH (6.8-8.0) within 2 h (Coria-Oriundo *et al.*, 2021). This study also revealed that β-(10-phenothyazyl)-propionic acid (PhCOOG) could be used as an efficient and affordable mediator in combination with recombinant SilA and could support the decolorization of remanzol brilliant blue R (an anthraquinone dye) by greater than 40% and 80% decolorization of xylidine ponceau (azo dye) (Coria-Oriundo *et al.*, 2021).

For biobleaching applications, *S. cyaneus* 3335 was reported as a source of purified laccase able to catalyze the biobleaching of eucalyptus kraft pulps in combination with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as redox mediator (Arias *et al.*, 2003). It was shown to achieve a decrease of 2.3 U in the kappa number, which is a measurement of potassium permanganate solution that is consumed by pulp (Li and Gellerstedt, 1997) and serves as an indication of lignin content or pulp biobleachability. A brightness increase of 2.2% was observed for this study (Arias *et al.*, 2003).

Additional successful laccase-mediator system was developed using *S. cyaneus* 3335, where the decolorization and detoxification of azo-dyes were achieved (Moya *et al.*, 2010). In this case, Methyl Orange and Orange II were decolorized by a 90% in the presence of the mediator acetosyringone; however, toxicity studies involving *Vibrio fischeri* viability analyses revealed that toxicity increased by 500% with Methyl Orange and 200% with Orange II after the treatment, while New Coccine and Chromotrope 2R were decolorized and decreased in toxicity by (300 and 185%, respectively (Moya *et al.*, 2010). Based on these findings, it is important to include appropriate toxicological analyses of the treatments as well as chromatographic analyses for better understanding of the process. It is also essential to highlight the importance of the cost

of laccase mediator systems. Mediators can be expensive and incompatible with industrial biodegradation processes (Debnath and Saha, 2020). This is a disadvantage that needs to be addressed in future process optimization.

2.6.3 *Streptomyces* immobilized biocatalyst systems

Enzyme immobilization of is also a promising method to consider for textile wastewater treatment. This has been demonstrated in pharmaceutical production (Barbuto Ferraiuolo *et al.*, 2021). Enzyme immobilization can be achieved by any of the following: entrapment, adsorption, covalent binding, self-immobilization, and encapsulation (Fernández-Fernández *et al.*, 2013). Whole cell immobilization approaches have distinct advantages since they allow high cell density during continuous operation and support process scale-up. Whole cell immobilization also can allow for more effective of nutrient access to the biocatalyst, substrate uptake, and improvement of catalytic activity (Rajoria and Roy, 2022).

Recently, it was shown who an immobilized laccase from *S. sviceus* KN3 could successfully mineralize and detoxify Congo Red (azo dye) (Chakravarthi, Bhoodevi *et al.*, 2021). In this study, the crude laccase extract (50 mg/L) was able to decolorize Congo Red by 69% after 48 h, while the purified and immobilized version (50 mg/L) showed a decolorization of 78% and 92% after 24 h, respectively. Another immobilized laccase method using *S. cyaneus* 3335 was evaluated for the decolorization of Amido Black 10B (61% decolorization), Reactive Black 5 (100% decolorization), Evans Blue (90% decolorization), and Remanzol Brilliant blue (100% decolorization) (Popović *et al.*, 2021). This study demonstrated that immobilized laccases from *S. cyaneus* 3335 can effectively catalyze delignification in a biobleaching process of paper pulping (Popović *et al.*, 2021).

2.7 Application of *Streptomyces* consortia

Most of the dye degradation studies featuring *Streptomyces* as a biocatalyst source describe the utility of recombinant enzymes or whole cell catalysts in pure culture. To maximize decolorization or degradation, consortia studies have also been performed. A *Streptomyces* consortium was used to biodegrade Reactive Blue 222, a reactive sulfonated di azo dye (Pillai, 2018). This process enhanced the biodegradation of the dye by 89% using two different *Streptomyces* spp.. This rationale for microbial consortium degradation of waste products has also been tested using *Streptomyces* spp. along with yeast species to bioconvert lignocellulosic components into biofuels (Wadler *et al.*, 2022), where it was observed that some individual strains were only able to biotransform about 40% of the soluble lignocellulosic components, while the mixed consortia increased up to 70% the degradation of the soluble lignocellulosic components.

2.7.1 Bioreactors

Optimization studies using bioreactors have been employed to evaluate the proposed biodegradation of waste at an industrial scale (Olmos *et al.*, 2013; Buntić *et al.*, 2016; Barbuto Ferraiuolo *et al.*, 2021). The optimization of laccase production from Actinobacteria was performed with *S. psammoticus* with different scale-up strategies, resulting in 215.6 U/g as the best titer in the presence of mediators to achieve decolorization of azo dyes (Niladevi and Prema, 2005; Niladevi *et al.*, 2008). The peroxidase-like activity production of *Streptomyces.* sp. strain BSII#1 was scaled up to 3 L culture volumes with an airlift bioreactor achieving 4.76 U/ml in the presence of veratryl alcohol as inducer. Bioreactor-based degradation of xylene, a toxic aromatic compound, by *S.* sp. AB1 was investigated, and it was shown that the biocatalyst was able to achieve high elimination levels (90%) of xylene from contaminated water (Chikhi *et al.*, 2016).

Solid-state fermentation has been reported as an ecofriendly tool in the biopulping process as a method for biological treatment of the wheat straw using *S. cyaneus* (Berrocal *et al.*, 2004). It was also shown that this microorganism was able to increase acid-soluble lignin from wood chips by reducing the energy required for the process by a 24% (Hernández *et al.*, 2005). Furthermore, *Streptomyces.* sp. MDG147 has been employed for the valorization of soda lignin from wheat straw to produce oleogels which are utilized in lubricant applications (Borrero-López *et al.*, 2018). The same strain, along with MDG301 was also used for the valorization of agricultural residues (barley and wheat straw) to generate APPL and alkali lignin, which after soda pulping exhibited adhesive properties (Blánquez *et al.*, 2022).

2.8 Conclusions and future directions

The textile and paper industry can lead to hazardous black liquor waste containing dyes from the industrial processing of synthetic and natural polymers. Therefore, safe degradation or decolorization applicable to industrial residues is needed (Saxena and Bharagava, 2020). *Streptomyces* spp. provides an opportunity for generating effective enzyme formulations and development of large-scale methods for microbial-based waste treatment. Identifying methods as novel alternatives requires expanding the available enzymatic repertoire and understanding the mechanisms degradation and bioconversion. Improvements in existing and forthcoming technologies can be achieved through rigorous biochemical characterization of enzymes of interest, expansion of the enzymatic repertoire for biodegradation, and through evaluation of application scalability and reproducibility.

Immobilized *Streptomyces* enzymes are promising for large-scale waste treatment. However, research is needed to apply those technologies to other biotechnological processes. To do so, it is critical to understand the microbial mechanisms that are employed by the potential

biocatalysts for waste compound degradation. Metabolomic and biochemical insights collected from studies of degradation, decolorization, and bioconversion need to be coupled to better understanding of the microbial metabolism that controls the biochemical processes. A way to advance a systematic understanding of the underlying microbial metabolic processes is through the implementation of functional -omics (e.g. transcriptomics, metatranscriptomics, genomics, proteomics and metagenomics) in conjunction with the waste compound degradation studies. These analysis approaches could be applied to the performance of pure cultures as well as when evaluating consortia. In addition, the design of mutagenesis experiments including genome editing and directed evolution could add to the understanding of the physiology and mechanisms of biodegradation, which is key to improving the application development for scalable use in both industries.

A strong case has been made for *Streptomyces* as a suitable source of enzymes that could be used for waste treatment. The main challenge remains in the development of large-scale production methods which can be achieved through a combination of strategic genetic engineering of *Streptomyces* biocatalysts to improve enzyme expression, activity, and robustness and by using our growing understanding of *Streptomyces* physiology to enhance growth and biocatalyst performance.

Author Contributions

MC and AG developed the review. MC wrote the original manuscript. MC and AG contributed to manuscript writing, editing, and reviewing.

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Compliance with Ethical Standards

Conflict of Interest: Mara F. Cuebas-Irizarry declares that she has no conflict of interest. Amy M. Grunden declares that she has no conflict of interest.

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CHAPTER 3

Characterization and Genome Analysis of *Streptomyces* spp. from Carpenter Bees (*Xylocopa*) with the Ability to Grow on Lignocellulosic Materials

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ABSTRACT

In this study, isolated bacterial strains from carpenter bees were characterized based on their ability to grow on lignocellulosic materials including BioChoice lignin, black liquor (a pulp milling stream), cellulose (filter paper), and xylans. Dye decolorization assays were also employed to screen for the ability of the isolates to degrade a variety of aromatic compounds that have implication for textile and dye waste remediation. Analysis of genomic sequences of two isolates identified as *Streptomyces* spp. that had been shown to grow on lignocellulosic materials, including lignin, is presented. The predicted enzymes and pathways for lignocellulose degradation were identified and discussed. *Streptomyces* spp. isolate 2-6, potentially belonging to a new genus based on phylogenetic analysis, seems to be the most promising isolate for lignin degradation and should be systematically explored for use in lignin and lignocellulose valorization applications.

3.1 Introduction

Rapid global urbanization in recent years is placing increased pressure on waste remediation infrastructure, as it is estimated that ~90 billion tons of solid waste will be generated per year through 2025 (Singh 2019; Sarsaiya et al. 2019). In the United States of America, the U.S. Environmental Protection Agency reported a total generation of municipal solid waste in

2018 of 292.4 million tons, an increase from the previous year. From the 2018 data, paper and paperboard waste represented the highest fraction of solid waste (23.05%, followed by food waste at 21.59% (US EPA 2022). These are examples of recalcitrant biomass that have the potential to be bioconverted into energy and commodity chemicals. A paper processing byproduct (black liquor), for example, is generated from the processing of lignocellulosic raw materials and is currently used as an energy source for the pulping plant operation. However, black liquor processing must be properly controlled because it is considered to be a regulated environmental pollutant (Mathews et al. 2015).

Black liquor is produced during the chemical processing of the pulping wood, and its major component is lignin, which is difficult to degrade (Mathews et al. 2019). It also contains other chemicals and lignocellulosic residuals, such as hemicellulose (Brown et al. 2021). Black liquor could be released to the environment as part of water effluents which can result in damage to aquatic life (Singh and Chandra 2019). Accidental discharges in the US have been reported and the consequences have been investigated (Kizha et al. 2016).

A possible solution to reduce the environmental impact of the lignocellulose-based waste, is the expansion of the application of lignocellulolytic microorganisms for conversion of lignocellulose waste into bioproducts and bioenergy (Sarsaiya et al. 2019). Biological treatments can provide a sustainable alternative for lignin depolymerization using microbial enzymes or whole cell approaches (de Carvalho 2016). Microbes have become a source of catalysts in the lignin depolymerization process (Bugg et al. 2011b; Debnath and Saha 2020; Atiwesh et al. 2021). A number of fungal enzymes have been characterized for their ability to degrade lignin (Andlar et al. 2018; del Cerro et al. 2021), and more recently, bacterial lignin degradation has been explored (Bugg et al. 2011a; de Gonzalo et al. 2016; Lee et al. 2019; Liu et al. 2022).

However, new sources of microbial enzymes are needed, and a focus on expanding the characterized repertoire of bacterial lignin-degrading enzymes is warranted since bacteria are known for having greater flexibility in their growth requirements such as a wide range of pH, temperature, salinity- properties that often translate to enzyme stability under a variety of conditions for proteins from these bacterial biocatalysts.

Here we evaluate the use of bacterial strains associated with carpenter bees to digest lignin from black liquor. While carpenter bees do not feed on wood for direct nutrient inputs, they bore into wood to build their nests, and therefore are in close contact with wood dust and shavings that could be broken down by microbes that are part of the carpenter bee microbiome. Previously, the microbiota for carpenter bees has been investigated to identify microbes that can contribute to bee's health (Subta et al. 2020). Understanding microbial health is important because carpenter bees are insect pollinators essential in the sexual reproduction of tropical plants (Subta et al. 2020). Other insect microbiota have been studied to isolate and identify microbes with lignin degradation capabilities including termites, mole crickets, and woodwasps (Brune 2014; Kim et al. 2014; Zhou et al. 2017). However, this is the first report focused on microbes isolated from carpenter bees with a framework targeting bacterial strains that can potentially use black liquor and lignin. We demonstrate that the resulting isolates have the growth characteristics and genetic potential that warrant further development for the valorization of lignocellulosic materials such as pulping milling stream products.

3.2 Methods

3.2.1 Isolation and screening of bacterial strains from carpenter bees for growth on lignocellulose components.

Insects (Xylocopa) were obtained from the North Carolina Arboretum were surface sterilized using 0.5% bleach and 70% ethanol as per (Arnold et al. 2003). Each insect was homogenized as described in Gupta et al. 2012. To select microbes that can grow on lignocellulose components, black liquor was used as the carbon source. The homogenate was plated on M9 minimal media consisting of a 1X solution from the following 5X stock solution prepared for 1L as follows: 30 Na_2HPO_4, 15 g KH_2PO_4, 5 g NH_4Cl, 2.5 g NaCl, 15 mg $CaCl_2$. The M9 final mixture for a final volume of 1L was prepared as follows (after autoclaving the water): 200 mL of the 5X solution of M9 described previously, 1 mL of 1M $MgSO_4$, 0.5% (w/v) vitamin B1 (thiamine) from a stock solution 1 mg/mL, 1% of black liquor as a sole carbon source, and a set of the plates were incubated at 25°C and another at 37°C. Further screening was performed using Luria Bertani (LB) consisting of tryptone (10 g), NaCl (5 g), and yeast extract (5 g) with 0.1% Congo Red. The presence of Congo Red helped determine whether the isolates can degrade the cellulose (Gupta et al. 2012). In addition, the isolates were streaked on M63 minimal media consisting of 10.72 g K_2HPO_4, 5.24 g KH_2PO_4, 2 g of $(NH_4)\ SO_4$. After autoclaving the following solutions or reagents were added: 0.5 mL of iron solution prepared using 1 mg of $FeSO_4$ dissolved in 1 mL of 0.01 M HCl; Trace elements SPV-4 with Fe^{III}-citrate substituted for Fe^{II}-chloride. Then the media was supplemented with either Biochoice lignin (BCL), carboxymethyl cellulose, beechwood xylan, xylose or pectin as the sole carbon source to assess the ability of these isolates to metabolize a wide variety of lignocellulose components.

3.2.2 Bacterial identification

3.2.2.1. Initial screening through 16S rRNA gene amplification and sequencing.

The 16S rRNA gene sequencing of the microbial isolates able to grow on lignin and black liquor as well as decolorize Congo Red was performed to determine their identity. The bacterial strains' genomic DNA was isolated using the Qiagen DNeasy® PowerSoil® Pro Kit per the manufacturer's instructions. The 16S rRNA gene was amplified using the polymerase chain reaction (PCR). The following primer set as referenced in (Ventorino et al. 2016) for bacterial identification: 5'-3': AGAGTTTGATCCTGGCTCAG (forward) and rD1: AAGGAGGTGATCCAGCC (reverse) was used to amplify the 16S rDNA gene. The resulting PCR products were analyzed on a 1% agarose gel. Then, the PCR products were purified with the Qiagen PCR clean-up kit (CA) as per the manufacturer's protocol. Sequencing was performed by Eton Bioscience (Durham, NC) with the primers indicated above. The sequencing results were analyzed using EZ-Taxon Database and Basic Local Alignment Search Tool (BLAST) (Mount 2007).

3.2.2.2. Determining evolutionary relationships of the isolates using phylogenetic analysis.

The most similar strains of *Streptomyces* sp. in EZ-taxon were used to infer the phylogenetic relationships of the closest strains, and *Bacillus subtilis* was used as the outgroup. The Multiple Sequence Alignment was performed using CLUSTAL W in SnapGene®. The neighbor-joining tree was constructed using the Kimura two-parameter in MEGA 11 (Tamura et al. 2021).

3.2.3 Culturing the bacterial isolates

Since the 16S rDNA sequencing identified the strains as *Streptomyces* sp., the microbes were grown following recommendations with a few modifications of (Kieser et al. 2000; Book et

al. 2014). The spores were prepared as indicated in (Kieser et al. 2000). Stocks were prepared by growing the isolates in minimal media with xylose. The spores were suspended in TE buffer (10 mM Tris-HCl containing 1 mM EDTA•Na_2) pH 8.0 and glycerol 50% (w/v). For spore activation, the glycerol stock was centrifuged to remove the glycerol and the buffer. By doing this, the spores were easily identified for a dark color at the bottom of the centrifuge tube. The resulting spores were suspended in 5 mL of TE buffer pH 8.0 and heat shocked at 50°C for 10 min. Double strength Germination Medium (1% yeast extract, 1% casamino acids, 1% $CaCl_2$, 0.01M) was added at equal volume. The sample was incubated overnight at 37°C as recommended for the reproducibility of the experiments (Kieser et al. 2000). The overnight culture was centrifuged and resuspended in TE buffer pH 8.0 or minimal media. The spore suspension was quantified at $OD_{450\ nm,}$ and the starting culture was calculated to be an initial $OD_{450\ nm}$ of 0.03-0.05 of absorbance. After the spores' activation, growth studies were performed in M63 minimal media at pH 7.0 per liter, 10.72 g of K_2HPO_4, 5.24 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.5 mL of iron solution containing 1 mg of $FeSO_4$ dissolved in 1 ml of 0.01 M HCl; 1 ml of thiamine (1 mg/ml), and 5 mL of the SPV-4 with Fe^{III}-citrate (trace elements solution) (Balows et al. 1992; Book et al. 2014).

3.2.4 Draft genome sequencing

3.2.4.1 Sequencing methods specifications

Pure cultures were grown in Double strength Germination Media and in solid media as described above. After robust growth occurred (~ 72 h), the biomass was transferred and suspended in a tube with cryopreservative (Microbank™, Pro-Lab Diagnostics UK, United Kingdom) following MicrobesNG strain submission procedures. The bacterial strains were then

shipped to MicrobesNG (IMI – School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom) for sequencing.

The genomic DNA extractions, DNA libraries and sequencing methods were performed by the MicrobesNG sequencing facility as follows. Genomic DNA libraries were prepared using a Nextera XT Library Prep Kit (Illumina, San Diego, USA) according to the manufacturer's protocol with the following modifications: targeted DNA was increased 2-fold, and PCR elongation time was increased to 45 seconds. Library preparation and DNA quantification were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Generated libraries were quantified using Kapa Biosystems Library Quantification Kit for Illumina. The libraries were sequenced using Illumina sequencers NovaSeq 6000 using a 250bp paired-end protocol. Pair end reads were trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger, Lohse and Usadel, 2014). De novo assembly was performed using SPAdes version 3.7 (Bankevich *et al.*, 2012). The contigs were annotated using RAST (Aziz et al. 2008). Protein-coding genes were annotated and grouped by functional classes using Cluster of Orthologous Groups (COG). The Genes related to lignin degradation were identified using CAZy and KEGG. The genes involved in aromatic intermediates metabolism were identified using RAST subsystem features.

3.2.4.1 CAZy database

Potential lignocellulose-degrading enzymes were accessed through the carbohydrate-active enzymes (CAZy) database through KBase (Arkin et al. 2018). This database provides a compilation of carbohydrate degrading or modifying enzymes and group these enzymes in families of structurally related enzymes (Andlar et al. 2018). These families are Glycoside or Glycosyl hydrolase (GH), Carbohydrate Binding Module (CBM), Glycosyl Transferase (GT),

Auxiliary Activity (AA), polysaccharide lyases (PL), and Carbohydrate Esterases (CE) (Lombard et al. 2014).

3.2.4.2 Genome annotation and distilling assembly using the Distilled and Refined Annotation of Metabolism (DRAM) tool

Functional categories specifically related to lignocellulose depolymerization for the *Streptomyces* spp. isolate 2-6 were assessed using DRAM (Distilled and Refined Annotation of Metabolism) (Shaffer et al. 2020). This tool enables the annotation of assembled genomes and includes a distill step that supports the curation of annotations into the functional categories. This was accomplished using the KBase platform (Arkin et al. 2018)

3.2.4.3 Putative Secretion Prediction (pSORTb)

The putative proteins for lignin degradation according to CaZY database results were analyzed for secretion using pSortB (http://psort.org/psort/), a bacterial protein subcellular localization predictor (Yu et al. 2010). It generates the localization prediction distinguishing between Gram positive and Gram negative bacteria with high precision (96.5%) (Yu et al. 2010; Mathews et al. 2019).

3.2.5 Analyses of extracellular lignin-related enzymes.

Samples are collected from growth on minimal media and lignin (0.2%) for 48-72 hours at 10,000 rpm for 5 min to separate cell pellets from the supernatant. A total of 10 mL of supernatant was concentrated using 0.22-μm polyethersulfone (PES) membrane filter. The resulting supernatant was collected and used as a crude enzyme preparation to measure enzymatic activity in response growth on black liquor or lignin. The lignin peroxidase (LiP) enzymatic activity was detected by measuring alcohol oxidation from veratryl (3,4-dimethoxybenzyl) to veratryl aldehyde as per (Camarero *et al.*, 1999). The enzyme reaction

mixture consisted of 500 µL of 100 mM sodium tartrate buffer at pH 3.8, 500 µL of 4 mM veratryl alcohol, and 100 µL of crude enzyme. The reaction was started by adding 100 µL of 2 mM H_2O_2 to the mixture and incubating the reaction for 5 min at 30°C. The reaction was monitored with the change in absorbance at 310 nm with a factor coefficient of ε=9,300M/cm. Laccase enzymatic activity was performed by measuring the oxidation of guaiacol as described by (Arora and Sandhu, 1985).

3.2.6 Dye decolorization assays

After 12 h of the microbial isolates growing in LB at 30°C the following dyes were added with their respective concentrations indicated in parentheses: Congo Red (CR; 50 mg/L), Bromocresol Green (BG; 50 mg/L), Remanzol Brilliant Blue (RBB; 25 mg/L), and Toluidine Blue (TB; 25 mg/L). The cultures were then returned to an incubator set at 30°C with shaking (200 rpm). Decolorization was assessed spectrophotometrically every 24 h using the specific absorbance for each dye. Decolorization was calculated as the percent (%) of initial absorbance at λ max at 470 nm for CR, 595 nm for RBB, 615 nm for BG, and 635 nm for TB. Percent of decolorization was calculated by subtracting the maximum absorbance from the initial absorbance and then dividing by the initial absorbance and multiplying by 100. Data represented as mean ± Standard Error.

3.2.7 Residual cellulose quantification

The quantification of residual cellulose was performed as described in (Book et al. 2014). The isolates were grown as mentioned above starting with an OD_{450nm} 0.3-0.05 spore suspension in 8 mL of M63 minimal media supplemented with 0.07 g of filter paper Whatman no. 1 (GE Healthcare Life sciences, Pittsburgh, PA). The cultures were processed after 10 d of incubation, and the residual cellulose was determined using the acid-detergent fiber method (Weimer et al. 1990). A solution of acid detergent (16 mL) was added to each test tube (Goering and Van Soest

1970). The tubes were sealed with rubber stoppers and autoclaved for 41 min at 121°C. After venting at room temperature, the samples were individually vacuum filtered through a pre-weighed Whatman GF/D, 2.7- μm pore size-glass microfiber filter (GE Heathcare Life Sciences, Pittsburgh, PA). The filters were washed using hot water and dried in an oven at 90°C overnight. The filters were reweighed after removing them from the oven. The net cellulose loss was quantified by subtracting the final residual cellulose mass from the initial cellulose mass. The resulting value was divided by the initial cellulose mass to correct and consider the percent of cellulose degraded and the loss sample.

3.2.8 Residual lignin quantification

3.2.8.1. Residual lignin quantification using the acid precipitation method.

Inoculated and uninoculated M63 media supplemented with 0.2% BioChoice lignin was incubated for 480 h at 30°C. Lignin was solubilized using 5 M NaOH, and cells were removed using a 0.2 μm nylon filter. Lignin was precipitated by acidifying it with 12N HCl. The resulting fractions were placed in pre-weighed conical tubes and centrifuged at 4,700 rpm for 15 min. The conical tubes were placed into an oven at 60°C for 24 h letting the lignin completely dry. Solid lignin was then weighed to calculate lignin reduction.

3.2.8.2. Residual lignin quantification with *E. coli*, and without *E.coli* as a pre-treatment, and after dialysis of lignin

BioChoice lignin is composed of Klason lignin 91.1% and acid soluble lignin (5.4%) for a total of 96.5% total lignin. The remaining carbon inputs consist of sugars: arabinan (0.26%), galactan (0.76%), glucan (0.12%), xylan (0.84%), and ash (1.36%) (Hu et al. 2016). To test if *Streptomyces* sp. 2-6 was metabolizing only the sugars instead of the lignin, multiple experimental treatments were performed. The first one used *E. coli* (strain BL21) as a pre-

treatment to remove residual sugars. *E. coli* strain BL21 was grown on the prepared media (M63 and BCL) for 48 h. Then the *E. coli* cells were inactivated by treatment with 5M NaOH, and were removed with filtration as described in the previous section. The pH of the pre-treated culture media was corrected to 7.0, and the media was inoculated with *Streptomyces* sp. 2-6. For the dialysis experiments, membranes of 3,000-5,000 and 6,000-8,000 molecular weight cutoff (MWCO), were used to dialyze lignin against sterile distilled water at 4°C to avoid contamination. The media was prepared as usual and was inoculated, and processed with the acid precipitation method as described previously.

3.3 Results

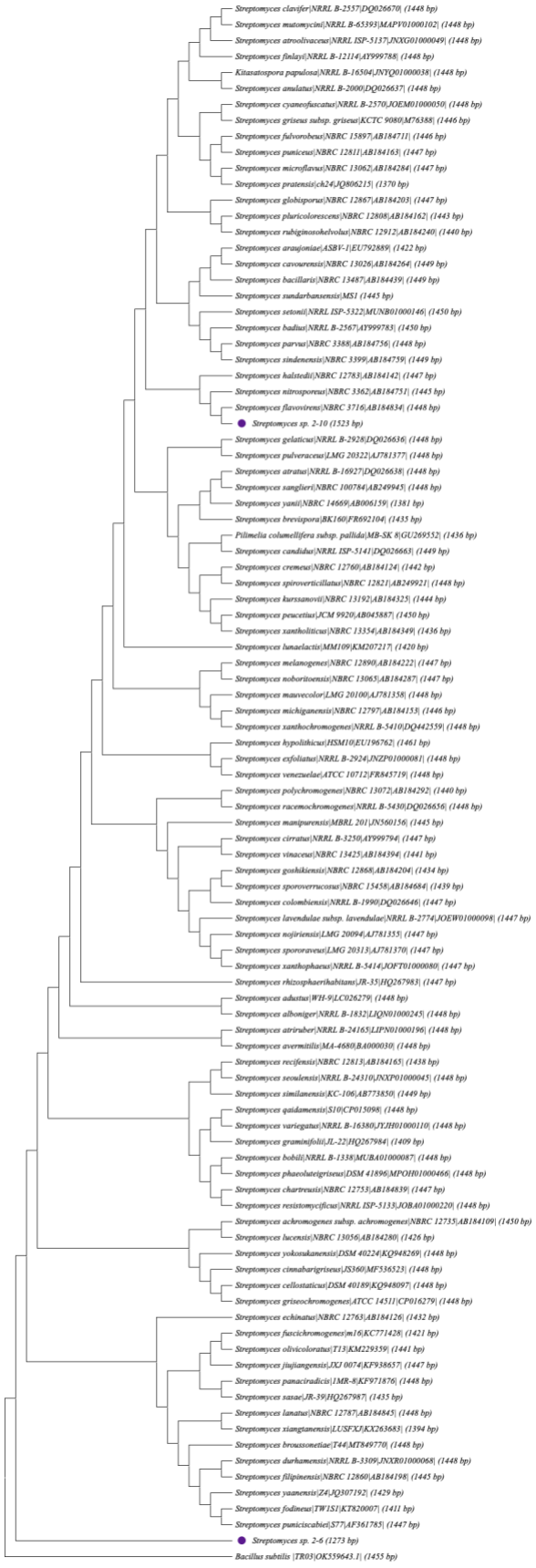
3.3.1 Bacterial isolate strain identification

Three isolates were selected for their capacity to grow in BioChoice Lignin, Black liquor and to decolorize Congo Red. These isolates were initially labeled as isolates 1-1, 2-6, and 2-10. Based on the initial 16S rRNA gene sequencing and BLAST analysis, all three strains were identified to be *Streptomyces* sp. (93% identity) for isolate 1-1, *Streptomyces lavendulaee* (90% identity) for isolate 2-6 and *Streptomyces pratensis* (96% identity) for isolate 2-10. *Bacillus subtilis* was selected as the outgroup comparator because it is phylogenetically outside of the *Streptomyces* genus, but is related enough for meaningful comparisons to the ingroup (Gram positive, spore former, able to decolorize dyes, and grow on lignocellulose compounds).

This preliminary strain identification informed the design of growth experiments to select candidates for further whole genome sequencing. After obtaining the genome sequencing results and performing phylogenetic analysis using the respective 16S rRNA gene sequences, isolate 2-6 was identified to be related to *Streptomyces equinatus* (99% identity) using the EZ-taxon database. For isolate 2-10, results of the EZ-taxon analysis identify it to be *Streptomyces*

flavovirens with 100% identity. However, when the 16S rRNA gene-based tree is constructed (**Figure 3-1**) with the most similar type-strains, *Streptomyces* sp. 2-6 clusters as a different group. The whole genome sequencing project can be found at this public link: https://microbesng.com/portal/projects/5FB7B9AB-2C4D-498A-A726-0973B49EAEC4/. The details of the draft genomes can be found in **Supplemental Table 3-1**.

Figure 3-1. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The evolutionary distances were computed using the Kimura 2-parameter method and the units of the number of base substitutions per site. This analysis involved 98 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1967 positions in the final set. Evolutionary analyses were conducted using MEGA 11 (Tamura et al. 2021)


Streptomyces clavifer|NRRL B-2557|DQ026670| (1448 bp)
Streptomyces mutomycini|NRRL B-65393|MAPV01000102| (1448 bp)
Streptomyces atroolivaceus|NRRL ISP-5137|JNXG01000049| (1448 bp)
Streptomyces finlayi|NRRL B-12114|AY999788| (1448 bp)
Kitasatospora papulosa|NRRL B-16504|JNYQ01000038| (1448 bp)
Streptomyces anulatus|NRRL B-2000|DQ026637| (1448 bp)
Streptomyces cyaneofuscatus|NRRL B-2570|JOEM01000050| (1448 bp)
Streptomyces griseus subsp. griseus|KCTC 9080|M76388| (1446 bp)
Streptomyces fulvorobeus|NBRC 15897|AB184711| (1446 bp)
Streptomyces puniceus|NBRC 12811|AB184163| (1447 bp)
Streptomyces microflavus|NBRC 13062|AB184284| (1447 bp)
Streptomyces pratensis|ch24|JQ806215| (1370 bp)
Streptomyces globisporus|NBRC 12867|AB184203| (1447 bp)
Streptomyces pluricolorescens|NBRC 12808|AB184162| (1443 bp)
Streptomyces rubiginosohelvolus|NBRC 12912|AB184240| (1440 bp)
Streptomyces araujoniae|ASBV-1|EU792889| (1422 bp)
Streptomyces cavourensis|NBRC 13026|AB184264| (1449 bp)
Streptomyces bacillaris|NBRC 13487|AB184439| (1449 bp)
Streptomyces sundarbansensis|MS1 (1445 bp)
Streptomyces setonii|NRRL ISP-5322|MUNB01000146| (1450 bp)
Streptomyces badius|NRRL B-2567|AY999783| (1450 bp)
Streptomyces parvus|NBRC 3388|AB184756| (1448 bp)
Streptomyces sindenensis|NBRC 3399|AB184759| (1449 bp)
Streptomyces halstedii|NBRC 12783|AB184142| (1447 bp)
Streptomyces nitrosporeus|NBRC 3362|AB184751| (1445 bp)
Streptomyces flavovirens|NBRC 3716|AB184834| (1448 bp)
Streptomyces sp. 2-10 (1523 bp)
Streptomyces gelaticus|NRRL B-2928|DQ026636| (1448 bp)
Streptomyces pulveraceus|LMG 20322|AJ781377| (1448 bp)
Streptomyces atratus|NRRL B-16927|DQ026638| (1448 bp)
Streptomyces sanglieri|NBRC 100784|AB249945| (1448 bp)
Streptomyces yanii|NBRC 14669|AB006159| (1381 bp)
Streptomyces brevispora|BK160|FR692104| (1435 bp)
Pilimelia columellifera subsp. pallida|MB-SK 8|GU269552| (1436 bp)
Streptomyces candidus|NRRL ISP-5141|DQ026663| (1449 bp)
Streptomyces cremeus|NBRC 12760|AB184124| (1442 bp)
Streptomyces spiroverticillatus|NBRC 12821|AB249921| (1448 bp)
Streptomyces kurssanovii|NBRC 13192|AB184325| (1444 bp)
Streptomyces peucetius|JCM 9920|AB045887| (1450 bp)
Streptomyces xantholiticus|NBRC 13354|AB184349| (1436 bp)
Streptomyces lunaelactis|MM109|KM207217| (1420 bp)
Streptomyces melanogenes|NBRC 12890|AB184222| (1447 bp)
Streptomyces noboritoensis|NBRC 13065|AB184287| (1447 bp)
Streptomyces mauvecolor|LMG 20100|AJ781358| (1448 bp)
Streptomyces michiganensis|NBRC 12797|AB184153| (1446 bp)
Streptomyces xanthochromogenes|NRRL B-5410|DQ442559| (1448 bp)
Streptomyces hypolithicus|HSM10|EU196762| (1461 bp)
Streptomyces exfoliatus|NRRL B-2924|JNZP01000081| (1448 bp)
Streptomyces venezuelae|ATCC 10712|FR845719| (1448 bp)
Streptomyces polychromogenes|NBRC 13072|AB184292| (1440 bp)
Streptomyces racemochromogenes|NRRL B-5430|DQ026656| (1448 bp)
Streptomyces manipurensis|MBRL 201|JN560156| (1445 bp)
Streptomyces cirratus|NRRL B-3250|AY999794| (1447 bp)
Streptomyces vinaceus|NBRC 13425|AB184394| (1441 bp)
Streptomyces goshikiensis|NBRC 12868|AB184204| (1434 bp)
Streptomyces sporoverrucosus|NBRC 15458|AB184684| (1439 bp)
Streptomyces colombiensis|NRRL B-1990|DQ026646| (1447 bp)
Streptomyces lavendulae subsp. lavendulae|NRRL B-2774|JOEW01000098| (1447 bp)
Streptomyces nojiriensis|LMG 20094|AJ781355| (1447 bp)
Streptomyces spororaveus|LMG 20313|AJ781370| (1447 bp)
Streptomyces xanthophaeus|NRRL B-5414|JOFT01000080| (1447 bp)
Streptomyces rhizosphaerihabitans|JR-35|HQ267983| (1447 bp)
Streptomyces adustus|WH-9|LC026279| (1448 bp)
Streptomyces alboniger|NRRL B-1832|LIQN01000245| (1448 bp)
Streptomyces atriruber|NRRL B-24165|LIPN01000196| (1448 bp)
Streptomyces avermitilis|MA-4680|BA000030| (1448 bp)
Streptomyces recifensis|NBRC 12813|AB184165| (1438 bp)
Streptomyces seoulensis|NRRL B-24310|JNXP01000045| (1448 bp)
Streptomyces similanensis|KC-106|AB773850| (1449 bp)
Streptomyces qaidamensis|S10|CP015098| (1448 bp)
Streptomyces variegatus|NRRL B-16380|JYJH01000110| (1448 bp)
Streptomyces graminifolii|JL-22|HQ267984| (1409 bp)
Streptomyces bobili|NRRL B-1338|MUBA01000087| (1448 bp)
Streptomyces phaeoluteigriseus|DSM 41896|MPOH01000466| (1448 bp)
Streptomyces chartreusis|NBRC 12753|AB184839| (1447 bp)
Streptomyces resistomycificus|NRRL ISP-5133|JOBA01000220| (1448 bp)
Streptomyces achromogenes subsp. achromogenes|NBRC 12735|AB184109| (1450 bp)
Streptomyces lucensis|NBRC 13056|AB184280| (1426 bp)
Streptomyces yokosukanensis|DSM 40224|KQ948269| (1448 bp)
Streptomyces cinnabarigriseus|JS360|MF536523| (1448 bp)
Streptomyces cellostaticus|DSM 40189|KQ948097| (1448 bp)
Streptomyces griseochromogenes|ATCC 14511|CP016279| (1448 bp)
Streptomyces echinatus|NBRC 12763|AB184126| (1432 bp)
Streptomyces fuscichromogenes|m16|KC771428| (1421 bp)
Streptomyces olivicoloratus|T13|KM229359| (1441 bp)
Streptomyces jiujiangensis|JXJ 0074|KF938657| (1447 bp)
Streptomyces panaciradicis|1MR-8|KF971876| (1448 bp)
Streptomyces saxae|JR-39|HQ267987| (1435 bp)
Streptomyces lanatus|NBRC 12787|AB184845| (1448 bp)
Streptomyces xiangtanensis|LUSFXJ|KX263683| (1394 bp)
Streptomyces broussonetiae|T44|MT849770| (1448 bp)
Streptomyces durhamensis|NRRL B-3309|JNXR01000068| (1448 bp)
Streptomyces filipinensis|NBRC 12860|AB184198| (1445 bp)
Streptomyces yaanensis|Z4|JQ307192| (1429 bp)
Streptomyces fodineus|TW1S1|KT820007| (1411 bp)
Streptomyces puniciscabiei|S77|AF361785| (1447 bp)
Streptomyces sp. 2-6 (1273 bp)
Bacillus subtilis |TR03|OK559643.1| (1455 bp)

3.3.2 Growth on lignocellulosic compounds as the sole carbon sources

The *Streptomyces* sp. isolates were grown with different lignocellulosic sources to assess their ability to use them as the sole carbon source. In **Table 3-1**, the isolates recovered from black liquor supplemented agar plates were verified for their ability to grow in 1% black liquor (BL), 0.2% lignin, 0.2% Carboxymethylcellulose (CMC), 0.2% Xylan from Beechwood, 0.2% Xylan from Corn, and LB to compare growth viability. *Paenibacillus glucanolyticus* was used as a positive control for growing on these lignocellulose sources as the sole carbon source as described previously (Mathews et al. 2016). All three *Streptomyces* spp. isolates were shown to be capable of growing well on 1% black liquor as well as 0.2% Biochoice lignin, CMC and corn xylan; however, none of the isolates grew on the beechwood xylan (**Table 3-1**).

Table 3-1. *Streptomyces* sp. isolates from BL are grown on different lignocellulose sources. All strains showed robust growth (>10^6 CFU/mL) in each respective media within less than a week. Strains were grown at 30°C.

Isolate	M63 1% Black Liquor	M63 0.2% Lignin	M63 0.2% CMC	M63 0.2% Xylan CC	M63 0.2% Xylan Beechwood	LB
1-1	+	+	+	+	-	+
2-6	+	+	+	+	-	+
2-10	+	+	+	+	-	+
P. glucanolyticus	+	+	+	+	+	+

The growth of the *Streptomyces* sp. isolates on lignin was further assessed by determining the amount of residual lignin remaining after a 408 h incubation period. As indicated in **Table 3-2A**, the isolate showing the most promising results was the *Streptomyces* sp. 2-6 isolate, which exhibited an 8.23% lignin reduction after 408 h. Subsequently, this isolate was selected for a growth experiment conducted over a longer period that is in line with lignin reduction studies

performed with other microbial isolates such as *P. glucanolyticus* (Mathews et al. 2016). As shown in **Table 3-2B**, the *Streptomyces* sp. 2-6 isolate reduced BioChoice lignin mass by 24%, which is a promising finding and compares favorably to lignin degradation results seen for other bacterial isolates (Mathews et al. 2016)

To assess the *Streptomyces* sp. strains for cellulose deconstruction potential, filter paper test growth experiments were performed. It was demonstrated that the *Streptomyces* sp. 2-10 isolate was able to degrade the filter paper to the greatest extent (22% loss of filter paper weight compared to 18% for *Streptomyces* sp. 2-6) (**Figure 3-3**).

In light of these results, the *Streptomyces* sp. 2-6 and 2-10 isolates were further characterized to determine their temperature optima and their ability to decolorize Congo red at different pH (**Table 3-3**). Their growth on different concentrations of Black liquor and pH was also assessed (**Table 3-4**). It should be noted that by increasing the concentration of black liquor in the media, the pH of the media is also increased. The growth studies with various carbon sources and at different temperatures and pH were performed using plate-based methods because these strains formed clumps in liquid media, as is often seen for *Streptomyces* sp. (Ventorino et al. 2016), which makes both qualitative and quantitative growth assessments difficult.

Table 3-2. **A.** Lignin reduction potential of the three *Streptomyces* sp. strains. The amount of lignin reduction was determined by calculating the percentage of the difference between inoculated and uninoculated samples. The isolates were grown in M63 supplemented with 2 mg/mL of BCL for 480 h. The results presented are the average of three biological replicates. **B.** Lignin reduction observed for *Streptomyces* sp. 2-6 grown on Biochoice lignin at 30°C for 720 h. The lignin reduction was determined by calculating the percentage of the difference between inoculated and uninoculated samples. The results presented are the average of three biological replicates.

A. Sample	Percent Lignin Reduction
Uninoculated	0
1-1	0.128
2-6	8.23
2-10	0.237

B. Sample	Percent Lignin Reduction
Uninoculated	0
2-6	24

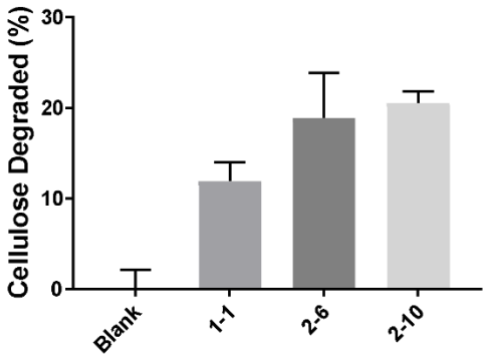


Figure 3-2. Degradation of filter paper by the *Streptomyces* sp. carpenter bee isolates. The acid detergent method was used to determine the residual cellulose after growth treatment. Growth was performed at 30ºC for 10 days. Data was collected for three biological replicates. The data are presented as the mean average, and the error bars indicate the standard deviation.

Table 3-3. Growth of the isolates at different pH and temperatures when cultured in LB Congo Red media. The ability of the isolates to decolorize Congo Red by producing a halo around the colonies was also determined.

	LB Congo Red											
Temperature	22	30	37	45	22	30	37	45	22	30	37	45
Isolates	**pH 4**				**pH 7**				**pH 10**			
Streptomyces sp. 2-6	- (N)	- (N)	- (N)	- (N)	+ (Y)	+ (Y)	+ (Y)	+ (N)	- (N)	- (N)	+ (Y)	- (N)
Streptomyces sp. 2-10	- (Y)	- (N)	- (N)	- (N)	+ (Y)	+ (Y)	+ (Y)	+ (N)	- (N)	- (N)	+ (Y)	+ (N)

(-) Indicates cultures that had limited growth on the plate (0 colonies). (+) Indicates cultures that had robust growth (> 10^6 CFU/mL). Y and N designations indicate whether Congo Red had been decolorized for each condition tested.

Table 3-4. Growth of the isolates at different temperatures in M63 media supplemented with Black liquor at 1, 5 or 10%.

	M63 and Black liquor											
Isolates	**1%**				**5%**				**10%**			
Temperature (°)	22	30	37	45	22	30	37	45	22	30	37	45
Streptomyces sp. 2-6	+	+	+	+	+	+	+	-	-	-	-	-
Streptomyces sp. 2-10	+	+	+	+	+	+	+	+	-	-	-	-

(-) Indicates cultures that had limited growth on the plate (0 colonies). (+) Indicates cultures that had robust growth (> 10^6 CFU/mL).

Based on the results presented in **Table 3-3**, it appears that *Streptomyces* sp. 2-6 and 2-10 are able to grow robustly (> 10^6 cells per mL) at all temperatures tested at pH 7 and at 37 °C at pH 10 but were unable to grow well at pH 4 regardless of the incubation temperature.

Interestingly, unlike *Streptomyces* sp. 2-6, *Streptomyces* sp. strain 2-10 was also able to grow well at pH 10 both at 37 °C and 45 °C. Congo Red decolorization was observed for *Streptomyces* sp. 2-6 at pH 7 for all tested temperatures but only at 37 °C when grown at a pH of 10. No Congo Red decolorizaton was detected when *Streptomyces* sp. strain 2-6 was grown at pH 4 at any incubation temperature tested. A similar Congo Red decolorization trend was seen for *Streptomyces* sp. strain 2-10; however, *Streptomyces* sp. strain 2-10 was able to decolorize Congo Red at pH 4 when the strain was incubated at 22 °C.

Results of the black liquor growth experiments shown in **Table 3-4** indicate that both *Streptomyces* spp. strains 2-6 and 2-10 are able to grow well at a 1% black liquor concentration at all temperatures tested. With 5% black liquor, *Streptomyces* sp. 2-6 only grew well at temperatures tested from 22 °C to 37 °C, while *Streptomyces* sp. 2-10 grew well on media supplemented with 5% black liquor at all tested incubation temperatures. Neither strain was able to grow robustly on media supplemented with 10% black liquor.

3.3.3. *Streptomyces* sp. strain 2-6 and 2-10 dye decolorization properties.

Streptomyces sp. 2-6, and 2-10 isolates were grown in rich media in the presence of compounds mimicking lignin (dyes) to screen for lignin-degrading enzymes. In this case, azo, thiazine, and trimethylmethane were selected to screen for potential enzymes able to catalyze reactions relevant to the textile industry since azo dyes belong to the largest family of dyes used in this industry (Chakravarthi et al. 2020; Ajaz et al. 2020). A resulting halo around the grown colony suggests the presence of enzymes that may catalyze reactions that could participate in degradation of recalcitrant compounds resulting in the decolorization of the present dye. This initial screening was used to determine which dyes should be included in quantitative dye

decolorization experiments. In **Figure 3-3**, it is shown that the isolates can decolorize up to 50% of the dye in 48 h in the case of Alizarin yellow; approximately 70% decolorization of Congo Red at 48 h was achieved by *Streptomyces* sp. 2-6, while *Streptomyces* sp. 2-10 showed maximum decolorization at 60 h. The lowest rate of decolorization was shown for bromocresol green. Unlike the other dyes, bromocresol green belongs to the trimethylmethane dye type, which suggests a different class of enzyme is needed to achieve higher rates of decolorization.

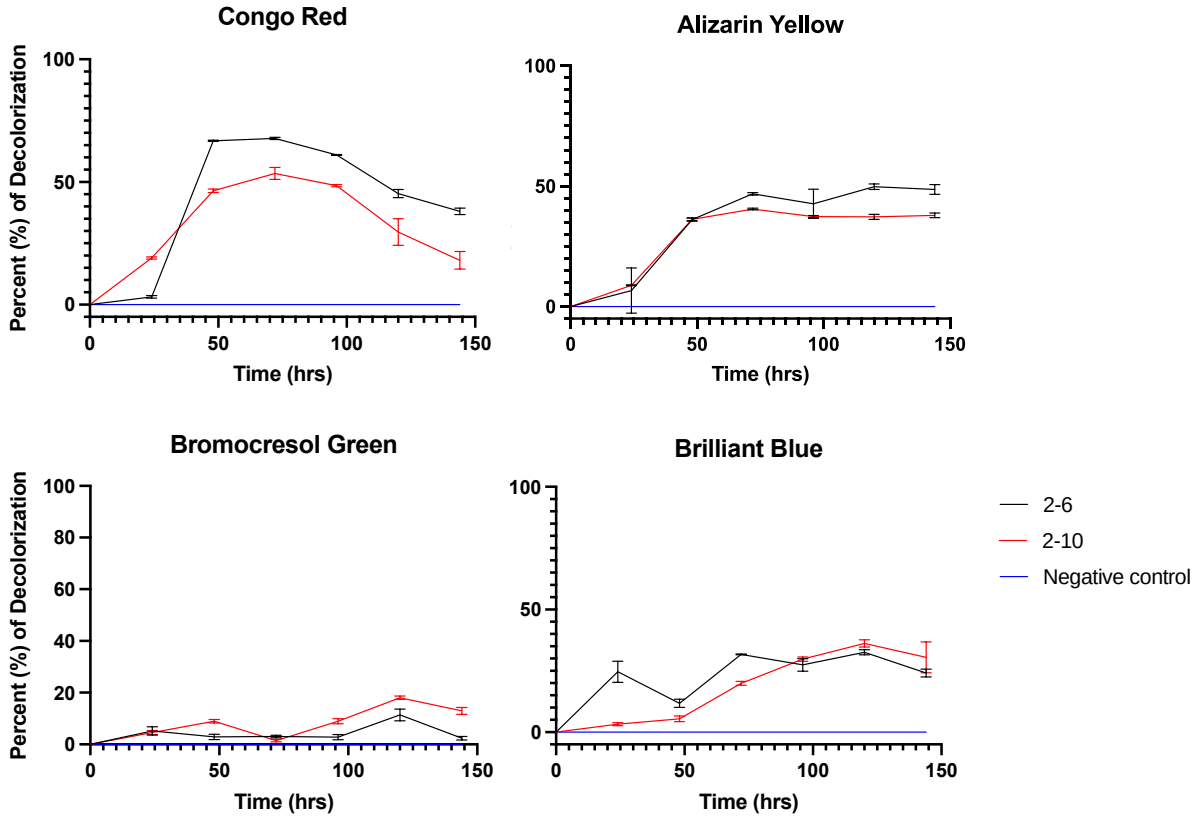


Figure 3-3 Decolorization percentage of Azo-dyes (Congo Red, Brilliant Blue, Alizarin yellow) and a triphenylmethane dye (Bromocresol Green) for *Streptomyces* sp. 2-6, and 2-10 cultures grown at 30°C in LB for 150 h without mediators. Isolates were grown in LB until reaching the mid-logarithmic phase and then the indicated dye was added to the culture (50 mg/L) and the cultures incubated for an additional 150 h. The negative control results were obtained from

uninoculated media to which the individual dyes were added. The data presented are the average of three separate experiments and the error bars indicate the calculated standard error.

Furthermore, decolorization was assessed for thiazine dyes (toluidine blue O), the results of which also suggest that a different class of enzyme than what is produced by *Streptomyces* sp. 2-6 and *Streptomyces* sp. 2-10 is needed to achieve the decolorization observed for the azo-dyes (**Figure 3-4**).

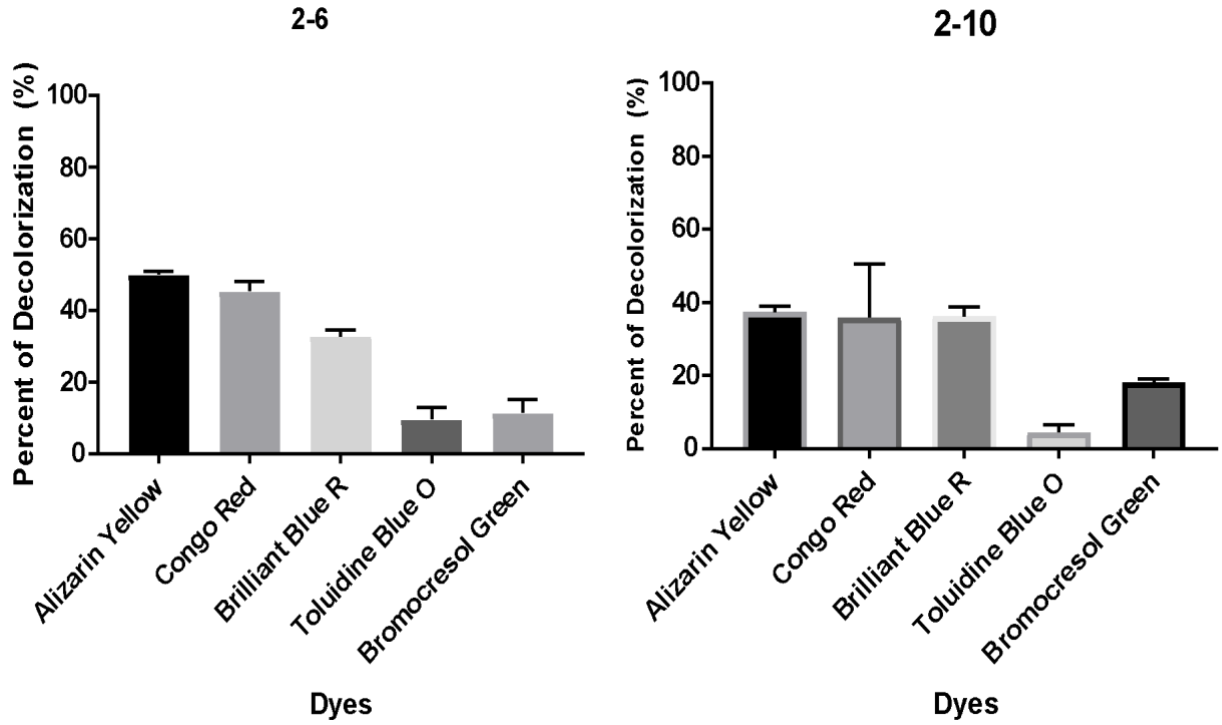


Figure 3-4. Decolorization percentage of Azo-dyes (Congo Red, Brilliant Blue, Alizarin yellow), triphenylmethane dye (Bromocresol Green), and thiazine dye (Toluidine blue) for *Streptomyces* sp. 2-6, and 2-10 cultures grown at 30°C in LB for 150 h without mediators. Isolates were grown in LB until reaching the mid-logarithmic phase. The indicated dye was then added to the culture (50 mg/L), and the cultures incubated for an additional 150 h. The negative control results were obtained from uninoculated media to which the individual dyes were added.

The data presented are the average of three separate experiments and the error bars indicate the calculated standard error.

3.3.4. Growth on lignocellulose sources.

3.3.4.1. Growth in black liquor, lignin and laccase activity evaluation.

Growth of *Streptomyces* sp. 2-6, and 2-10 was assessed in several ways, and it was found that counting cells using a light microscope and Petroff-Hauser counter provided the most reproducible results in determining how well the strains could grow on the lignocellulose compounds as the sole carbon source in minimal media (**Figure 3-5A** and **B**, **Figure 3-6**). In addition, enzymatic activity for laccase was assessed. Differences in laccase activity were observed at 48 and 72 h when *Streptomyces* sp. 2-6 was grown on lignin (**Figure 3-6B**). The activity level detected in this study is similar to laccase activity levels reported in the literature for other *Streptomyces* sp. using the same assay (Riyadi et al. 2020).

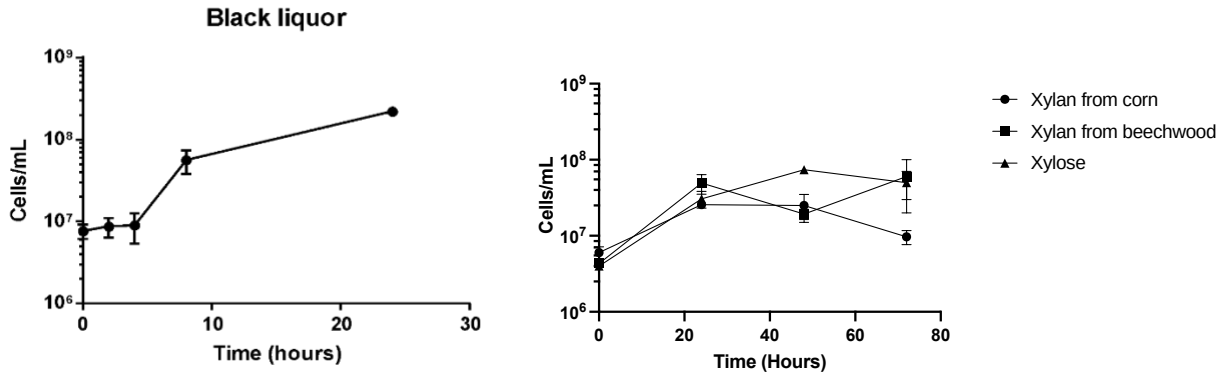


Figure 3-5. A. Growth curve for *Streptomyces* sp. 2-6 cultured on minimal media with 10% black liquor as the sole carbon source and incubated at 30ºC with shaking (150 rpm) for 72 h. **B.** Growth curve for *Streptomyces* sp. 2-6 cultured in minimal media with 0.2% xylan from corn, xylan from beechwood, or xylose as the sole carbon source and incubated at 30ºC with shaking (150 rpm) for 72 h. The data presented are the average of three biological replicates and the error bars represent the standard deviation.

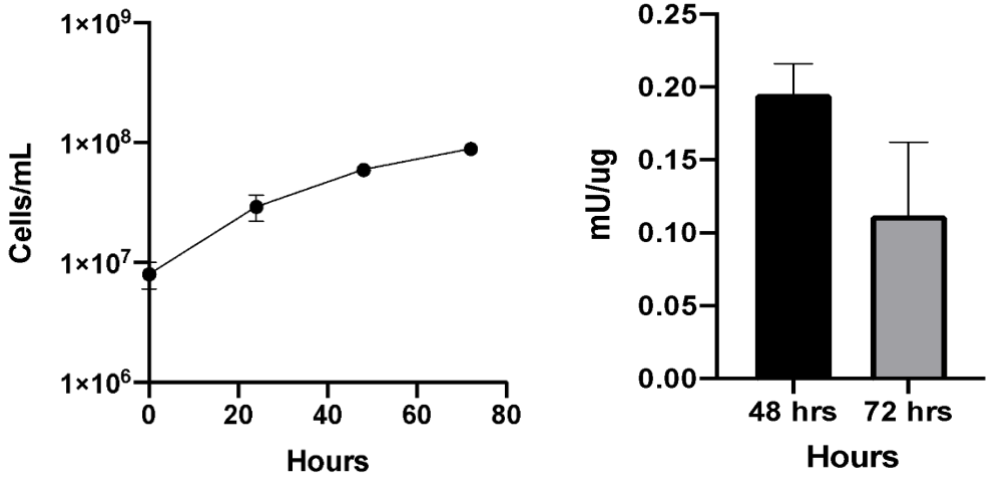


Figure 3-6. A. Growth curve for *Streptomyces* sp. 2-6 cultured on minimal media with 0.2% (w/v) of lignin as a carbon source and incubated at 30ºC with shaking (150 rpm) for 72 h. The data presented are the average of three biological replicates and the error bars represent the standard deviation. **B.** Laccase activity detected for *Streptomyces* sp. 2-6 cultured on minimal media with 0.2% (w/v) of lignin as a carbon source. The data presented are the average of three biological replicates and the error bars represent standard deviation.

3.3.4.2. Growth studies in to demonstrate BioChoice lignin reduction.

With the intention of increasing the overall amount of lignin reduction, a previously characterized bacteria in the laboratory, *Paenibacillus glucanolyticus* was added in combination with *Streptomyces* sp. 2-6 in a culture with lignin provided as the sole carbon source. In order to quantify the use of lignin, the acid detergent method was used to calculate the percent of change of inoculated samples and uninoculated samples. The *Streptomyces* sp. 2-6 isolate demonstrated higher reduction alone than with the addition of *P. glucanolyticus* (**Table 3-5**).

Table 3-5. BioChoice lignin reduction in cultures inoculated with *Streptomyces* sp. 2-6 or inoculated with the combination of *Streptomyces* sp. 2-6 and *P. glucanolyticus.*

Experiment	Percent of change
Uninoculated	0%
Streptomyces sp. 2-6	34%
2-6 + *P. glucanolyticus*	20%

The cultures were grown in in M63 minimal medium with 0.2% (w/v) of Biochoice Lignin at 30ºC with shaking (150 rpm) for 420 h. The data presented are the result of three biological repeats.

Because BioChoice lignin might be contaminated with a small percentage of sugars that could be used as the primary carbon source, *E. coli* was inoculated into in the media with Biochoice lignin to remove any residual sugars prior to inoculation with the test strains. Another method to avoid sugar contamination from the lignin source was to dialyze the lignin before inoculating with the test strains. It is hypothesized that there may be resting cell effect occurring here (Geisel et al. 2011) since the cells had seemingly entered into a stationary phase (**Figure 3-7A**); however, when the percent of lignin mass change was analyzed, there are statistically significant differences seen (Figure 3-7B). The resting cell process allows the bacteria to live and continue metabolizing under stress conditions (Geisel et al. 2011). These results suggest that *Streptomyces* sp. 2-6 is capable of metabolizing different molecular weights of lignin.

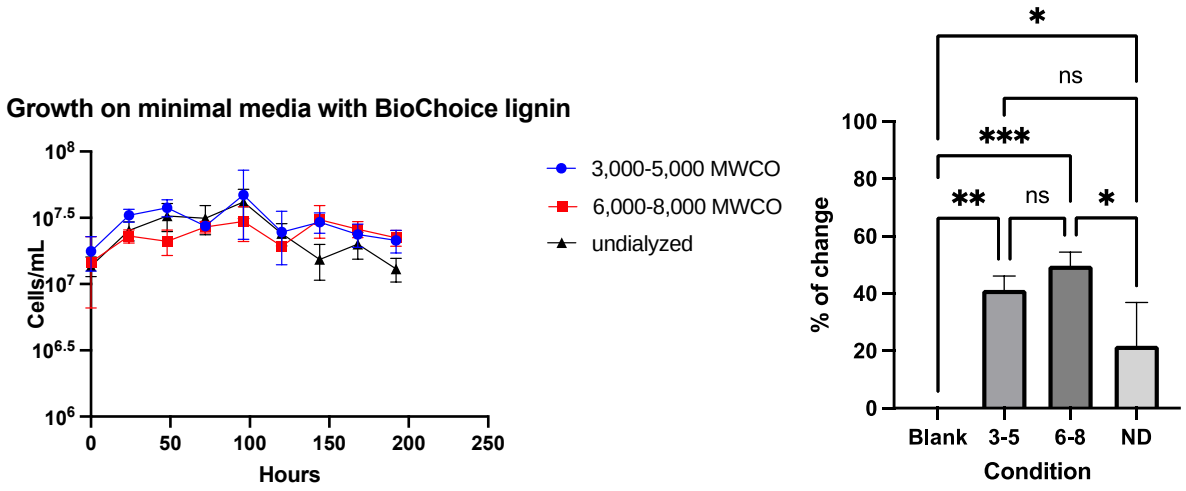


Figure 3-7 A. Growth of *Streptomyces* sp. 2-6 in cultures supplemented with dialyzed lignin as the carbon source. Error bars represent standard deviation. **B**. Percent reduction in lignin mass after incubation with *Streptomyces* sp. 2-6 for 400 h. Blank refers to uninoculated minimal media; 3-5 indicates inoculated with 3,000-5,000 MWCO dialyzed lignin; 6-8 indicates cultures inoculated with 6,000-8,000 MWCO and ND is Non-dialyzed. Two-way ANOVA was performed *** P-value (<0.05), No significance is indicated with (ns).

3.3.4. Prediction of metabolic pathways encoded by *Streptomyces* sp. 2-6 and 2-10 that could be involved in lignocellulose degradation

For this study *Streptomyces* spp. Strains 2-6 and 2-10 were selected for whole genome sequencing because of their apparent ability to grow on lignocellulose sources with *Streptomyces* sp. 2-6 performing better lignin reduction, and *Streptomyces* sp. 2-10 degrading cellulose to a greater extent (see **Table 3-2** and **Figure 3-2**, respectively). The sequenced genomes were examined for the presence of lignocellulose metabolizing homologs using the Distilled and Refined Annotation of Metabolism tool (Shaffer et al. 2020), KEGG annotation server, and Rapid Annotation using sybsystems Technology (RAST) (Aziz et al. 2008).

Most enzymes related to lignocellulose breakdown are hydrolytic enzymes that have been characterized for the degradation of cellulose, hemicellulose, and pectin in a synergistic manner

with other hydrolases (Palmqvist and Hahn-Hägerdal 2000). For cellulose degradation, enzymes such as cellulases can hydrolyze β-1,4-glycosidic linkages that connect glucose units in the cellulose fiber (Andlar et al. 2018). The enzymes capable of hydrolyzing β-1,4-glycosidic bonds are endo-β-1,4-glucanases, exoglucanases, and β-glucosidases (Liang et al. 2022).

Hemicellulose is a polysaccharide that contains sugars and sugar acids such as xylose, mannose, galactose, glucose, arabinose, 4-O-methylglucoronic, galacturonic, and glucuronic acids forming a web by links between β-1,4- and β-1,3-glycosidic bonds, but xylan is its main component (Andlar et al. 2018). The enzymes that have a role in the use of xylan have been identified, and the most common are endo-β-1,4-xylanase, exo-1,4-β-xylosidase, xylanase, xylan 1,4- β-xylosidase (Kim et al. 2014). The number of these enzymes can differ between genomes. This might explain the efficiency of the isolates to be capable of using these substrates.

Enzymes involved in lignin deconstruction have also been identified. Enzymes that participate in degrading or modifying lignin are categorized under auxiliary activity (AA) families in the Carbohydrate-Active enZYmes (CAZy) database because of their redox catalytic activity (Lombard *et al.*, 2014). Auxiliary activity enzymes present in the genomes of *Streptomyces* sp. 2-6 and 2-10 are listed in **Supplemental Table 3-3**. Other enzymes involved in lignocellulose degradation could be glycosyl hydrolases and glycosyl transferases. These were also identified using the CAZy database and can be found with their predictive role in lignocellulose metabolism in **Supplemental Table 3-4 and 3-5**. These enzymes have been described in *Streptomyces albus* (Vela Gurovic et al. 2021).

In the case of lignin metabolism, *Streptomyces* sp. 2-6 has two different multicopper oxidases and one dye-peroxidase. *Streptomyces* sp. 2-10, contains three multicopper oxidases and one dye-peroxidase. Multicopper oxidases and DyP-type peroxidases have been widely

discussed in the literature for their role in lignin degradation in different systems (Lu et al. 2014; Sugano and Yoshida 2021). These enzymes have been identified and characterized for other *Streptomyces* spp. Enzymes like multicopper oxidases are considered to be essential oxidases in different biological systems due to their role in defense mechanisms, biosynthetic processes, polymerization, and detoxification of plant phenolic compounds (Lu et al. 2014). Enzymes such as multicopper oxidases or ferroxidases, oxidases, reductases are present in the draft genomes of *Streptomyces* sp. 2-6 and 2-10 and localization and secretion were predicted using pSortB (**Supplemental Table 3-3**).

Peroxidases are involved in the initial depolymerization stage of lignin degradation (Bugg et al. 2011a). The auxiliary activity oxidases function by generating hydrogen peroxides that are used by peroxidases for degradation of aromatic compounds, and lignin (Kameshwar and Qin 2017). Enzymes such as quinone oxidoreductases might also participate in lignin-degradation since these enzymes have been reported in fungi to be involved in lignin degradation (Bugg et al. 2011b). NADPH:quinone oxidoreductase has been described in the bacterium *Pandorea* ISTKB and the fungus *Trametes versicolor* to participate in degrading lignin via the Fenton reaction (Lee et al. 2007).

Some pathways that could be involved in lignin degradation were identified in both the *Streptomyces* sp. 2-6 and 2-10 draft genomes. The summary of the relevant pathways with identified enzymes is shown in **Figure 3-8**. When comparing the genomes of *Streptomyces* sp. 2-6 and 2-10, one of the differences is the presence of protocatechuate 3,4-dioxygenases (*S.* sp. 2-6), which are enzymes involved in xenobiotic aromatic compound degradation pathways, but also have been recently reported to be involved in low molecular lignin utilization by *Streptomyces* (Tan et al. 2022). This was also seen in the *Pandorea* sp. ISTKB genome (Kumar

et al. 2018). However, very little is known about the role of hydrocarbon metabolism in these pathways for lignin degradation.

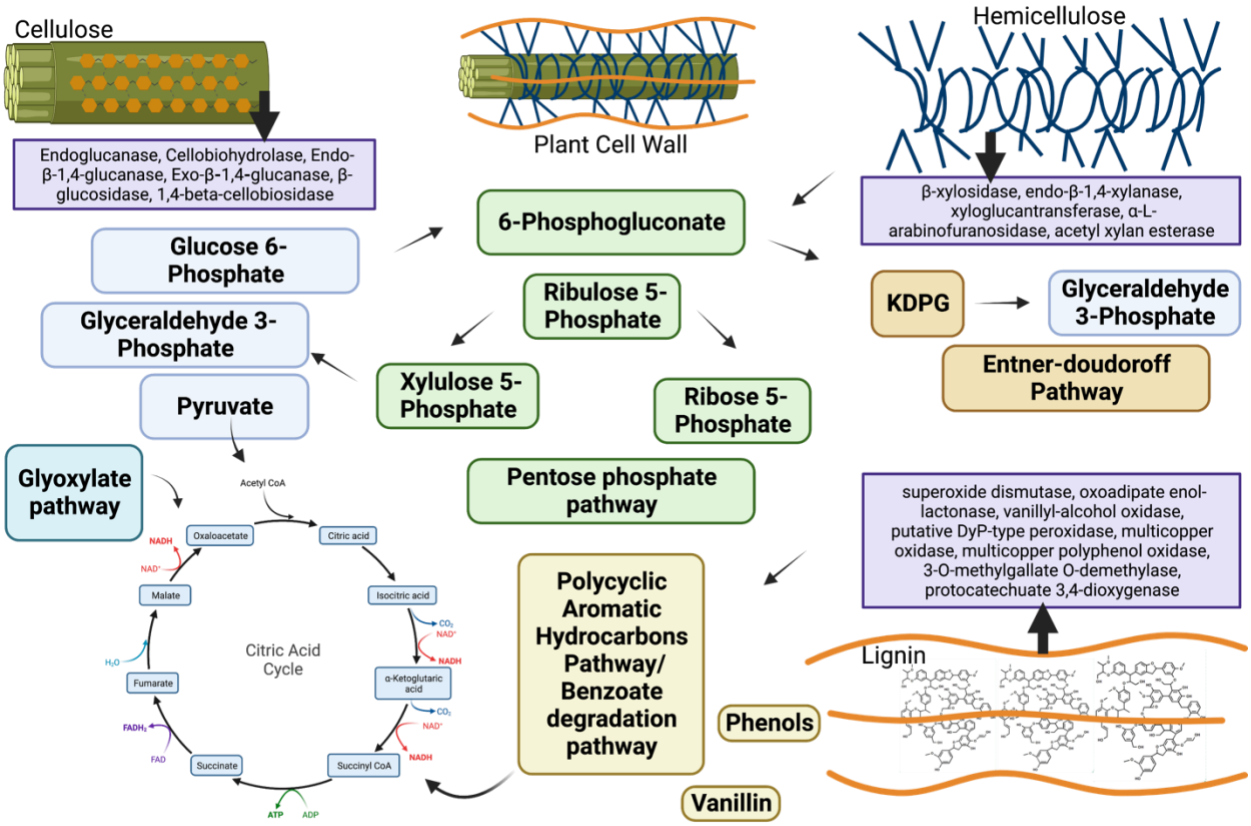


Figure 3-8. Predicted metabolic model with putative annotated enzymes from *Streptomyces*. sp. 2-6 and 2-10 based on *in silico* genome sequence analysis. The model was created based on KEGG pathways using the data provided by annotation using KAAS (KEGG Automatic Annotation Server). Figure was created using BioRender.com

3.4 Discussion

Industrially relevant bacterial isolates from insect sources have been addressed in multiple organisms from termites, ants, bees, camel crickets, etc. (Watanabe et al. 2003;

Kameshwar and Qin 2017; Mathews et al. 2019; Chevrette et al. 2019; Subta et al. 2020). However, this is the first time that microbial isolates from carpenter bees were assessed for their ability to metabolize black liquor and lignin. The results presented here suggest that the carpenter bee microflora should be more systematically studied as a source of bacterial strains for biotechnological applications.

To determine the ability of the bacterial isolates to grow on lignocellulose sources, the isolates were grown in minimal media with Biochoice lignin, xylans, cellulose (filter paper), and black liquor as the sole carbon sources. The isolates were identified as potential new species of *Streptomyces* sp. given that the initial identification was <95% match of 16S rRNA gene for known strains. However, this particular genus has a complicated phylogeny with many branches that some consider may include a different genus (Li et al. 2021). The presence of different genus can be observed in the phylogenetic tree (**Figure 3-1**).

Streptomyces sp. also have high recombination rates, which makes it harder to differentiate between strains at the interspecies level (Doroghazi and Buckley 2014). Being able to distinguish between strains at the interspecies level is very important when trying to understand the production of secondary metabolites by members of this genus (Cheng et al. 2016) because the production of secondary metabolites is not dependent on primary metabolism, which means that it is not essential. Therefore, the production of secondary metabolites can change within species, becoming strain specific (Doroghazi and Metcalf 2013). This suggests that the phylogenetic pattern is based on the capacity of the organism to adapt, which if secondary metabolism provides a benefit, it can result in the diversification and speciation of microbes (Choudoir et al. 2018). Therefore, whole genome sequencing of *Streptomyces* strains helps to avoid this issue.

From analysis using the ANI calculator, the most similar species to *Streptomyces* sp. 2-6 and 2-10 seem to originate from lignocellulose deconstruction prospecting projects (Book et al. 2014). However, none of the isolates for genome sequencing were matched with a valid deposited, characterized strains for lignocellulose degradation, which means that the closest relative strains were never published as a novel species and a type strain is not characterized such as when novel species are published in the International Journal of Systematic and Evolutionary Microbiology. The closest relative to *Streptomyces* sp. 2-10, has been characterized for lignocellulose degradation, though it was isolated from wood wasps (Book et al. 2014). This is important when proposing microbes for biotechnological purposes since the strain and its genome data should be accessible to the public.

Analysis of the *Streptomyces* sp. 2-6 vs *Streptomyces* sp. 2-10 genomes shows that the genomes encode a different array of CAZy annotated enzymes, including Auxiliary enzymes (**Supplemental Table 3-3**). This might explain why one strain is a better performer than the other in the utilization of carbohydrates. *Streptomyces* sp. 2-10 has more annotated hits with carbohydrate-utilization proteins than does *Streptomyces* sp. 2-6, which seemingly metabolizes lignin better. For example, *Streptomyces* sp. 2-6 contains 15 hits for GH13 family while *Streptomyces* sp. 2-10 has 11 hits. *Streptomyces* sp. 2-10 contains hits for 9 families of carbohydrate esterases which catabolize complex carbohydrates, while *Streptomyces* sp. 2-6 only has hits for 5 families. *Streptomyces* sp. 2-10 contains matches to 18 families of polysaccharide lyases, while *Streptomyces* sp. 2-6 only has matches to 4, and 3 of those are not annotated in the *Streptomyces* sp. 2-10 genome **(Supplemental Figure 3-3).** Notably enzymes such as multicopper oxidases that have been implicated in lignin deconstruction were found in both genomes; however, expression of these enzymes might require regulatory control, and it has

been reported that lignocellulose degradation can be effected by carbon catabolite repression (Liang et al. 2022).

In addition to the lignocellulose metabolizing potential revealed through the genome analyses and growth studies, the ability of the *Streptomyces* sp. 2-6 and 2-10 strains to decolorize different types of dyes suggests that they may have utility for textile applications in addition to pulp milling stream treatment. Currently, the textile industry faces challenges in detoxifying effluents (Mahmood et al. 2016; Blánquez et al. 2019; Kumar et al. 2021). The presence of these dyes in waters is dangerous to species in the environment and can affect human health (Al-Tohamy et al. 2022).

The pathways for lignin degradation are currently only partly defined. There are many review articles that propose enzymes for lignocellulose depolymerization (Xu et al. 2018; Riyadi et al. 2020; Higuchi et al. 2022; Dexter et al. 2022); however, there is still a need for careful biochemical characterization of enzymes capable of deconstructing lignin, especially from bacterial sources. To understand the physiological aspects and mechanisms required to achieve biological lignin degradation, there is a need to better understand the function of these enzymes against different substrates and their expression patterns when the microbial source is grown in the presence of different lignocellulose substrates.

3.5 Conclusions

The presence of enzymes that have identity to those known to participate in lignocellulose degradation suggest that *Streptomyces* spp. have the capacity to breakdown these. This and other recent studies (e.g. Tan et al. 2022) provide compelling evidence that *Streptomyces* sp. strains could have a direct role in lignin degradation or depolymerization. To

confirm this, the enzymes from these *Streptomyces* sp. strains will need to be purified and fully characterized. Interestingly, the presence of these enzymes as indicated through genome sequence analysis and initial activity screens could confer certain benefits to the carpenter bee, an avenue of investigation that should be explored. Draft genome sequences of *Streptomyces* spp. can be used in combination with biochemical studies to determine potential roles of these bacteria in lignocellulose degradation and valorization as well as the bioconversion of pulp milling side-streams.

Data availability

The whole-genome sequencing project is available in GenBank under the accession number PRJNA857064. The raw sequencing reads can be found in GenBank under the accession number SAMN29607947 and SAMN29607948.

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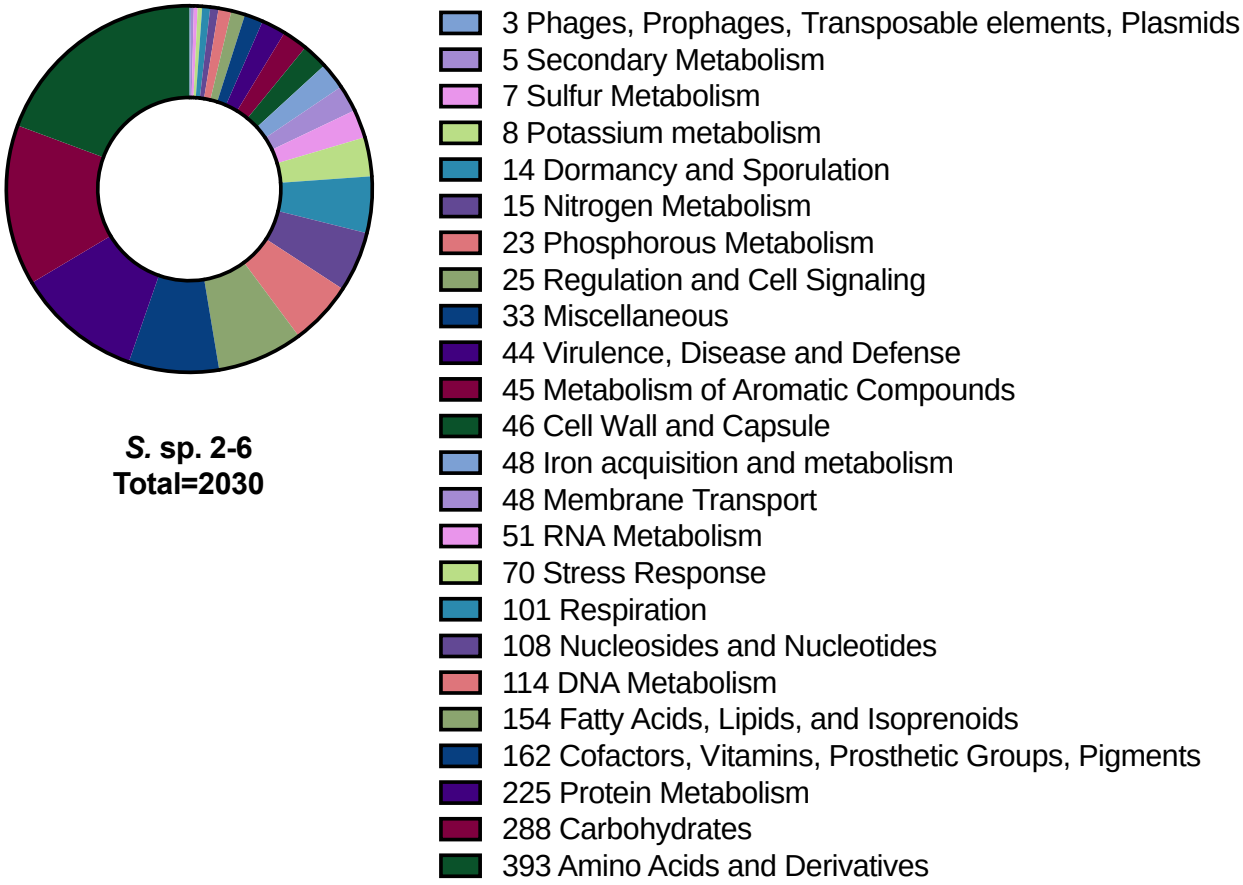
Supplemental Table 3-1. Draft genome features of *Streptomyces* sp. 2-6 and *S. flavorivens* 2-10 from BioProject PRJNA857064.

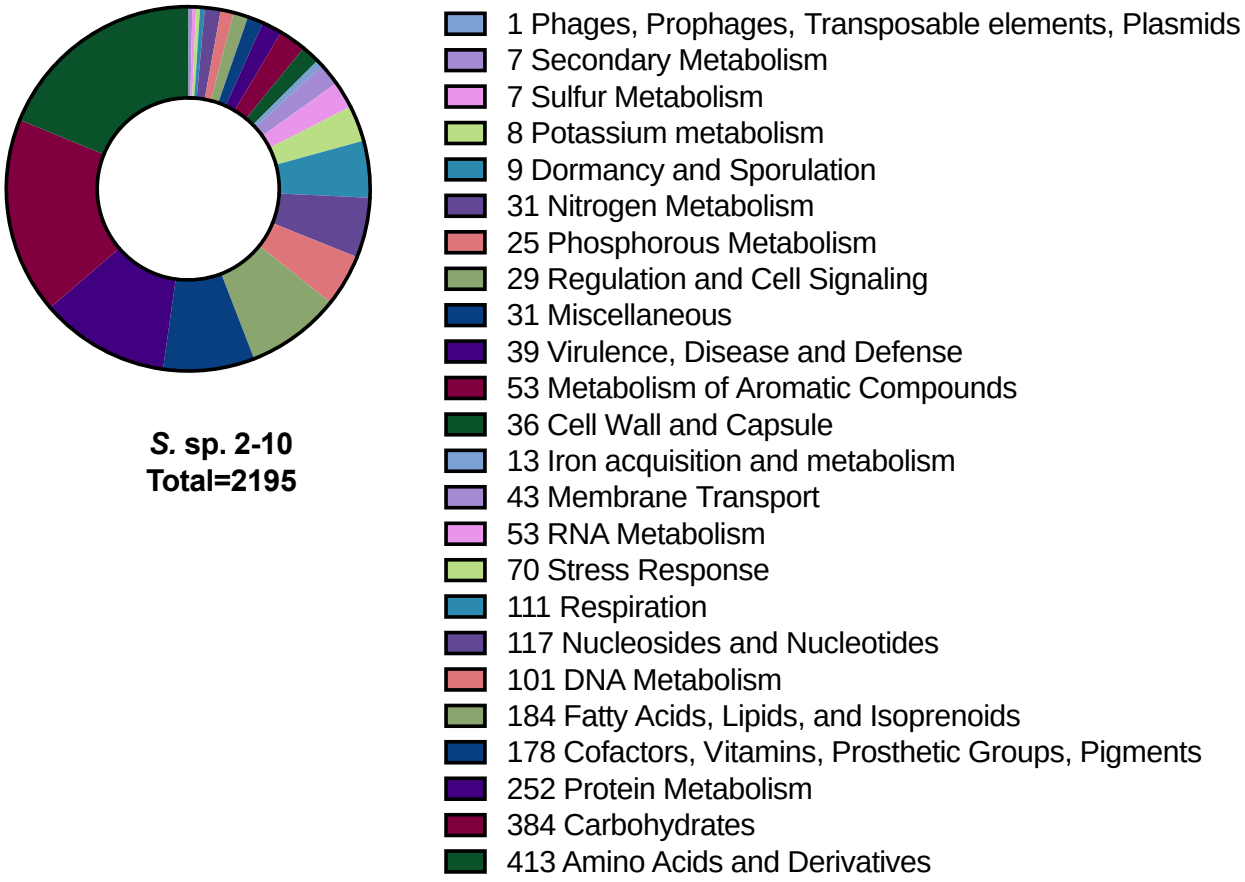
Feature	***Streptomyces* sp. 2-6**	***S. flavovirens* 2-10**
Genome size (bp)	8,166,232 bp	7, 457, 959 bp
Total GC content	73.22%	71.78%
Total of contigs	198	138
Protein Coding Genes	6803	6595
Non-coding gene	110	84
RNAs	81	72
tRNA	101	78
tmRNA	1	1
CRISPR	3	2
N_{50}	156960	245002
L50	17	11
ANI	92.36% *Streptomyces seoulensis*	95% *Streptomyces* sp. AAxE
Accession number	SAMN29607947	SAMN29607948

Supplemental Table 3-2. Number of sequences per subsystem feature of the genomes.

Feature	***Streptomyces* sp. 2-6**	***Streptomyces* sp. 2-10**
Cell Division and Cell Cycle	0	0
Motility and Chemotaxis	0	0
Photosynthesis	0	0
Phages, Prophages, Transposable elements, Plasmids	3	1
Secondary Metabolism	5	7
Sulfur Metabolism	7	7
Potassium metabolism	8	8
Dormancy and Sporulation	14	9
Nitrogen Metabolism	15	31
Phosphorous Metabolism	23	25
Regulation and Cell Signaling	25	29
Miscellaneous	33	31
Virulence, Disease and Defense	44	39
Metabolism of Aromatic Compounds	45	53
Cell Wall and Capsule	46	36
Iron acquisition and metabolism	48	13
Membrane Transport	48	43
RNA Metabolism	51	53
Stress Response	70	70
Respiration	101	111
Nucleosides and Nucleotides	108	117
DNA Metabolism	114	101
Fatty Acids, Lipids, and Isoprenoids	154	184
Cofactors, Vitamins, Prosthetic Groups, Pigments	162	178
Protein Metabolism	225	252
Carbohydrates	288	384
Amino Acids and Derivatives	393	413
Total	2,030	2,195



Supplemental Figure 3-1. Annotated genome of *Streptomyces.* sp. 2-6 using RAST. The functional summary is shown.



Supplemental Figure 3-2. Annotated genome of *Streptomyces*. sp. 2-10 using RAST. The functional summary is shown.

Supplemental Table 3-3. Alignment scores with Auxiliary activity enzymes identified from CAZy and their scores from PSORTb. Alignments were performed in KBase.

ALIGNMENT COVERAGE (HIT SEQ)	GENE ID	FUNCTION	GENOME	ALN_LEN	i-EVALUE	BIT SCORE	H_BEG-H_END	Signal Peptide and localization prediction
AA1	HILGIKNC_02541	Multicopper oxidase MmcO	2_10	345 (96.4%)	2.60E-67	226.5	89-515	Cell wall, Extracellular, spore
	HILGIKNC_02809	Multicopper oxidase mco	2_10	348 (97.2%)	2.60E-32	111.4	82-477	Cell wall, Extracellular, spore
	KPOIMGEJ_02627	Multicopper oxidase MmcO	2-6	345 (96.4%)	7.10E-63	211.9	102-554	Cell wall, Extracellular
AA1_2	HILGIKNC_02541	Multicopper oxidase MmcO	2_10	213 (63.2%)	2.10E-44	150.5	94-343	Cell wall, Extracellular, spore
AA1_3	HILGIKNC_02541	Multicopper oxidase MmcO	2_10	250 (80.1%)	1.60E-46	157.5	63-343	Cell wall, Extracellular, spore
AA3	HILGIKNC_00311	Choline oxidase	2_10	314 (50.8%)	5.50E-88	294.5	9-514	no signal peptide
	HILGIKNC_04263	Cholesterol oxidase	2_10	320 (51.8%)	3.40E-49	166.3	22-565	no signal peptide
	KPOIMGEJ_04669	putative GMC-type oxidoreductase	2-6	313 (50.6%)	2.40E-102	341.9	2-516	Cytoplasmic, No signal peptide
	KPOIMGEJ_06248	Choline oxidase	2-6	316 (51.1%)	9.10E-92	307	3-508	No signal peptide, cytoplasmic membrane
	KPOIMGEJ_00265	Cholesterol oxidase	2-6	322 (52.1%)	4.80E-50	169.2	51-598	no signal peptide, extracellular
AA3_1	HILGIKNC_00311	Choline oxidase	2_10	315 (57.5%)	2.40E-29	100.9	7-306	no signal peptide
AA3_2	HILGIKNC_00311	Choline oxidase	2_10	329 (57.9%)	6.70E-84	281.2	13-318	no signal peptide
AA3_3	HILGIKNC_00311	Choline oxidase	2_10	315 (53.4%)	2.40E-40	137	13-306	no signal peptide
AA6	HILGIKNdC_04004	NAD(P)H dehydrogenase (quinone)	2_10	193 (99.0%)	1.30E-58	196.3	7-197	no signal peptide

Supplemental Table 3-3. (Continued)

AA7	HILGIKNC_03549	Mitomycin radical oxidase	2_10	281 (61.4%)	1.70 E-66	224.4	12-290	no signal peptide
	KPOIMGEJ_05270	6-hydroxy-D-nicotine oxidase	2-6	453 (98.9%)	2.60 E-85	286.4	63-493	Signal peptide detected, Cell wall, Extracellular, cytoplasmic membrane, unknown final prediction
	KPOIMGEJ_06812	putative FAD-linked oxidoreductase YvdP	2-6	455 (99.3%)	2.40 E-128	428.3	80-520	Signal peptide detected, Extracellular
	KPOIMGEJ_05918	putative FAD-linked oxidoreductase YvdP	2-6	456 (99.6%)	2.30 E-102	342.6	28-473	no signal peptide, Cytoplasmic
	KPOIMGEJ_05923	putative FAD-linked oxidoreductase YvdP	2-6	452 (98.7%)	1.00 E-99	333.9	21-461	no signal peptide, Cytoplasmic
	KPOIMGEJ_05920	putative FAD-linked oxidoreductase YvdP	2-6	449 (98.0%)	1.10 E-95	320.6	70-507	Signal peptide detected, cytoplasmic membrane, cell wall, extracellular, unknown final prediction
AA10	HILGIKNC_03915	GlcNAc-binding protein A	2_10	177 (99.4%)	3.00 E-57	192.6	28-194	Signal peptide detected, cytoplasmic membrane, cell wall, extracellular, unknown prediction
	HILGIKNC_06206	GlcNAc-binding protein A	2_10	177 (99.4%)	1.40 E-51	174.1	29-194	Signal peptide detected, cytoplasmic membrane, cell wall, extracellular, unknown prediction
	HILGIKNC_05989	GlcNAc-binding protein A	2_10	177 (99.4%)	2.40 E-45	153.8	36-212	Signal peptide detected, Cytoplasmic membrane
	HILGIKNC_05040	Chitin-binding protein CbpD	2_10	178 (100.0%)	4.90 E-43	146.3	37-220	Signal peptide detected, Extracellular
	HILGIKNC_01113	Lytic cellulose monooxygenase	2_10	177 (99.4%)	2.30 E-42	144.1	34-224	Signal peptide detected, Extracellular
	KPOIMGEJ_01603	GlcNAc-binding protein A	2-6	177 (99.4%)	3.30 E-53	179.5	30-199	No signal peptide, Extracellular
	KPOIMGEJ_04874	GlcNAc-binding protein A	2-6	177 (99.4%)	7.20 E-45	152.3	50-227	Signal peptide detected, Cytoplasmic Membrane, Cell Wall, Extracellular, Final prediction unknown.

Supplemental Table 3-4. Functional Annotations assigned to *Streptomyces* sp. 2-6 using the DRAM tool

gene_id	gene_description	module	header	subheader	Counts
GH76	alpha-1,6-mannanase (EC 3.2.1.101); alpha-glucosidase (EC 3.2.1.20)	Glycoside Hydrolases	CAZY	Alpha-mannan Backbone and Oligo Cleavage (Hemicellulose)	2
GH38	alpha-mannosidase (EC 3.2.1.24); mannosyl-oligosaccharide alpha-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide alpha-1,3-1,6-mannosidase (EC 3.2.1.114); alpha-2-O-mannosylglycerate hydrolase (EC 3.2.1.170)	Glycoside Hydrolases	CAZY	Alpha-mannan Oligo Cleavage (Hemicellulose)	2
GH92	mannosyl-oligosaccharide alpha-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide alpha-1,3-mannosidase (EC 3.2.1.-); mannosyl-oligosaccharide alpha-1,6-mannosidase (EC 3.2.1.-); alpha-mannosidase (EC 3.2.1.24); alpha-1,2-mannosidase (EC 3.2.1.-); alpha-1,3-mannosidase (EC 3.2.1.-); alpha-1,4-mannosidase (EC 3.2.1.-); mannosyl-1-phosphodiester alpha-1,P-mannosidase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Alpha-mannan Oligo Cleavage (Hemicellulose)	8
GH6	endoglucanase (EC 3.2.1.4); cellobiohydrolase (EC 3.2.1.91)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Backbone Cleavage	5
GH5	endo-beta-1,4-glucanase / cellulase (EC 3.2.1.4); endo-beta-1,4-xylanase (EC 3.2.1.8); beta-glucosidase (EC 3.2.1.21); beta-mannosidase (EC 3.2.1.25); beta-glucosylceramidase (EC 3.2.1.45); glucan beta-1,3-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73); exo-beta-1,4-glucanase / cellodextrinase (EC 3.2.1.74); glucan endo-1,6-beta-glucosidase (EC 3.2.1.75); mannan endo-beta-1,4-mannosidase (EC 3.2.1.78); cellulose beta-1,4-cellobiosidase (EC 3.2.1.91); steryl beta-glucosidase (EC 3.2.1.104); endoglycoceramidase (EC 3.2.1.123); chitosanase (EC 3.2.1.132); beta-primeverosidase (EC 3.2.1.149); xyloglucan-specific endo-beta-1,4-glucanase (EC 3.2.1.151); endo-beta-1,6-galactanase (EC 3.2.1.164); hesperidin 6-O-alpha-L-rhamnosyl-beta-glucosidase (EC 3.2.1.168); beta-1,3-mannanase (EC 3.2.1.-); arabinoxylan-specific endo-beta-1,4-xylanase (EC 3.2.1.-); mannan transglycosylase (EC 2.4.1.-); lichenase / endo-beta-1,3-1,4-glucanase (EC 3.2.1.73); beta-glycosidase (EC 3.2.1.-); endo-beta-1,3-glucanase / laminarinase (EC 3.2.1.39); beta-N-acetylhexosaminidase (EC 3.2.1.52); chitosanase (EC 3.2.1.132); beta-D-galactofuranosidase (EC 3.2.1.146)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Backbone Cleavage, Amorphous Cellulose Oligo Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose), Xylan Backbone Cleavage (Hemicellulose), Beta-mannan Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Backbone Cleavage (Hemicellulose), Chitin Oligo Cleavage	7

Supplemental Table 3-4. (continued)

GH12	endoglucanase (EC 3.2.1.4); xyloglucan hydrolase (EC 3.2.1.151); beta-1,3-1,4-glucanase (EC 3.2.1.73); xyloglucan endotransglycosylase (EC 2.4.1.207)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Backbone Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose)	1
GH10	endo-1,4-beta-xylanase (EC 3.2.1.8); endo-1,3-beta-xylanase (EC 3.2.1.32); tomatinase (EC 3.2.1.-); xylan endotransglycosylase (EC 2.4.2.-); endo-beta-1,4-glucanase (EC 3.2.1.4)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Backbone Cleavage, Xyloglucan Oligo Cleavage (Hemicellulose), Xylan Backbone Cleavage (Hemicellulose)	1
GH1	beta-glucosidase (EC 3.2.1.21); beta-galactosidase (EC 3.2.1.23); beta-mannosidase (EC 3.2.1.25); beta-glucuronidase (EC 3.2.1.31); beta-xylosidase (EC 3.2.1.37); beta-D-fucosidase (EC 3.2.1.38); phlorizin hydrolase (EC 3.2.1.62); exo-beta-1,4-glucanase (EC 3.2.1.74); 6-phospho-beta-galactosidase (EC 3.2.1.85); 6-phospho-beta-glucosidase (EC 3.2.1.86); strictosidine beta-glucosidase (EC 3.2.1.105); lactase (EC 3.2.1.108); amygdalin beta-glucosidase (EC 3.2.1.117); prunasin beta-glucosidase (EC 3.2.1.118); vicianin hydrolase (EC 3.2.1.119); raucaffricine beta-glucosidase (EC 3.2.1.125); thioglucosidase (EC 3.2.1.147); beta-primeverosidase (EC 3.2.1.149); isoflavonoid 7-O-beta-apiosyl-beta-glucosidase (EC 3.2.1.161); ABA-specific beta-glucosidase (EC 3.2.1.175); DIMBOA beta-glucosidase (EC 3.2.1.182); beta-glycosidase (EC 3.2.1.-); hydroxyisourate hydrolase (EC 3.-.-.-)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Oligo Cleavage, Mixed-Linkage glucans Oligo Cleavage (Hemicellulose), Beta-galactan (pectic galactan) Oligo Cleavage	10
GH3	beta-glucosidase (EC 3.2.1.21); xylan 1,4-beta-xylosidase (EC 3.2.1.37); beta-glucosylceramidase (EC 3.2.1.45); beta-N-acetylhexosaminidase (EC 3.2.1.52); alpha-L-arabinofuranosidase (EC 3.2.1.55); glucan 1,3-beta-glucosidase (EC 3.2.1.58); glucan 1,4-beta-glucosidase (EC 3.2.1.74); isoprimeverose-producing oligoxyloglucan hydrolase (EC 3.2.1.120); coniferin beta-glucosidase (EC 3.2.1.126); exo-1,3-1,4-glucanase (EC 3.2.1.-); beta-N-acetylglucosaminide phosphorylases (EC 2.4.1.-)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Oligo Cleavage, Xylan Oligo Cleavage (Hemicellulose), Mixed-Linkage glucans Oligo Cleavage (Hemicellulose), Arabinan Oligo Cleavage, Chitin Oligo Cleavage	11

Supplemental Table 3-4. (continued)

GH16	xyloglucan:xyloglucosyltransferase (EC 2.4.1.207); keratan-sulfate endo-1,4-beta-galactosidase (EC 3.2.1.103); endo-1,3-beta-glucanase / laminarinase (EC 3.2.1.39); endo-1,3(4)-beta-glucanase (EC 3.2.1.6); licheninase (EC 3.2.1.73); beta-agarase (EC 3.2.1.81); kappa;-carrageenase (EC 3.2.1.83); xyloglucanase (EC 3.2.1.151); endo-beta-1,3-galactanase (EC 3.2.1.181); [retaining] beta-porphyranase (EC 3.2.1.178); hyaluronidase (EC 3.2.1.35); endo-beta-1,4-galactosidase (EC 3.2.1.-); chitin beta-1,6-glucanosyltransferase (EC 2.4.1.-); beta-transglycosidase (EC 2.4.1.-); beta-glycosidase (EC 3.2.1.-); endo-beta-1,3-galactanase (EC 3.2.1.181)	Glycoside Hydrolases	CAZY	Amorphous Cellulose Oligo Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Oligo Cleavage (Hemicellulose), Sulf-Polysachharides Backbone Cleavage	3
GH2	beta-galactosidase (EC 3.2.1.23) ; beta-mannosidase (EC 3.2.1.25); beta-glucuronidase (EC 3.2.1.31); alpha-L-arabinofuranosidase (EC 3.2.1.55); mannosylglycoprotein endo-beta-mannosidase (EC 3.2.1.152); exo-beta-glucosaminidase (EC 3.2.1.165); alpha-L-arabinopyranosidase (EC 3.2.1.-); beta-galacturonidase (EC 3.2.1.-); beta-xylosidase (EC 3.2.1.37); beta-D-galactofuranosidase (EC 3.2.1.146);	Glycoside Hydrolases	CAZY	Amorphous Cellulose Oligo Cleavage, Xyloglucan Oligo Cleavage (Hemicellulose), Beta-mannan Oligo Cleavage (Hemicellulose), Pectin Oligo Cleavage, Beta-galactan (pectic galactan) Oligo Cleavage, Arabinose Oligo cleavage	4
GH51	endoglucanase (EC 3.2.1.4); endo-beta-1,4-xylanase (EC 3.2.1.8); beta-xylosidase (EC 3.2.1.37); alpha-L-arabinofuranosidase (EC 3.2.1.55); lichenase / endo-beta-1,3-1,4-glucanase (EC 3.2.1.73)	Glycoside Hydrolases	CAZY	Arabinan Oligo Cleavage	1
GH54	alpha-L-arabinofuranosidase (EC 3.2.1.55); beta-xylosidase (EC 3.2.1.37).	Glycoside Hydrolases	CAZY	Arabinan Oligo Cleavage	1
GH127	beta-L-arabinofuranosidase (EC 3.2.1.185); 3-C-carboxy-5-deoxy-L-xylose (aceric acid) hydrolase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Arabinose Oligo cleavage	2
GH146	beta-L-arabinofuranosidase (EC 3.2.1.185)	Glycoside Hydrolases	CAZY	Arabinose Oligo cleavage	1
GH35	beta-galactosidase (EC 3.2.1.23); exo-beta-glucosaminidase (EC 3.2.1.165); exo-beta-1,4-galactanase (EC 3.2.1.-); beta-1,3-galactosidase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Beta-galactan (pectic galactan) Oligo Cleavage	6
GH18	chitinase (EC 3.2.1.14); lysozyme (EC 3.2.1.17); endo-beta-N-acetylglucosaminidase (EC 3.2.1.96); peptidoglycan hydrolase with endo-beta-N-acetylglucosaminidase specificity (EC 3.2.1.-); Nod factor hydrolase (EC 3.2.1.-); xylanase inhibitor; concanavalin B; narbonin	Glycoside Hydrolases	CAZY	Chitin Backbone Cleavage	7

Supplemental Table 3-4. (continued)

GH23	lysozyme type G (EC 3.2.1.17); peptidoglycan lyase (EC 4.2.2.n1) also known in the literature as peptidoglycan lytic transglycosylase; chitinase (EC 3.2.1.14)	Glycoside Hydrolases	CAZY	Chitin Backbone Cleavage	6
CE4	acetyl xylan esterase (EC 3.1.1.72); chitin deacetylase (EC 3.5.1.41); chitooligosaccharide deacetylase (EC 3.5.1.-); peptidoglycan GlcNAc deacetylase (EC 3.5.1.-); peptidoglycan N-acetylmuramic acid deacetylase (EC 3.5.1.-).	Carbohydrate Esterases	CAZY	Chitin Oligo Cleavage	26
GH20	beta-hexosaminidase (EC 3.2.1.52); lacto-N-biosidase (EC 3.2.1.140); beta-1,6-N-acetylglucosaminidase (EC 3.2.1.-); beta-6-SO3-N-acetylglucosaminidase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Chitin Oligo Cleavage	8
AA10	(formerly CBM33) proteins are copper-dependent lytic polysaccharide monooxygenases (LPMOs); some proteins have been shown to act on chitin, others on cellulose; lytic cellulose monooxygenase (C1-hydroxylating) (EC 1.14.99.54); lytic cellulose monooxygenase (C4-dehydrogenating)(EC 1.14.99.56); lytic chitin monooxygenase (EC 1.14.99.53)	Auxiliary Activities	CAZY	Crystalline Cellulose Backbone Cleavage, Crystalline Cellulose Backbone Cleavage, Chitin Oligo Cleavage	6
GH48	reducing end-acting cellobiohydrolase (EC 3.2.1.176); endo-beta-1,4-glucanase (EC 3.2.1.4); chitinase (EC 3.2.1.14)	Glycoside Hydrolases	CAZY	Crystalline Cellulose Backbone Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose)	1
GH29	alpha-L-fucosidase (EC 3.2.1.51); alpha-1,3/1,4-L-fucosidase (EC 3.2.1.111)	Glycoside Hydrolases	CAZY	Fucose Oligo Cleavage	2
GH95	alpha-L-fucosidase (EC 3.2.1.51); alpha-1,2-L-fucosidase (EC 3.2.1.63); alpha-L-galactosidase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Fucose Oligo Cleavage	0
GH158	endo-beta-1,3-glucanase (EC 3.2.1.39)	Glycoside Hydrolases	CAZY	Mixed-Linkage glucans Backbone Cleavage (Hemicellulose)	1
GH64	beta-1,3-glucanase (EC 3.2.1.39)	Glycoside Hydrolases	CAZY	Mixed-Linkage glucans Backbone Cleavage (Hemicellulose)	2
GH55	exo-beta-1,3-glucanase (EC 3.2.1.58); endo-beta-1,3-glucanase (EC 3.2.1.39)	Glycoside Hydrolases	CAZY	Mixed-Linkage glucans Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Oligo Cleavage (Hemicellulose)	3
GH4	maltose-6-phosphate glucosidase (EC 3.2.1.122); alpha-glucosidase (EC 3.2.1.20); alpha-galactosidase (EC 3.2.1.22); 6-phospho-beta-glucosidase (EC 3.2.1.86); alpha-glucuronidase (EC 3.2.1.139); alpha-galacturonase (EC 3.2.1.67); palatinase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Pectin Oligo Cleavage, Alpha-galactans Oligo Cleavage	4
AA1	Laccase / p-diphenol:oxygen oxidoreductase / ferroxidase (EC 1.10.3.2); ; ferroxidase (EC 1.10.3.-); Laccase-like multicopper oxidase (EC 1.10.3.-)	Auxiliary Activities	CAZY	Polyphenolics Cleavage	1

Supplemental Table 3-4. (continued)

AA2	manganese peroxidase (EC 1.11.1.13); versatile peroxidase (EC 1.11.1.16); lignin peroxidase (EC 1.11.1.14); peroxidase (EC 1.11.1.-)	Auxiliary Activities	CAZY	Polyphenolics Cleavage	4
AA4	vanillyl-alcohol oxidase (EC 1.1.3.38)	Auxiliary Activities	CAZY	Polyphenolics Cleavage	2
GH13	alpha-amylase (EC 3.2.1.1); pullulanase (EC 3.2.1.41); cyclomaltodextrin glucanotransferase (EC 2.4.1.19); cyclomaltodextrinase (EC 3.2.1.54); trehalose-6-phosphate hydrolase (EC 3.2.1.93); oligo-alpha-glucosidase (EC 3.2.1.10); maltogenic amylase (EC 3.2.1.133); neopullulanase (EC 3.2.1.135); alpha-glucosidase (EC 3.2.1.20); maltotetraose-forming alpha-amylase (EC 3.2.1.60); isoamylase (EC 3.2.1.68); glucodextranase (EC 3.2.1.70); maltohexaose-forming alpha-amylase (EC 3.2.1.98); maltotriose-forming alpha-amylase (EC 3.2.1.116); branching enzyme (EC 2.4.1.18); trehalose synthase (EC 5.4.99.16); 4-alpha-glucanotransferase (EC 2.4.1.25); maltopentaose-forming alpha-amylase (EC 3.2.1.-) ; amylosucrase (EC 2.4.1.4) ; sucrose phosphorylase (EC 2.4.1.7); malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141); isomaltulose synthase (EC 5.4.99.11); malto-oligosyltrehalose synthase (EC 5.4.99.15); amylo-alpha-1,6-glucosidase (EC 3.2.1.33); alpha-1,4-glucan: phosphate alpha-maltosyltransferase (EC 2.4.99.16); 6'-P-sucrose phosphorylase (EC 2.4.1.-); amino acid transporter	Glycoside Hydrolases	CAZY	Starch Backbone Cleavage	30
GH15	glucoamylase (EC 3.2.1.3); glucodextranase (EC 3.2.1.70); alpha,alpha-trehalase (EC 3.2.1.28); dextran dextrinase (EC 2.4.1.2)	Glycoside Hydrolases	CAZY	Starch Oligo Cleavage	8
PL8	hyaluronate lyase (EC 4.2.2.1); chondroitin AC lyase (EC 4.2.2.5); xanthan lyase (EC 4.2.2.12); chondroitin ABC lyase (EC 4.2.2.20)	Polysaccharide Lyases	CAZY	Sulf-Polysachharides Backbone Cleavage	4
GH11	endo-beta-1,4-xylanase (EC 3.2.1.8); endo-beta-1,3-xylanase (EC 3.2.1.32)	Glycoside Hydrolases	CAZY	Xylan Backbone Cleavage (Hemicellulose)	2
GH30	endo-beta-1,4-xylanase (EC 3.2.1.8); beta-glucosidase (3.2.1.21); beta-glucuronidase (EC 3.2.1.31); beta-xylosidase (EC 3.2.1.37); beta-fucosidase (EC 3.2.1.38); glucosylceramidase (EC 3.2.1.45); beta-1,6-glucanase (EC 3.2.1.75); glucuronoarabinoxylan endo-beta-1,4-xylanase (EC 3.2.1.136); endo-beta-1,6-galactanase (EC:3.2.1.164); [reducing end] beta-xylosidase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Xylan Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Backbone Cleavage (Hemicellulose)	1

Supplemental Table 3-4. (continued)

GH115	xylan alpha-1,2-glucuronidase (3.2.1.131); alpha-(4-O-methyl)-glucuronidase (3.2.1.-)	Glycoside Hydrolases	CAZY	Xylan Oligo Cleavage (Hemicellulose)	2
GH43	beta-xylosidase (EC 3.2.1.37); alpha-L-arabinofuranosidase (EC 3.2.1.55); xylanase (EC 3.2.1.8); alpha-1,2-L-arabinofuranosidase (EC 3.2.1.-); exo-alpha-1,5-L-arabinofuranosidase (EC 3.2.1.-); [inverting] exo-alpha-1,5-L-arabinanase (EC 3.2.1.-); beta-1,3-xylosidase (EC 3.2.1.-); [inverting] exo-alpha-1,5-L-arabinanase (EC 3.2.1.-); [inverting] endo-alpha-1,5-L-arabinanase (EC 3.2.1.99); exo-beta-1,3-galactanase (EC 3.2.1.145); beta-D-galactofuranosidase (EC 3.2.1.146)	Glycoside Hydrolases	CAZY	Xyloglucan Oligo Cleavage (Hemicellulose), Arabinan Backbone Cleavage, Arabinan Oligo Cleavage	4
GH31	alpha-glucosidase (EC 3.2.1.20); alpha-galactosidase (EC 3.2.1.22); alpha-mannosidase (EC 3.2.1.24); alpha-1,3-glucosidase (EC 3.2.1.84); sucrase-isomaltase (EC 3.2.1.48) (EC 3.2.1.10); alpha-xylosidase (EC 3.2.1.177); alpha-glucan lyase (EC 4.2.2.13); isomaltosyltransferase (EC 2.4.1.-); oligosaccharide alpha-1,4-glucosyltransferase (EC 2.4.1.161); sulfoquinovosidase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY	Xyloglucan Oligo Cleavage (Hemicellulose), Xylan Oligo Cleavage (Hemicellulose), Alpha-galactans Oligo Cleavage, Alpha-galactans Oligo Cleavage, Mucin Oligo Cleavage	6
AA3	cellobiose dehydrogenase (EC 1.1.99.18); glucose 1-oxidase (EC 1.1.3.4); aryl alcohol oxidase (EC 1.1.3.7); alcohol oxidase (EC 1.1.3.13); pyranose oxidase (EC 1.1.3.10)	Auxiliary Activities	CAZY		6
AA5	Oxidase with oxygen as acceptor (EC 1.1.3.-); galactose oxidase (EC 1.1.3.9); glyoxal oxidase (EC 1.2.3.15); alcohol oxidase (EC 1.1.3.13)	Auxiliary Activities	CAZY		2
AA7	glucooligosaccharide oxidase (EC 1.1.3.-); chitooligosaccharide oxidase (EC 1.1.3.-)	Auxiliary Activities	CAZY		15
AA8	Iron reductase domain	Auxiliary Activities	CAZY		1
CE1	acetyl xylan esterase (EC 3.1.1.72); cinnamoyl esterase (EC 3.1.1.-); feruloyl esterase (EC 3.1.1.73); carboxylesterase (EC 3.1.1.1); S-formylglutathione hydrolase (EC 3.1.2.12); diacylglycerol O-acyltransferase (EC 2.3.1.20); trehalose 6-O-mycolyltransferase (EC 2.3.1.122)	Carbohydrate Esterases	CAZY		12
CE10	arylesterase (EC 3.1.1.-); carboxyl esterase (EC 3.1.1.3); acetylcholinesterase (EC 3.1.1.7); cholinesterase (EC 3.1.1.8); sterol esterase (EC 3.1.1.13); brefeldin A esterase (EC 3.1.1.-).	Carbohydrate Esterases	CAZY		26

Supplemental Table 3-4. (continued)

CE14	N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-alpha-D-glucopyranoside deacetylase (EC 3.5.1.89); diacetylchitobiose deacetylase (EC 3.5.1.-); mycothiol S-conjugate amidase (EC 3.5.1.-)	Carbohydrate Esterases	CAZY		9
CE3	acetyl xylan esterase (EC 3.1.1.72).	Carbohydrate Esterases	CAZY		4
CE7	acetyl xylan esterase (EC 3.1.1.72); cephalosporin-C deacetylase (EC 3.1.1.41).	Carbohydrate Esterases	CAZY		2
CE9	N-acetylglucosamine 6-phosphate deacetylase (EC 3.5.1.25); N-acetylglucosamine 6-phosphate deacetylase (EC 3.5.1.80)	Carbohydrate Esterases	CAZY		2
CBM11	Modules of approx. 180-200 residues. The CBM11 of Clotridium thermocellum Cel26A-Cel5E has been shown to bind both beta-1,4-glucan and beta-1,3-1,4-mixed linked glucans.	Carbohydrate-Binding Modules	CAZY		2
CBM13	CBM13 Modules of approx. 150 residues which always appear as a threefold internal repeat. The only apparent exception to this, xylanase II of Actinomadura sp. FC7 (GenBank U08894), is in fact not completely sequenced. These modules were first identified in several plant lectins such as ricin or agglutinin of Ricinus communis which bind galactose residues. The three-dimensional structure of a plant lectin has been determined and displays a pseudo-threefold symmetry in accord with the observed sequence threefold repeat. These modules have since been found in a number of other proteins of various functions including glycoside hydrolases and glycosyltransferases. While in the plant lectins this module binds mannose, binding to xylan has been demonstrated in the Streptomyces lividans xylanase A and arabinofuranosidase B. Binding to GalNAc has been shown for the corresponding module of GalNAc transferase 4. For the other proteins, the binding specificity of these modules has not been established. The pseudo three-fold symmetry of the CBM13 module has now been confirmed in the 3-D structure of the intact, two-domain, xylanase of Streptomyces olivaceoviridis.	Carbohydrate-Binding Modules	CAZY		26
CBM16	CBM16 Carbohydrate-binding module 16. Binding to cellulose and glucomannan demonstrated [B. Bae et al (2008) J Biol Chem. 283:12415-25 (PMID: 18025086)]	Carbohydrate-Binding Modules	CAZY		7

Supplemental Table 3-4. (continued)

CBM2	CBM2 Modules of approx. 100 residues and which are found in a large number of bacterial enzymes. The cellulose-binding function has been demonstrated in many cases. Several of these modules have been shown to also bind chitin or xylan.	Carbohydrate-Binding Modules	CAZY		34
CBM20	CBM20 The granular starch-binding function has been demonstrated in several cases. Interact strongly with cyclodextrins. Often designated as starch-binding domains (SBD).	Carbohydrate-Binding Modules	CAZY		5
CBM25	CBM25 Starch-binding function demonstrated in one case.	Carbohydrate-Binding Modules	CAZY		3
CBM26	CBM26 Starch-binding function demonstrated in two cases.	Carbohydrate-Binding Modules	CAZY		2
CBM3	CBM3 Modules of approx. 150 residues found in bacterial enzymes. The cellulose-binding function has been demonstrated in many cases. In one instance binding to chitin has been reported.	Carbohydrate-Binding Modules	CAZY		2
CBM32	CBM32 Binding to galactose and lactose has been demonstrated for the module of Micromonospora viridifaciens sialidase (PMID: 16239725). Binding to polygalacturonic acid has been shown for a Yersinia member (PMID: 17292916). Binding to LacNAc (beta-D-galactosyl-1,4-beta-D-N-acetylglucosamine) has been shown for an N-acetylglucosaminidase from Clostridium perfingens (PMID: 16990278).	Carbohydrate-Binding Modules	CAZY		22
CBM35	CBM35 Modules of approx. 130 residues. A module that is conserved in three Cellvibrio xylan-degrading enzymes binds to xylan and the interaction is calcium dependent, while a module from a Cellvibrio mannanase binds to decorated soluble mannans and mannooligosaccharides. A module in a Phanerochaete chrysosporium galactan 1,3-beta-galactosidase binds to beta-galactan.	Carbohydrate-Binding Modules	CAZY		7
CBM41	CBM41 Modules of approx. 100 residues found in primarily in bacterial pullulanases. The N-terminal module from Thermotoga maritima Pul13 has been shown to bind to the alpha-glucans amylose, amylopectin, pullulan, and oligosaccharide fragments derived from these polysaccharides (Lammerts van Bueren et al. (2004) Biochemistry 43:15633-42) (PMID: 15581376).	Carbohydrate-Binding Modules	CAZY		4

Supplemental Table 3-4. (continued)

CBM44	CBM44 The C-terminal CBM44 module of the Clostridium thermocellum enzyme has been demonstrated to bind equally well cellulose and xyloglucan	Carbohydrate-Binding Modules	CAZY		2
CBM47	Modules of approx 150 residues. Fucose-binding activity demonstrated	Carbohydrate-Binding Modules	CAZY		11
CBM48	Modules of approx. 100 residues with glycogen-binding function, appended to GH13 modules. Also found in the beta subunit (glycogen-binding) of AMP-activated protein kinases (AMPK)	Carbohydrate-Binding Modules	CAZY		10
CBM50	CBM50 Modules of approx. 50 residues found attached to various enzymes from families GH18, GH19, GH23, GH24, GH25 and GH73, i.e. enzymes cleaving either chitin or peptidoglycan. Binding to chitopentaose demonstrated in the case of Pteris ryukyuensis chitinase A [Ohnuma T et al. (2008) J. Biol. Chem. 283:5178-87 (PMID: 18083709)]. CBM50 modules are also found in a multitude of other enzymes targeting the petidoglycan such as peptidases and amidases. These enzymes are not reported in the list below.	Carbohydrate-Binding Modules	CAZY		2
CBM51	CBM51 Modules of approx. 150 residues found attached to various enzymes from families GH2, GH27, GH31, GH95, GH98 and GH101 . Binding to galactose and to blood group A/B-antigens demonstrated in the case of C. perfringens GH95CBM51 and GH98CBM51 respectively [Gregg KJ et al. (2008) J. Biol. Chem. 283:12604-13 PMID: 18292090].	Carbohydrate-Binding Modules	CAZY		3
CBM6	CBM6 Modules of approx. 120 residues. The cellulose-binding function has been demonstrated in one case on amorphous cellulose and beta-1,4-xylan. Some of these modules also bind beta-1,3-glucan, beta-1,3-1,4-glucan, and beta-1,4-glucan.	Carbohydrate-Binding Modules	CAZY		8
CBM61	CBM61 Modules of approx. 150 residues found appended to GH16, GH30, GH31, GH43, GH53 and GH66 catalytic domains. A beta-1,4-galactan binding function has been demonstrated for the CBM61 of Thermotoga maritima GH53 galactanase [PMID: 20826814].	Carbohydrate-Binding Modules	CAZY		2
CBM62	CBM62 The CBM62 module of Clostridium thermocellum Cthe_2193 protein binds galactose moieties found on xyloglucan, arabinogalactan and galactomannan.	Carbohydrate-Binding Modules	CAZY		2

Supplemental Table 3-4. (continued)

GH114	GH114 endo-alpha-1,4-polygalactosaminidase (EC 3.2.1.109)	Glycoside Hydrolases	CAZY		4
GH25	GH25 lysozyme (EC 3.2.1.17)	Glycoside Hydrolases	CAZY		4
GH33	GH33 sialidase or neuraminidase (EC 3.2.1.18); trans-sialidase (EC 2.4.1.-); anhydrosialidase (EC 4.2.2.15); Kdo hydrolase (EC 3.2.1.-); 2-keto-3-deoxynononic acid hydrolase / KDNase (EC 3.2.1.-)	Glycoside Hydrolases	CAZY		2
GH46	GH46 chitosanase (EC 3.2.1.132)	Glycoside Hydrolases	CAZY		2
GH65	GH65 alpha,alpha-trehalase (EC 3.2.1.28); maltose phosphorylase (EC 2.4.1.8); trehalose phosphorylase (EC 2.4.1.64); kojibiose phosphorylase (EC 2.4.1.230); trehalose-6-phosphate phosphorylase (EC 2.4.1.216); nigerose phosphorylase (EC 2.4.1 .279); 3-O-alpha-glucopyranosyl-L-rhamnose phosphorylase (EC 2.4.1. 282); 2-O-alpha-glucopyranosyl glycerol: phosphate β-glucosyl transferase (EC 2.4.1.-); alpha-glucosyl-1,2-β-galactosyl-L-hydroxylysine alpha-glucosidase (EC 3.2.1.107)	Glycoside Hydrolases	CAZY		4
GH75	GH75 chitosanase (EC 3.2.1.132)	Glycoside Hydrolases	CAZY		2
GH77	GH77 amylomaltase or 4-alpha-glucanotransferase (EC 2.4.1.25)	Glycoside Hydrolases	CAZY		2
GH85	GH85 endo-beta-N-acetylglucosaminidase (EC 3.2.1.96)	Glycoside Hydrolases	CAZY		2
GH87	GH87 mycodextranase (EC 3.2.1.61); alpha-1,3-glucanase (EC 3.2.1.59)	Glycoside Hydrolases	CAZY		2

Supplemental Table 3-4. (continued)

GT1	UDP-glucuronosyl transferase (EC 2.4. 1.17); zeatin O-β-xylosyl transferase (EC 2.4.2.40); 2-hydroxy acyl sphingosine 1-β-galactosyl transferase (EC 2.4.1.45); N-acylsphingosine galactosyl transferase (EC 2.4.1.47); flavonol 3-O-glucosyl transferase (EC 2.4.1.91); anthocyanidin 3-O-glucosyl transferase (EC 2.4.1.115); sinapate 1-glucosyl transferase (EC 2.4.1.120); indole-3-acetate β-glucosyltransferase (EC 2.4. 1.121); flavonol L-rhamnosyl transferase (EC 2.4.1.159); sterol glucosyl transferase (EC 2.4.1.173); UDP-Glc: 4-hydroxybenzoate 4-O-β-glucosyltransferase (EC 2.4.1. 194); zeatin O-β-glucosyl transferase (EC 2.4.1.203); limonoid glucosyl transferase (EC 2.4.1.210); UDP-GlcA: baicalein 7-O-β-glucuronosyl transferase (EC 2.4.1.253); ecdysteroid UDP-glucosyl transferase (EC 2.4.1.-); salicylic acid β-glucosyltransferase (EC 2.4.1.-); anthocyanin 3-O-galactosyltransferase (EC 2.4.1.-); anthocyanin 5-O-glucosyl transferase (EC 2.4.1.-); dTDP-β-2-deoxy-L-fucose: alpha-L-2-deoxyfucosyl transferase (EC 2.4.1.-); UDP-β-L-rhamnose: alpha-L-rhamnosyl transferase (EC 2.4.1.-); zeaxanthin glucosyl transferase (EC 2. 4.1.-); flavone 8-C-glycosyltransferase	GlycosylTransferases	CAZY		6
GT2	cellulose synthase (EC 2.4.1.12); chitin synthase (EC 2.4.1.16); dolichyl-phosphate β-D-mannosyltransferase (EC 2.4.1.83); dolichyl-phosphate β-glucosyltransferase (EC 2.4.1.117); N-acetylglucosaminyl transferase (EC 2. 4.1.-); N-acetyl galactosaminyl transferase (EC 2.4.1.-); hyaluronan synthase (EC 2.4.1.212); chitin oligosaccharide synthase (EC 2.4.1.-); β-1,3-glucan synthase (EC 2.4.1.34); β-1,4-mannan synthase (EC 2.4.1.-); β-mannosylphosphodecaprenol-mannooligosaccharide alpha-1,6-mannosyltransferase (EC 2.4.1.199); UDP-Galf: rhamnopyranosyl-N-acetyl glucosaminyl-PP-decaprenol β-1,4/1,5-galactofuranosyl transferase (EC 2.4.1. 287); UDP-Galf: galactofuranosyl-galactofuranosyl-rhamnosyl-N-acetylglucosaminyl-PP-decaprenol beta-1,5/1,6-galactofuranosyl transferase (EC 2.4.1.288); dTDP-L-Rha: N-acetylglucosaminyl-PP-decaprenol alpha-1,3-L-rhamnosyltransferase (EC 2.4.1.289)	GlycosylTransferases	CAZY		21

Supplemental Table 3-4. (continued)

GT20	α,α-trehalose-phosphate synthase [UDP-forming] (EC 2.4.1. 15); Glucosylglycerol-phosphate synthase (EC 2.4.1.213); trehalose-6-P phosphatase (EC 3.1.3.12); GDP-valeniol: validamine 7-phosphate valeniolyltransferase (EC 2.-.-.-)	GlycosylTransferases	CAZY		2
GT21	UDP-Glc: ceramide β-glucosyl transferase (EC 2.4.1.80)	GlycosylTransferases	CAZY		2
GT28	1,2-diacylglycerol 3-β-galactosyl transferase (EC 2.4.1.46); 1,2-diacyl glycerol 3-β-glucosyltransferase (EC 2.4.1.157); UDP-GlcNAc: Und-PP-MurAc-pentapeptide β-N-acetyl glucosaminyltransferase (EC 2.4.1. 227); digalactosyldiacylglycerol synthase (EC 2.4.1.241)	GlycosylTransferases	CAZY		3
GT35	glycogen or starch phosphorylase (EC 2.4.1.1).	GlycosylTransferases	CAZY		2
GT39	Dol-P-Man: alpha-mannosyl transferase (EC 2.4.1.109)	GlycosylTransferases	CAZY		6
GT4	sucrose synthase (EC 2.4.1.13); sucrose-phosphate synthase (EC 2.4.1. 14); α-glucosyltransferase (EC 2.4.1. 52); lipopolysaccharide N-acetyl glucosaminyl transferase (EC 2.4.1. 56); phosphatidylinositol-mannosyl transferase (EC 2.4.1.57); Man1 GlcNAc2-PP-dolichol α-1,3-mannosyl transferase (EC 2.4.1.132); GDP-Man: Man3GlcNAc2-PP-dolichol/Man4 GlcNAc2-PP-dolichol α-1,2-mannosyl transferase (EC 2.4.1 .131); digalactosyldiacyl glycerol synthase (EC 2.4.1.141); 1,2-diacyl glycerol 3-glucosyltransferase (EC 2.4. 1.157); diglucosyldiacylglycerol synthase (EC 2.4.1.208); trehalose phosphorylase (EC 2.4.1. 231); NDP-Glc: α-glucose α-glucosyl transferase/ α,α-trehalose synthase (EC 2.4.1.245); GDP-Man: Man2GlcNAc2-PP-dolichol α-1,6-mannosyl transferase (EC 2.4.1.257); UDP-GlcNAc: 2-deoxystreptamine α-N-acetyl glucosaminyl transferase (EC 2.4.1. 283); UDP-GlcNAc: ribostamycin α-N-acetyl glucosaminyl transferase (EC 2.4.1.285); UDP-Gal α-galactosyl transferase (EC 2.4.1.-); UDP-Xyl α-xylosyltransferase (EC 2.4.2.-); UDP-GlcA α-glucuronyl transferase (EC 2.4.1.-); UDP-Glc α-glucosyl transferase (EC 2.4.1.-); UDP-GalNAc: GalNAc-PP-Und α-1,3-N-acetylgalactosaminyltransferase (EC 2.4.1.306); UDP-GalNAc: N,N'-diacetyl bacillosaminyl-PP-Und α-1,3-N-acetylgalactosaminyltransferase	GlycosylTransferases	CAZY		28

Supplemental Table 3-4. (continued)

GT5	UDP-Glc: glycogen glucosyltransferase (EC 2.4.1.11); ADP-Glc: starch glucosyltransferase (EC 2.4.1.21); NDP-Glc: starch glucosyltransferase (EC 2.4.1.242); UDP-Glc: alpha-1,3-glucan synthase (EC 2.4.1.183) UDP-Glc: alpha-1,4-glucan synthase (EC 2.4.1.-)	GlycosylTransferases	CAZY		7
GT51	murein polymerase (EC 2.4.1.129).	GlycosylTransferases	CAZY		10
GT76	Dol-P-Man: alpha-1,6-mannosyltransferase (EC 2.4.1.-)	GlycosylTransferases	CAZY		4
GT81	NDP-Glc: glucosyl-3-phosphoglycerate synthase (EC 2.4.1.-); NDP-Man: mannosyl-3-phosphoglycerate synthase (EC 2.4.1.-);	GlycosylTransferases	CAZY		2
GT82	UDP-GalNAc: beta-1,4-N-acetylgalactosaminyltransferase (EC 2.4.1.-)	GlycosylTransferases	CAZY		0
GT83	undecaprenyl phosphate-alpha-L-Ara4N: 4-amino-4-deoxy-beta-L-arabinosyltransferase (EC 2.4.2.43); dodecaprenyl phosphate-beta-galacturonic acid: lipopolysaccharide core alpha-galacturonosyl transferase (EC 2.4.1.-)	GlycosylTransferases	CAZY		5
GT87	polyprenol-P-Man: alpha-1,2-mannosyltransferase (EC 2.4.1.-)	GlycosylTransferases	CAZY		7
GT9	lipopolysaccharide N-acetyl glucosaminyltransferase (EC 2.4.1.56); heptosyltransferase (EC 2.4.-.-).	GlycosylTransferases	CAZY		4
K14727	oxoadipate enol-lactonase / 4-carboxymuconolactone decarboxylase [EC:3.1.1.24 4.1.1.44]	Catechol ortho-cleavage, catechol => 3-oxoadipate	hydrocarbon degradation		2
K01826	5-carboxymethyl-2-hydroxymuconate isomerase [EC:5.3.3.10][RN:R04379]	Homoprotocatechuate degradatio, homoprotocatechuate => 2-oxohept-3-enedioate	hydrocarbon degradation		2
K04102	4,5-dihydroxyphthalate decarboxylase [EC:4.1.1.55] [RN:R01635]	Phthalate degradation, phthalate => protocatechuate	hydrocarbon degradation		1
K00055	xylB; aryl-alcohol dehydrogenase [EC:1.1.1.90] [RN:R01763]	Toluene degradation, toluene => benzoate	hydrocarbon degradation		1
K00141	xylC; benzaldehyde dehydrogenase (NAD) [EC:1.2.1.28] [RN:R01419]	Toluene degradation, toluene => benzoate	hydrocarbon degradation		1

Supplemental Table 3-4. (continued)

K00529	3-phenylpropionate/cinnamic acid dioxygenase [EC:1.14.12.19] [RN:R06783]	Trans-cinnamate degradation, trans-cinnamate => acetyl-CoA	hydrocarbon degradation	4
K05710	3-phenylpropionate/cinnamic acid dioxygenase [EC:1.14.12.19] [RN:R06783]	Trans-cinnamate degradation, trans-cinnamate => acetyl-CoA	hydrocarbon degradation	1
K05712	mhpA; 3-(3-hydroxy-phenyl)propionate hydroxylase [EC:1.14.13.127] [RN:R06787]	Trans-cinnamate degradation, trans-cinnamate => acetyl-CoA	hydrocarbon degradation	3
K00055	E1.1.1.90; aryl-alcohol dehydrogenase [EC:1.1.1.90] [RN:R05282 R05348 R05347]	Xylene degradation, xylene => methylbenzoate	hydrocarbon degradation	1
K00141	xylC; benzaldehyde dehydrogenase (NAD) [EC:1.2.1.28] [RN:R05289 R05663 R05664]	Xylene degradation, xylene => methylbenzoate	hydrocarbon degradation	1
K15066	ligM; vanillate/3-O-methylgallate O-demethylase [EC:2.1.1.341]	lignin subunit degradation	hydrocarbon degradation	1
K00448	pcaG; protocatechuate 3,4-dioxygenase, alpha subunit [EC:1.13.11.3]	protocatechuate degradation, protocatechuate => 3-oxoadipate	hydrocarbon degradation	1
K00449	pcaH; protocatechuate 3,4-dioxygenase, beta subunit [EC:1.13.11.3]	protocatechuate degradation, protocatechuate => 3-oxoadipate	hydrocarbon degradation	1
K01607	pcaC; 4-carboxymuconolactone decarboxylase [EC:4.1.1.44]	protocatechuate degradation, protocatechuate => 3-oxoadipate	hydrocarbon degradation	3
K01857	pcaB; 3-carboxy-cis,cis-muconate cycloisomerase [EC:5.5.1.2]	protocatechuate degradation, protocatechuate => 3-oxoadipate	hydrocarbon degradation	1

Supplemental Table 3-4. (continued)

K14727	pcaL; 3-oxoadipate enol-lactonase / 4-carboxymuconolactone decarboxylase [EC:3.1.1.24 4.1.1.44]	protocatechuate degradation, protocatechuate => 3-oxoadipate	hydrocarbon degradation	2
K10218	ligK; 4-hydroxy-4-methyl-2-oxoglutarate aldolase [EC:4.1.3.17]	protocatechuate degradation, protocatechuate => oxaloacteate + pyruvate	hydrocarbon degradation	1
K10220	ligJ; 4-oxalmesaconate hydratase [EC:4.2.1.83]	protocatechuate degradation, protocatechuate => oxaloacteate + pyruvate	hydrocarbon degradation	1
K05810	polyphenol oxidase	polyphenols	Polyphenolics Cleavage	1

Supplemental Table 3-5. Functional Annotations assigned to *Streptomyces* sp. 2-10 using the DRAM tool

gene_id	gene_description	module	subheader	Count
GH76	GH76 alpha-1,6-mannanase (EC 3.2.1.101); alpha-glucosidase (EC 3.2.1.20)	Glycoside Hydrolases	Alpha-mannan Backbone and Oligo Cleavage (Hemicellulose),	2
GH38	GH38 alpha-mannosidase (EC 3.2.1.24); mannosyl-oligosaccharide alpha-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide alpha-1,3-1,6-mannosidase (EC 3.2.1.114); alpha-2-O-mannosylglycerate hydrolase (EC 3.2.1.170); mannosyl-oligosaccharide alpha-1,3-mannosidase (EC 3.2.1.-)	Glycoside Hydrolases	Alpha-mannan Oligo Cleavage (Hemicellulose)	2
GH92	GH92 mannosyl-oligosaccharide alpha-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide alpha-1,3-mannosidase (EC 3.2.1.-); mannosyl-oligosaccharide alpha-1,6-mannosidase (EC 3.2.1.-); alpha-mannosidase (EC 3.2.1.24); alpha-1,2-mannosidase (EC 3.2.1.-); alpha-1,3-mannosidase (EC 3.2.1.-); alpha-1,4-mannosidase (EC 3.2.1.-); mannosyl-1-phosphodiester alpha-1,P-mannosidase (EC 3.2.1.-)	Glycoside Hydrolases	Alpha-mannan Oligo Cleavage (Hemicellulose)	8
GH6	GH6 endoglucanase (EC 3.2.1.4); cellobiohydrolase (EC 3.2.1.91)	Glycoside Hydrolases	Amorphous Cellulose Backbone Cleavage	5
GH5	GH5 endo-beta-1,4-glucanase / cellulase (EC 3.2.1.4); endo-beta-1,4-xylanase (EC 3.2.1.8); beta-glucosidase (EC 3.2.1.21); beta-mannosidase (EC 3.2.1.25); beta-glucosylceramidase (EC 3.2.1.45); glucan beta-1,3-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73); exo-beta-1,4-glucanase / cellodextrinase (EC 3.2.1.74); glucan endo-1,6-beta-glucosidase (EC 3.2.1.75); mannan endo-beta-1,4-mannosidase (EC 3.2.1.78); cellulose beta-1,4-cellobiosidase (EC 3.2.1.91); steryl beta-glucosidase (EC 3.2.1.104); endoglycoceramidase (EC 3.2.1.123); chitosanase (EC 3.2.1.132); beta-primeverosidase (EC 3.2.1.149); xyloglucan-specific endo-beta-1,4-glucanase (EC 3.2.1.151); endo-beta-1,6-galactanase (EC 3.2.1.164); hesperidin 6-O-alpha-L-rhamnosyl-beta-glucosidase (EC 3.2.1.168); beta-1,3-mannanase (EC 3.2.1.-); arabinoxylan-specific endo-beta-1,4-xylanase (EC 3.2.1.-); mannan transglycosylase (EC 2.4.1.-); lichenase / endo-beta-1,3-1,4-glucanase (EC 3.2.1.73); beta-glycosidase (EC 3.2.1.-); endo-beta-1,3-glucanase / laminarinase (EC 3.2.1.39); beta-N-acetylhexosaminidase (EC 3.2.1.52); chitosanase (EC 3.2.1.132); beta-D-galactofuranosidase (EC 3.2.1.146);	Glycoside Hydrolases	Amorphous Cellulose Backbone Cleavage, Amorphous Cellulose Oligo Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose), Xylan Backbone Cleavage (Hemicellulose), Beta-mannan Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Backbone Cleavage (Hemicellulose), Chitin Oligo Cleavage	7
GH12	GH12 endoglucanase (EC 3.2.1.4); xyloglucan hydrolase (EC 3.2.1.151); beta-1,3-1,4-glucanase (EC 3.2.1.73); xyloglucan endotransglycosylase (EC 2.4.1.207)	Glycoside Hydrolases	Amorphous Cellulose Backbone Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose)	1
GH10	GH10 endo-1,4-beta-xylanase (EC 3.2.1.8); endo-1,3-beta-xylanase (EC 3.2.1.32); tomatinase (EC 3.2.1.-); xylan endotransglycosylase (EC 2.4.2.-); endo-beta-1,4-glucanase (EC 3.2.1.4)	Glycoside Hydrolases	Amorphous Cellulose Backbone Cleavage, Xyloglucan Oligo Cleavage,Xylan Backbone Cleavage (Hemicellulose)	1

Supplemental Table 3-5. (Continued)

GH1	GH1 beta-glucosidase (EC 3.2.1.21); beta-galactosidase (EC 3.2.1.23); beta-mannosidase (EC 3.2.1.25); beta-glucuronidase (EC 3.2.1.31); beta-xylosidase (EC 3.2.1.37); beta-D-fucosidase (EC 3.2.1.38); phlorizin hydrolase (EC 3.2.1.62); exo-beta-1,4-glucanase (EC 3.2.1.74); 6-phospho-beta-galactosidase (EC 3.2.1.85); 6-phospho-beta-glucosidase (EC 3.2.1.86); strictosidine beta-glucosidase (EC 3.2.1.105); lactase (EC 3.2.1.108); amygdalin beta-glucosidase (EC 3.2.1.117); prunasin beta-glucosidase (EC 3.2.1.118); vicianin hydrolase (EC 3.2.1.119); raucaffricine beta-glucosidase (EC 3.2.1.125); thioglucosidase (EC 3.2.1.147); beta-primeverosidase (EC 3.2.1.149); isoflavonoid 7-O-beta-apiosyl-beta-glucosidase (EC 3.2.1.161); ABA-specific beta-glucosidase (EC 3.2.1.175); DIMBOA beta-glucosidase (EC 3.2.1.182); beta-glycosidase (EC 3.2.1.-); hydroxyisourate hydrolase (EC 3.-.-.-)	Glycoside Hydrolases	Amorphous Cellulose Oligo Cleavage, Mixed-Linkage glucans Oligo Cleavage (Hemicellulose), Beta-galactan (pectic galactan) Oligo Cleavage	10
GH3	GH3 beta-glucosidase (EC 3.2.1.21); xylan 1,4-beta-xylosidase (EC 3.2.1.37); beta-glucosylceramidase (EC 3.2.1.45); beta-N-acetylhexosaminidase (EC 3.2.1.52); alpha-L-arabinofuranosidase (EC 3.2.1.55); glucan 1,3-beta-glucosidase (EC 3.2.1.58); glucan 1,4-beta-glucosidase (EC 3.2.1.74); isoprimeverose-producing oligoxyloglucan hydrolase (EC 3.2.1.120); coniferin beta-glucosidase (EC 3.2.1.126); exo-1,3-1,4-glucanase (EC 3.2.1.-); beta-N-acetylglucosaminide phosphorylases (EC 2.4.1.-)	Glycoside Hydrolases	Amorphous Cellulose Oligo Cleavage, Xylan Oligo Cleavage (Hemicellulose), Mixed-Linkage glucans Oligo Cleavage (Hemicellulose), Arabinan Oligo Cleavage, Chitin Oligo Cleavage	11
GH16	GH16 xyloglucan:xyloglucosyltransferase (EC 2.4.1.207); keratan-sulfate endo-1,4-beta-galactosidase (EC 3.2.1.103); endo-1,3-beta-glucanase / laminarinase (EC 3.2.1.39); endo-1,3(4)-beta-glucanase (EC 3.2.1.6); licheninase (EC 3.2.1.73); beta-agarase (EC 3.2.1.81); kappa;-carrageenase (EC 3.2.1.83); xyloglucanase (EC 3.2.1.151); endo-beta-1,3-galactanase (EC 3.2.1.181); [retaining] beta-porphyranase (EC 3.2.1.178); hyaluronidase (EC 3.2.1.35); endo-beta-1,4-galactosidase (EC 3.2.1.-); chitin beta-1,6-glucanosyltransferase (EC 2.4.1.-); beta-transglycosidase (EC 2.4.1.-); beta-glycosidase (EC 3.2.1.-); endo-beta-1,3-galactanase (EC 3.2.1.181)	Glycoside Hydrolases	Amorphous Cellulose Oligo Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Oligo Cleavage (Hemicellulose), Sulf-Polysachharides Backbone Cleavage	3
GH2	GH2 beta-galactosidase (EC 3.2.1.23) ; beta-mannosidase (EC 3.2.1.25); beta-glucuronidase (EC 3.2.1.31); alpha-L-arabinofuranosidase (EC 3.2.1.55); mannosylglycoprotein endo-beta-mannosidase (EC 3.2.1.152); exo-beta-glucosaminidase (EC 3.2.1.165); alpha-L-arabinopyranosidase (EC 3.2.1.-); beta-galacturonidase (EC 3.2.1.-); beta-xylosidase (EC 3.2.1.37); beta-D-galactofuranosidase (EC 3.2.1.146);	Glycoside Hydrolases	Amorphous Cellulose Oligo Cleavage, Xyloglucan Oligo Cleavage (Hemicellulose), Beta-mannan Oligo Cleavage (Hemicellulose), Pectin Oligo Cleavage, Beta-galactan (pectic galactan) Oligo Cleavage, Arabinose Oligo cleavage	4
GH51	GH51 endoglucanase (EC 3.2.1.4); endo-beta-1,4-xylanase (EC 3.2.1.8); beta-xylosidase (EC 3.2.1.37); alpha-L-arabinofuranosidase (EC 3.2.1.55); lichenase / endo-beta-1,3-1,4-glucanase (EC 3.2.1.73)	Glycoside Hydrolases	Arabinan Oligo Cleavage	1
GH54	GH54 alpha-L-arabinofuranosidase (EC 3.2.1.55); beta-xylosidase (EC 3.2.1.37).	Glycoside Hydrolases	Arabinan Oligo Cleavage	1

Supplemental Table 3-5. (Continued)

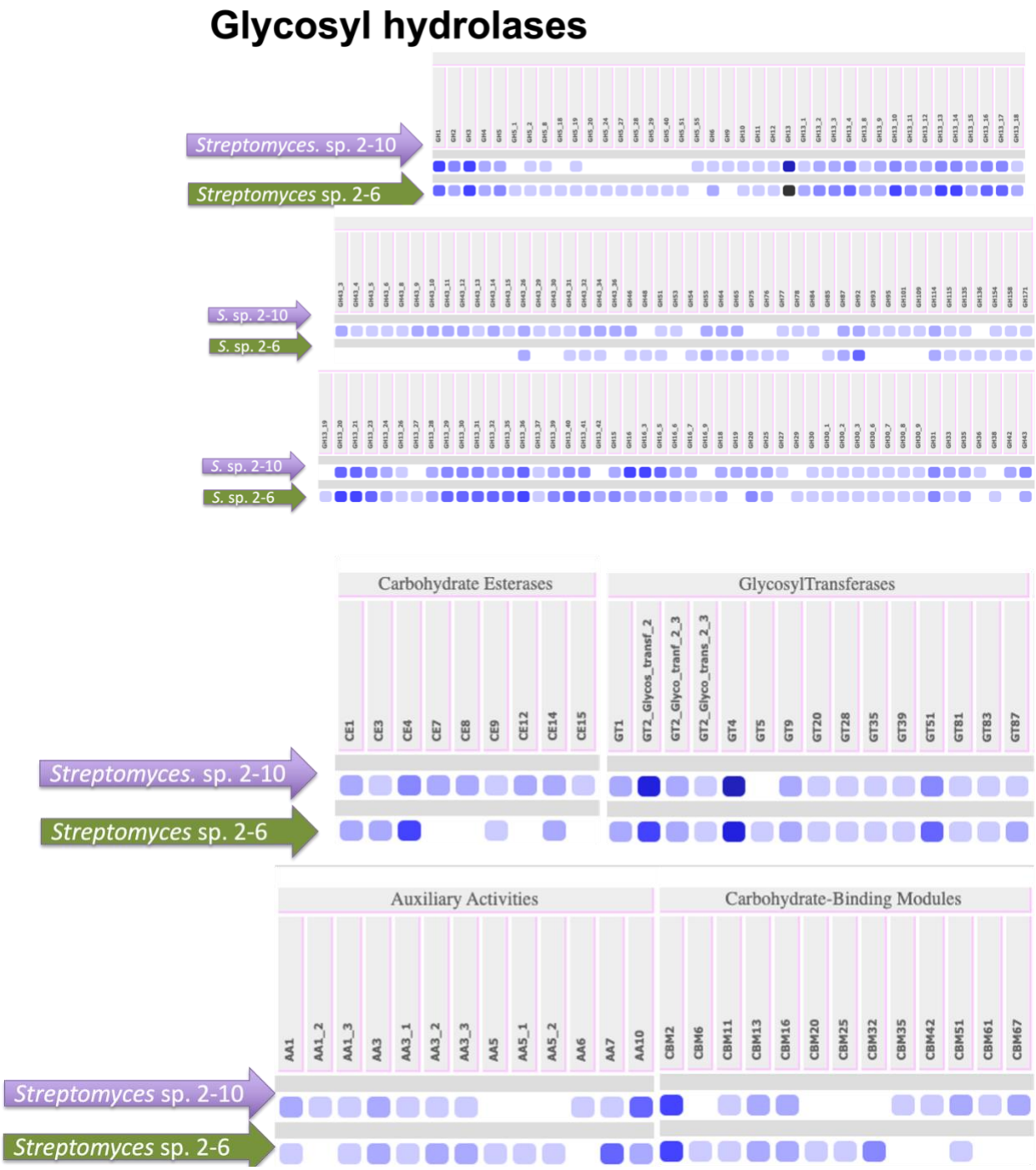
GH127	GH127 beta-L-arabinofuranosidase (EC 3.2.1.185); 3-C-carboxy-5-deoxy-L-xylose (aceric acid) hydrolase (EC 3.2.1.-)	Glycoside Hydrolases	Arabinose Oligo cleavage	2
GH146	GH146 beta-L-arabinofuranosidase (EC 3.2.1.185)	Glycoside Hydrolases	Arabinose Oligo cleavage	1
GH35	GH35 beta-galactosidase (EC 3.2.1.23); exo-beta-glucosaminidase (EC 3.2.1.165); exo-beta-1,4-galactanase (EC 3.2.1.-); beta-1,3-galactosidase (EC 3.2.1.-)	Glycoside Hydrolases	Beta-galactan (pectic galactan) Oligo Cleavage	6
GH18	GH18 chitinase (EC 3.2.1.14); lysozyme (EC 3.2.1.17); endo-beta-N-acetylglucosaminidase (EC 3.2.1.96); peptidoglycan hydrolase with endo-beta-N-acetylglucosaminidase specificity (EC 3.2.1.-); Nod factor hydrolase (EC 3.2.1.-); xylanase inhibitor; concanavalin B; narbonin	Glycoside Hydrolases	Chitin Backbone Cleavage	7
GH23	GH23 lysozyme type G (EC 3.2.1.17); peptidoglycan lyase (EC 4.2.2.n1) also known in the literature as peptidoglycan lytic transglycosylase; chitinase (EC 3.2.1.14)	Glycoside Hydrolases	Chitin Backbone Cleavage	6
CE4	CE4 acetyl xylan esterase (EC 3.1.1.72); chitin deacetylase (EC 3.5.1.41); chitooligosaccharide deacetylase (EC 3.5.1.-); peptidoglycan GlcNAc deacetylase (EC 3.5.1.-); peptidoglycan N-acetylmuramic acid deacetylase (EC 3.5.1.-).	Carbohydrate Esterases	Chitin Oligo Cleavage	26
GH20	GH20 beta-hexosaminidase (EC 3.2.1.52); lacto-N-biosidase (EC 3.2.1.140); beta-1,6-N-acetylglucosaminidase (EC 3.2.1.-); beta-6-SO3-N-acetylglucosaminidase (EC 3.2.1.-)	Glycoside Hydrolases	Chitin Oligo Cleavage	8
AA10	AA10 (formerly CBM33) proteins are copper-dependent lytic polysaccharide monooxygenases (LPMOs); some proteins have been shown to act on chitin, others on cellulose; lytic cellulose monooxygenase (C1-hydroxylating) (EC 1.14.99.54); lytic cellulose monooxygenase (C4-dehydrogenating)(EC 1.14.99.56); lytic chitin monooxygenase (EC 1.14.99.53)	Auxiliary Activities	Crystalline Cellulose Backbone Cleavage, Crystalline Cellulose Backbone Cleavage, Chitin Oligo Cleavage	6
GH48	GH48 reducing end-acting cellobiohydrolase (EC 3.2.1.176); endo-beta-1,4-glucanase (EC 3.2.1.4); chitinase (EC 3.2.1.14)	Glycoside Hydrolases	Crystalline Cellulose Backbone Cleavage, Xyloglucan Backbone Cleavage (Hemicellulose)	1
GH29	GH29 alpha-L-fucosidase (EC 3.2.1.51); alpha-1,3/1,4-L-fucosidase (EC 3.2.1.111)	Glycoside Hydrolases	Fucose Oligo Cleavage	2
GH158	GH158 endo-beta-1,3-glucanase (EC 3.2.1.39)	Glycoside Hydrolases	Mixed-Linkage glucans Backbone Cleavage (Hemicellulose)	1
GH64	GH64 beta-1,3-glucanase (EC 3.2.1.39)	Glycoside Hydrolases	Mixed-Linkage glucans Backbone Cleavage (Hemicellulose)	2
GH55	GH55 exo-beta-1,3-glucanase (EC 3.2.1.58); endo-beta-1,3-glucanase (EC 3.2.1.39)	Glycoside Hydrolases	Mixed-Linkage glucans Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Oligo Cleavage (Hemicellulose)	3
GH4	GH4 maltose-6-phosphate glucosidase (EC 3.2.1.122); alpha-glucosidase (EC 3.2.1.20); alpha-galactosidase (EC 3.2.1.22); 6-phospho-beta-glucosidase (EC 3.2.1.86); alpha-glucuronidase (EC 3.2.1.139); alpha-galacturonase (EC 3.2.1.67); palatinase (EC 3.2.1.-)	Glycoside Hydrolases	Pectin Oligo Cleavage, Alpha-galactans Oligo Cleavage	4

Supplemental Table 3-5. (Continued)

AA1	AA1 Laccase / p-diphenol:oxygen oxidoreductase / ferroxidase (EC 1.10.3.2); ; ferroxidase (EC 1.10.3.-); Laccase-like multicopper oxidase (EC 1.10.3.-)	Auxiliary Activities	Polyphenolics Cleavage	1
AA2	AA2 manganese peroxidase (EC 1.11.1.13); versatile peroxidase (EC 1.11.1.16); lignin peroxidase (EC 1.11.1.14); peroxidase (EC 1.11.1.-)	Auxiliary Activities	Polyphenolics Cleavage	4
AA4	AA4 vanillyl-alcohol oxidase (EC 1.1.3.38)	Auxiliary Activities	Polyphenolics Cleavage	2
GH13	GH13 alpha-amylase (EC 3.2.1.1); pullulanase (EC 3.2.1.41); cyclomaltodextrin glucanotransferase (EC 2.4.1.19); cyclomaltodextrinase (EC 3.2.1.54); trehalose-6-phosphate hydrolase (EC 3.2.1.93); oligo-alpha-glucosidase (EC 3.2.1.10); maltogenic amylase (EC 3.2.1.133); neopullulanase (EC 3.2.1.135); alpha-glucosidase (EC 3.2.1.20); maltotetraose-forming alpha-amylase (EC 3.2.1.60); isoamylase (EC 3.2.1.68); glucodextranase (EC 3.2.1.70); maltohexaose-forming alpha-amylase (EC 3.2.1.98); maltotriose-forming alpha-amylase (EC 3.2.1.116); branching enzyme (EC 2.4.1.18); trehalose synthase (EC 5.4.99.16); 4-alpha-glucanotransferase (EC 2.4.1.25); maltopentaose-forming alpha-amylase (EC 3.2.1.-) ; amylosucrase (EC 2.4.1.4) ; sucrose phosphorylase (EC 2.4.1.7); malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141); isomaltulose synthase (EC 5.4.99.11); malto-oligosyltrehalose synthase (EC 5.4.99.15); amylo-alpha-1,6-glucosidase (EC 3.2.1.33); alpha-1,4-glucan: phosphate alpha-maltosyltransferase (EC 2.4.99.16); 6'-P-sucrose phosphorylase (EC 2.4.1.-); amino acid transporter	Glycoside Hydrolases	Starch Backbone Cleavage	30
GH15	GH15 glucoamylase (EC 3.2.1.3); glucodextranase (EC 3.2.1.70); alpha,alpha-trehalase (EC 3.2.1.28); dextran dextrinase (EC 2.4.1.2)	Glycoside Hydrolases	Starch Oligo Cleavage	8
PL8	PL8 hyaluronate lyase (EC 4.2.2.1); chondroitin AC lyase (EC 4.2.2.5); xanthan lyase (EC 4.2.2.12); chondroitin ABC lyase (EC 4.2.2.20)	Polysaccharide Lyases	Sulf-Polysachharides Backbone Cleavage	4
GH11	GH11 endo-beta-1,4-xylanase (EC 3.2.1.8); endo-beta-1,3-xylanase (EC 3.2.1.32)	Glycoside Hydrolases	Xylan Backbone Cleavage (Hemicellulose)	2
GH30	GH30 endo-beta-1,4-xylanase (EC 3.2.1.8); beta-glucosidase (3.2.1.21); beta-glucuronidase (EC 3.2.1.31); beta-xylosidase (EC 3.2.1.37); beta-fucosidase (EC 3.2.1.38); glucosylceramidase (EC 3.2.1.45); beta-1,6-glucanase (EC 3.2.1.75); glucuronoarabinoxylan endo-beta-1,4-xylanase (EC 3.2.1.136); endo-beta-1,6-galactanase (EC:3.2.1.164); [reducing end] beta-xylosidase (EC 3.2.1.-)	Glycoside Hydrolases	Xylan Backbone Cleavage (Hemicellulose), Mixed-Linkage glucans Backbone Cleavage (Hemicellulose)	1
GH115	GH115 xylan alpha-1,2-glucuronidase (3.2.1.131); alpha-(4-O-methyl)-glucuronidase (3.2.1.-)	Glycoside Hydrolases	Xylan Oligo Cleavage (Hemicellulose)	2
GH43	GH43 beta-xylosidase (EC 3.2.1.37); alpha-L-arabinofuranosidase (EC 3.2.1.55); xylanase (EC 3.2. 1.8); alpha-1,2-L-arabinofuranosidase (EC 3.2.1.-); exo-alpha-1,5-L-arabinofuranosidase (EC 3.2.1.-); [inverting] exo-alpha-1,5-L-arabinanase (EC 3.2.1.-); beta-1,3-xylosidase (EC 3.2.1.-); [inverting] exo-alpha-1,5-L-arabinanase (EC 3.2.1.-); [inverting] endo-alpha-1,5-L-arabinanase (EC 3.2.1.99); exo-beta -1,3-galactanase (EC 3.2.1.145); beta-D-galacto furanosidase (EC 3.2.1.146)	Glycoside Hydrolases	Xyloglucan Oligo Cleavage (Hemicellulose), Arabinan Backbone Cleavage, Arabinan Oligo Cleavage	4

Supplemental Table 3-5. (Continued)

GH31	GH31 alpha-glucosidase (EC 3.2.1.20); alpha-galactosidase (EC 3.2.1.22); alpha-mannosidase (EC 3.2.1.24); alpha-1,3-glucosidase (EC 3.2.1.84); sucrase-isomaltase (EC 3.2.1.48) (EC 3.2.1.10); alpha-xylosidase (EC 3.2.1.177); alpha-glucan lyase (EC 4.2.2.13); isomaltosyltransferase (EC 2.4.1.-); oligosaccharide alpha-1,4-glucosyltransferase (EC 2.4.1.161); sulfoquinovosidase (EC 3.2.1.-)	Glycoside Hydrolases	Xyloglucan Oligo Cleavage (Hemicellulose), Xylan Oligo Cleavage (Hemicellulose), Alpha-galactans Oligo Cleavage, Alpha-galactans Oligo Cleavage, Mucin Oligo Cleavage	6



Supplemental Figure 3-3. Comparison of identified CAZymes between the two genomes. Figure generated in K-base

CHAPTER 4

Analysis of BioChoice lignin and black liquor utilization by *Streptomyces* sp. 2-6

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Highlights

- Growing *Streptomyces* sp. 2-6 isolated from carpenter bees on BioChoice lignin changes the molecular weight of the lignin fraction
- Organic acids and other biodegradation products are produced from culturing *Streptomyces* sp. 2-6 on BioChoice lignin and black liquor.

Abstract

Lignocellulose is an abundant feedstock for producing fuels and chemicals. These value-added products are generated primarily through the bioconversion of the cellulose and hemicellulose fractions of lignocellulose. The remaining constituent, lignin, is a recalcitrant amorphous compound that is hard to breakdown. In addition, industrial processes such as wood pulping for paper production use lignocellulose as raw material and generate side streams (black liquor) that are rich in lignin. Microbes isolated from lignocellulose-rich environments and that are screened for lignocellulose deconstruction are potentially good options for development as biocatalysts for lignocellulose degradation. Here the utilization of lignin, lignin from black liquor, and black liquor by *Streptomyces* sp. 2-6, an isolate from wood-boring carpenter bees, was assessed. Gel Permeation Chromatography and Gas Chromatography-Mass Spectroscopy were used to examine the changes in molecular weight of lignin, and production of the low molecular weight organic products, respectively, that result from the incubation of *Streptomyces* sp. 2-6 with lignin or black liquor. An increase in the molecular weight of lignin isolated from

cultures inoculated with *Streptomyces* sp. 2-6 was observed for cultures containing either lignin or lignin extracted from black liquor as the carbon source. Compounds of industrial relevance such as the organic acids (acetic, pentanoic, propanoic, and benzoic acids), as well as bio-degradation compounds such as azoxybenzene, phenols, and phthalic acid were identified as products after growth of *Streptomyces* sp. 2-6 on lignin and black liquor. The results presented in this study suggest that *Streptomyces* sp. 2-6 is primarily metabolizing low molecular weight lignin (mono- and oligomers).

Keywords: Black liquor, Lignin, *Streptomyces* spp.

4.1 Introduction

Lignocellulose, composed of cellulose, hemicellulose, and lignin, is the most abundant renewable feedstock on earth and can be used for the production of fuels and commodity chemicals (Barcelos et al. 2020). The most difficult component of lignocellulose to breakdown is lignin owing to its amorphous structure (Liu *et al.*, 2016), and the presence of lignin prevents efficient enzymatic conversion of cellulose and hemicellulose into fermentable sugars (Wu et al. 2022). Therefore, lignin removal processes are needed when using lignocellulose as feedstocks, such as for bioethanol production, which results in large quantities of lignin being produced as low value side streams (Li *et al.*, 2015). Similarly, in the paper industry, lignin (and its breakdown products) constitutes a large portion of a by-product of the pulping process in the form of black liquor (Chandra et al. 2011). In addition to lignin, black liquor contains small quantities of hemicellulose, organic acids, phenolics, resins, fatty acids, inorganic compounds and tannins (Zaied and Bellakhal 2009).

In paper manufacture, the black liquor side stream is processed using a capital-intensive recovery boiler to provide energy as steam and recover chemicals from the pulping process

(Mathews et al. 2015), and for most paper production facilities, the limiting factor in paper pulp production is the recovery boiler capacity (Axelsson et al. 2006). If other uses can be found for the lignin in black liquor, it could help the paper industry by developing value-added processes that could result in the valorization of lignin present in the black liquor side stream.

Microbial treatments could be applied to produce high-value products such as chemicals, surfactants, biopolymers, and pharmaceuticals from industrial streams such as black liquor in a cost effective way (Lad et al. 2022). Recent studies have evaluated the utilization of paper mill sludge for bioconversion into fuels and chemicals (Ouadi et al. 2019; Du et al. 2020; Brown et al. 2021). In particular the gut microbiota of insects that are exposed to wood like termites, beetles, crickets have been explored as sources for lignocellulose-degrading bacteria (Brune 2014; Gales et al. 2018; Mathews et al. 2019). Other experiments specifically targeting insect gut microbiota have investigated making use of whole insect gut microbiomes as an inoculum for the fermentation of lignocellulose biomass in batch bioreactors (Gales et al. 2018).

In the present work, we examined the degradation of lignin and black liquor by the Gram-positive bacterium *Streptomyces* sp. 2-6, an isolate from carpenter bees. In this study, the *Streptomyces* sp. 2-6 isolate was inoculated into minimal media supplemented with BioChoice lignin and black liquor, respectively, as the carbon source. The depolymerization of lignin was assessed using Gel Permeation chromatography. A survey of organic compounds that are potentially produced as a result the metabolism of the targeted strain when it is cultured on lignin or black liquor was developed using Gas chromatography-mass spectrometry (GC-MS).

4.2 Methods

4.2.1. Bacterial cultivation on lignin and black liquor

Spores of *Streptomyces* sp. 2-6 were activated after centrifuging a glycerol stock (TE buffer pH 8.0 and glycerol 50% (w/v), and suspending them in 5 mL of TE buffer pH 8.0, followed by heat shock at 50°C for 10 min. An equal volume of double strength germination medium (1% yeast extract, 1% casamino acids, 1% $CaCl_2$, 0.01M) was added (Kieser et al. 2000). The overnight culture was centrifuged and washed with minimal media. The spore suspension was quantified at $OD_{450\ nm,}$ and the starting culture was calculated to be an initial $OD_{450\ nm}$ of 0.03-0.05 of absorbance. After the spores' activation, growth studies were performed in M63 minimal media at pH 7.0. The M63 minimal media contains, per L, 10.72 g of K_2HPO_4, 5.24 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.5 mL of iron solution (1 mg of $FeSO_4$ dissolved in 1 ml of 0.01 M HCl); 1 ml of thiamine (1 mg/ml), and 5 mL of the SPV-4 with Fe^{III}-citrate (trace elements solution) (Balows et al. 1992; Book et al. 2014). The minimal media was supplemented with 0.2% Biochoice lignin or 10% Black liquor.

4.2.1. Residual lignin quantification

Lignin was solubilized using 5 M NaOH, and cells were removed using a 0.2 μM nylon filter. Lignin was precipitated by acidifying the culture media with 12N HCl. Resulting fractions were placed in pre-weighed conical tubes and centrifuged at 4,700 rpm for 15 min. The conical tubes were placed into an oven at 60°C for 24 h to allow the lignin to dry completely. Solid lignin was then weighed to calculate the lignin reduction and was saved for acetylation for Gel-Permeation Chromatography (GPC).

4.2.3. Lignin extraction from black liquor

The uninoculated and inoculated minimal media supplemented with black liquor was filtered using 0.2 μM PES filters to remove bacterial cells. The lignin was extracted through precipitation as described in (Jardim et al. 2020). The samples were precipitated using HCl (0.1N) that was added until a pH of 2.5 was reached. Samples were centrifuged at 4,400 rpm for 30 min to precipitate the lignin. An additional wash with HCl pH 2.5 was done (3 mM HCl) followed by centrifugation for 30 min at 4,400 rpm. The resulting supernatant was discarded. Samples were washed using acetic acid at pH 3.5 (0.1 mM), followed by a final centrifugation for 30 min at 4,400 rpm. Then 5 ml of water were added to the precipitated lignin, and the samples were frozen overnight or until the freeze-dry step for Gel-Permeation Chromatography.

4.2.4. Gel-Permeation Chromatography.

Dried and acetylated lignin was dissolved in tetrahydrofuran (THF) as per (Glasser *et al.*, 1993). The samples were filter sterilized with a 0.2 μM nylon filter. A Waters GPC instrument equipped with a UV detector (set to 280_{nm}) was used to analyze the molecular weight of the lignin material. The solvent THF was used at a flow rate of 0.7 mL min^{-1} at 35°C. From the samples, 50 μL were injected onto two Styragel linear columns Styragel HR 1 7.8 x 300mm and Styragel HR 5E 7.8 x 300 mm (Waters, MA). The samples were eluted on the columns for 25 min and the absorbance detection was performed at 254 and 280 nm. For calibration, monodispersed polystyrene standards were used (Jiang et al. 2017).

4.2.5. Detection and quantification of low molecular weight lignin with UV-Vis spectrometry.

Acid precipitated lignin was quantified at 205 nm to detect aromatic groups of lignin (You et al. 2016) using a Genesys 50 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). Samples were diluted 1:20 prior to determining the absorbance.

4.2.6. Identification of organic products by GC-MS analysis.

To analyze the samples using GC-MS, the lignin was extracted in a multi-step process. The method used was modified from (Mathews et al. 2013) as described below. Uninoculated and inoculated cultures from black liquor and lignin were centrifuged at 4,700 rpm for 30 min after 400 h of growth, and the supernatant was acidified with 12 N HCl to pH 1-2. The resulting supernatant was mixed with three volumes of ethyl acetate in a separation funnel. Since lignin is a natural emulsifier, water was used to break the emulsion if one developed. The organic layer was collected, and water was removed with Na_2SO_4 by filtering the samples through a Whatman no.1 filter and adding the reagent to the filter and the bottom of the flask. The filtered ethyl acetate was concentrated using a rotovap until a volume of ~5 mL was attained.

The samples were run by injecting 1 mL in an Agilent 7280A GC system coupled with an Agilent 5977E MS and Agilent G4567A ALS autosampler (Santa Clara, CA, USA). The separation was carried out with the HP-5MS UI capillary column (30 m x 0.25 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 65°C for 3 min and increased to 105°C by 11.5 °C min^{-1} and held for 5 min. The transfer line was maintained at 280°C. A solvent delay of 11.5 min was selected. Electron ionization mass spectra were recorded at 30-500 (*m/z*) at electron energy of 70 eV. The resulting

compounds were identified by comparing retention times of purchased standards or data in the NIST library.

4.3. Results and discussion

4.3.1. Changes in lignin molecular weight during growth of *Streptomyces* sp. 2-6 on BioChoice lignin

Changes in the molecular weight of lignin were evaluated using GPC for cultures that were inoculated with *Streptomyces* sp. 2-6 and for uninoculated control cultures. GPC is a widely used technique that enables reproducible separation of polymers based on size (molecular weight) (Mathews et al. 2015). As shown in **Table 4-1**, the lignin dry weight was reduced by 39%, which is an indication that lignin was at least partially broken down or metabolized by *Streptomyces* sp. 2-6. The recovered yield of BioChoice lignin from the uninoculated culture is an average of 80%, while the percentage of the BioChoice lignin recovered from the cultures inoculated with *Streptomyces* sp. 2-6 dropped to 52% on average. These results indicate that *Streptomyces* sp. 2-6 is able to breakdown and/or metabolize some portion of the lignin; however, it should be noted that there is also some loss of lignin during sample processing. To provide confidence that the observed total lignin reduction is the result of microbial metabolism and not sampling process loss, the experiment included three biological replicates, and the reduction in lignin weight for the inoculated sample compared to the uninoculated sample was observed each time for each replicate.

Interestingly the GPC analysis revealed that there is an increase of molecular weight of the recovered lignin from the *Streptomyces* sp. 2-6 inoculated sample (red trace) compared to the lignin recovered from the uninoculated sample (blue trace) and the BioChoice lignin control (green trace, **Figure 4-1**). In gel-permeation chromatography, larger molecules elute earlier

because they sieve around the small pores in the chromatography matrix instead of traveling through the pores as occurs for smaller molecules (Zwilling et al. 2021). The gel-permeation chromatograph (**Figure 4-1**) shows decreased retention time and greater absorbance intensity (higher peaks) for the larger fractions of the lignin recovered from the cultures inoculated with *Streptomyces* sp. 2-6 compared to the lignin extracted from the uninoculated cultures. A comparison of the chromatograms of the lignin recovered from the *Streptomyces* sp. 2-6 inoculated samples and the BioChoice lignin control indicated that lignin masses are similar but that higher amounts of the larger lignin fractions are present in the sample inoculated with *Streptomyces* sp. 2-6 (**Figure 4-1**).

As noted in **Table 4-1**, the number average molecular weight (M_n) is also higher with the bacterial treated lignin, but the polydispersity index is lower than in the uninoculated sample. The increase of molecular weight observed for the recovered lignin could result from a scenario in which the bacteria can initially break down low molecular weight lignin but are then unable to metabolize the larger lignin molecules and the higher molecular weight fraction of the lignin remains behind and is recoverable (Zhao et al. 2016). Alternatively, it is also possible that *Streptomyces* sp. 2-6 promotes the modification or rearrangement of the lignin structure in a way that increases its average molecular weight, an interpretation that can be attributed to the observations of the repolymerization process of laccases in the absence of mediators (Majumdar et al. 2014; Zhao et al. 2016; Chan et al. 2020).

Table 4-1 Lignin weight resulting from *Streptomyces* sp. cultured with 200 mg BCL after 408 h of growth in minimal media.

Lignin sample	Mass (mg)	%Δ	Yield (%)	M_n[a](Da)	M_w[b](Da)	%Δ	M_w/M_n (PDI)[c]
BCL control			-	1228	4244		3.45
Uninoculated	153 ± 13.7		80 ± 6.52	1087	4104	-	3.78
Streptomyces sp. 2-6	93 ± 28.0	-39	52±14.72	1394	4687	+14.20	3.36

[a]M_n is molecular average, [b]M_w is molecular weight, and [c]PDI is polydispersity index. The BCL control refers to a sample processed in the same way as the culture samples for the GPC analysis. The averaged lignin mass (three biological replicates) is provided. The standard deviation of the yield percent is also provided.

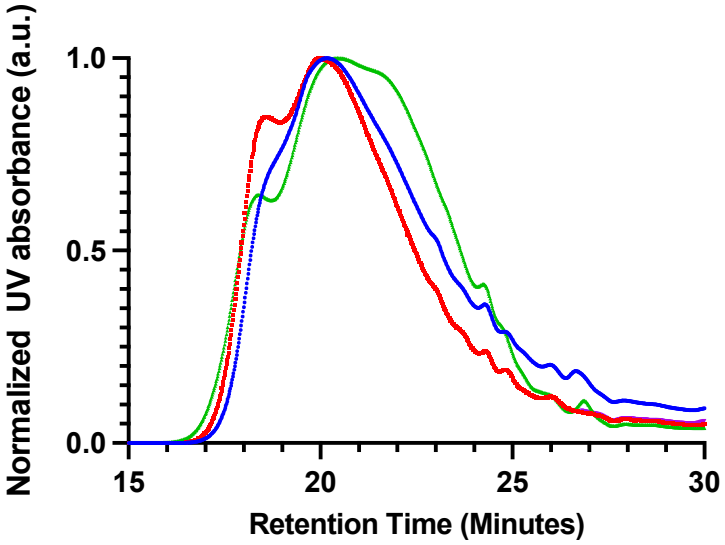

Figure 4-1 Distribution analysis of lignin molecular weight as resolved by GPC. The chromatography spectrum of the lignin recovered from the culture inoculated with *Streptomyces* sp. 2-6 is shown as the red trace. The blue line depicts the spectra for the lignin recovered from the uninoculated sample. The green trace shows the BioChoice lignin control. The absorbance was measured at 280 nm for all the samples. The averaged trace of three biological replicates is shown for each sample type. The molecular weight curves were normalized to 1 as measured by GPC. Lower retention time corresponds to higher molecular weight of the resolved compounds.

Similar to our study findings, Zhao *et al.*, 2016, also found an increase in the molecular weight of the recovered lignin after incubation of lignin with a bacterial biocatalyst, *Rhodococcus opacus* PD630. In their study, five different treatments of lignin were evaluated. The first treatment (I) was lignin without cells, the second treatment (II) was *Rhodococcus opacus* PD630 cells but no lignin, treatment (III) included lignin with the bacteria and the addition of laccase, while treatment (IV) was the lignin with bacteria, laccase, and iron (Fe^{2+}), and treatment (V) consisted of lignin, bacteria, laccase, iron (Fe^{2+}), and hydrogen peroxide (H_2O_2) (Zhao et al. 2016). An increase in molecular weight of the recovered lignin in their study was observed for treatment (III) and (IV) where the laccase was present either with or without iron supplementation, respectively. A recovered lignin M_w of 2.68×10^4 was reported for treatment (III) and 3.30×10^4 for treatment (IV), whereas a M_w of only 1.90×10^4 was reported for treatment (V) which included the addition of the mediator hydrogen peroxide along with the bacteria, laccase and iron. Note that hydrogen peroxide is known to promote the activity of laccase towards depolymerization (Zhao et al. 2016; Perna et al. 2020). For treatment (II) with cells but without the laccase, a slight decrease of the molecular weight of the isolated lignin (a M_w of 7.58×10^3) compared to 8.08×10^3 for treatment (I) without the bacteria was observed, suggesting that *R. opacus* PD630 has limitations in depolymerizing lignin. The authors attributed the observed increase of the lignin molecular weight to two possible reasons: 1) laccase may improve the bacterial metabolism of low molecular lignin, resulting in the disproportionate recovery of higher molecular weight lignin, or 2) laccase may be catalyzing lignin repolymerization in the absence of added mediator (Zhao et al. 2016).

It should be noted that in our study, the difference between the loss in lignin mass, and yield may also occur because of limitations in the column capacity, as larger molecules are

unable to pass through the column and as a result are not shown in the chromatogram. In addition, there is a small amount of lignin that is acid soluble that had been recovered from the samples during processing that would not have been loaded onto another chromatography such as HPLC where carbohydrates can be measured, and proteins can be accounted as part of the sample in the liquid fraction as products (Sluiter et al. 2008) . The only way the acid soluble lignin was analyzed was with UV-Vis spectroscropy. **Table 4-2**, shows the mass of lignin estimated to be in present in the acid treatment step of the lignin precipitation method for the uninoculated samples and those treated with *Streptomyces* sp. 2-6. Unfortunately, at this point in the process, one of the uninoculated samples was lost. Therefore, only standard deviation values are provided for the sample inoculated with bacteria.

Table 4-2. Acid soluble lignin mass assessed using UV-Vis spectroscopy. The averaged results of two biological replicates for the uninoculated samples, and three biological replicates for the samples inoculated with *Streptomyces* sp. 2-6 are presented. Standard deviation is provided for the samples treated with *Streptomyces* sp. 2-6.

Lignin sample	Mass (mg)
Uninoculated	2.69
Streptomyces sp. 2-6	2.17 ± 0.4328

4.3.2. Changes of lignin molecular weight during growth on lignin extracted from black liquor

As shown in **Figure 4-2**, there is little difference observed in the size distribution of the recovered lignin from the *Streptomyces* sp. 2-6 inoculated culture supplemented with black liquor compared to the uninoculated culture. It does appear that there is a slight shift to lower molecular weight lignin compared to the black liquor control for both the inoculated and

uninoculated samples. It should be noted that the composition of black liquor is complex and includes organic acids and sugars in addition to the lignin fraction (Brown et al. 2021) and as such, it is difficult to fully understand the biotic influence of *Streptomyces* sp. 2-6 on the fate of the lignin in black liquor supplemented cultures without being able to account for the effects of the other black liquor constituents.

Similar to what was observed for the GPC analysis of samples of BioChoice lignin incubated with *Streptomyces* sp. 2-6, there is an increase in molecular weight of the lignin extracted from the black liquor samples treated with the biocatalyst (**Table 4-3)**. These results suggest that either preferential metabolism of the lower molecular weight lignin and/or repolymerization of lignin occurred for black liquor-derived lignin treated with *Streptomyces* sp. 2-6 as was seen for the BioChoice lignin experiment (**Table 4-1** and **Figure 4-1**).

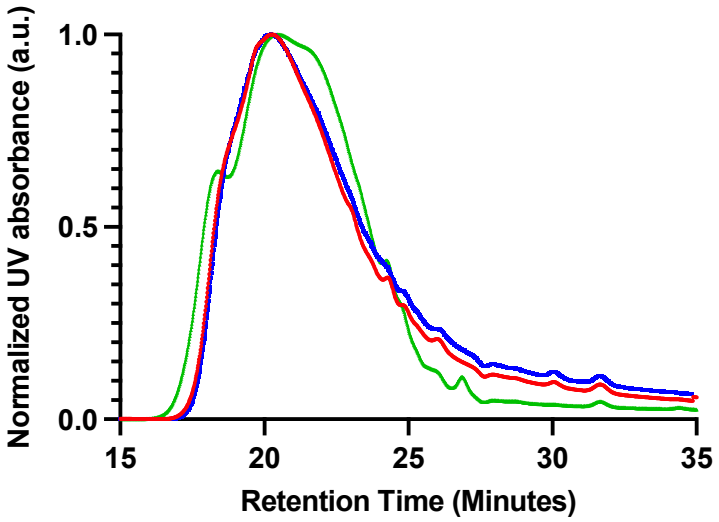

Figure 4-2 Distribution analysis of lignin molecular weight from black liquor samples as resolved by GPC. The chromatography spectrum of the lignin recovered from the culture supplemented with black liquor and inoculated with *Streptomyces* sp. *2-6* is shown as the red trace. The blue line depicts the spectra for the lignin recovered from the uninoculated sample. The green trace shows the black liquor lignin fraction control. The absorbance was measured at 280 nm for all the samples. The averaged trace of three biological replicates is shown for each sample type. The molecular weight curves were normalized to 1 as measured by GPC. Lower retention time corresponds to higher molecular weight of resolved compounds.

Table 4-3 Lignin weight results from *Streptomyces* sp. cultures supplemented with 2mg ml^{-1}

Black liquor sample	M_n (Da)	M_w (Da)	M_w/M_n (PDI)
BCL control	1228	4244	3.45
Uninoculated	983	3251	3.30
Streptomyces sp. 2-6	1082	3519	3.25

BCL control refers to a sample ran along with the others in the GPC for reference as an external control. The average of three samples is shown.

4.3.3 Prediction of lignin degradation potential of *Streptomyces* sp. 2-6 through genome analysis

Several recent studies have combined genomic data and analytical methods such as GC-MS to predict pathways that might be involved in bacterial lignin utilization (Riyadi et al. 2020; Tan et al. 2022). These studies predict that the first steps of lignin degradation are performed by extracellular enzymes that can achieve depolymerization (Janusz et al. 2017). Based on our analysis of the draft genome of *Streptomyces.* sp. 2-6, a list of annotated genes that may encode for extracellular lignin depolymerizng enzymes was identified and is presented in **Table 4-4.** Interestingly, the repertoire of genes putatively encoding for lignin degrading enzymes identified in the genome of *Streptomyces* sp. 2-6 is similar to the suite of genes identified in a recent study describing the lignin degradation potential of *Streptomyces thermocarboxydus* strain DF3-3 (Tan et al. 2022). All of the enzymes presented in **Table 4-4** were also identified in *S. thermocarboxydus* strain DF3-3. These genes are needed to be aligned with query sequences of lignin enzymes from bacterial query sequences to confirm. However, a few gene homologs including those encoding for extradiol dioxygenases, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase, carbonate dehydratases, 2-propenyl-6-methoxyphenol hydroxylase, and a *yfiH*

encoded laccase that were identified in *S. thermocarboxydus* strain DF3-3 have not been identified in the genome of *Streptomyces* sp. 2-6 (Tan et al. 2022).

Table 4-4. Predicted genes from *Streptomyces* sp. 2-6 that could encode for proteins that share homology with known lignin degrading or modifying activities

Gene ID	Gene name	Encoded protein
01701	*ubiD*	UbiD-like decarboxylase
00042, 01996	*ubiX*	Flavin prenyltransferase
04549		Pentachlorophenol 4-monooxygenase
01411	*ahpD*	Alkyl hydroperoxide reductase
02627	*mmcO*	Multicopper oxidase
04966	*dyP*	Dye-decolorizing peroxidase
02462, 02584		Catalase
02650		Catalase-peroxidase
05185	*katE*	Catalase HPII
01651		Phenoxybenzoate dioxygenase subunit beta
04789	*aldH*	Aldehyde dehydrogenase
02078		Pimeloyl-[acyl-carrier protein] methyl ester esterase
02664, 03177, 06011, 06521	*pikC*	Cytochrome P450 monooxygenase
03107		Polyphenol oxidase

These genes might have a role in the initial stages in lignin degradation. For this task enzymes such as multicopper oxidases, dye-decolorizing peroxidases, dehydrogenases, catalases, are expected to participate in this process. However, these enzymes are not fully characterized in many systems (Janusz et al. 2017; Chan et al. 2020).

4.3.4. Identification of products generated from the growth of *Streptomyces* sp. 2-6 in minimal media supplemented with BioChoice lignin and black liquor

Unlike GPC, GC-MS is a useful technique to detect and analyze low molecular weight compounds that are released from lignin (Raj et al. 2007; Mathews et al. 2013; Mathews et al.

2016; Yang et al. 2017; Riyadi et al. 2020) and black liquor (Mathews et al. 2013; Sapapporn et al. 2018; Navas et al. 2021). The products generated from BioChoice lignin and black liquor supplemented cultures are shown in **Table 4-5**, and **Table 4-6**, respectively. Representative chromatograms for Biochoice lignin and black liquor control and bacteria treated samples are shown in **Supplemental Figure 4-4,** and **4-5**, respectively.

The presence or absence of compounds in the inoculated versus uninoculated samples could indicate generation of a lignin-derived catabolite or depletion of a lignin-derived metabolite by *Streptomyces* sp. 2-6, respectively, when it is grown on BioChoice lignin or black liquor. Compounds known to be involved in microbial lignin metabolism that are shown in **Table 4-5** and **Table 4-6** will be discussed in turn below, as are compounds that were detected either only in the inoculated samples or only in the uninoculated samples.

As shown in previous studies, some microorganisms are able to grow on and degrade the lignin-derived compound acetovanillone (Navas et al. 2021; Dexter et al. 2022). The oxidation of lignin can produce major aromatic monomers such as aromatic aldehydes (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde), aromatic acids (vanillic acid, syringic acid, and *p*-hydroxybenzoic acid), acetovanillone, acetosyringone, syringyl-type acetophenone derivative, and 4, hydroxyacetophenone (Zhu et al. 2020; Higuchi et al. 2022).

In this study, acetovanillone (24.374 min) is detected in both the inoculated and uninoculated black liquor samples (**Table 4-5**) but not in the BioChoice lignin supplemented cultures (**Table 4-4**). However, the acetovanillone degradation product, vanillin (21.933 min), is seen for the inoculated and uninoculated black liquor and Biochoice lignin samples. The catabolism of acetovanillone has been investigated for the bacterium *Sphingobium* sp. strain SYK-6 (Higuchi et al. 2022); however, the products of that they observed in their study *(cis, cis-*

muconic acid, phosphorylated and dephosphorylated acetovanillone/acetosyringone, vanilloyil acetic acid/3-(4-hydroxy-3,5-dimethoxyphenyl)-3-oxopropanoic acid) were not detected in this study, suggesting that *Streptomyces.* sp. 2-6 is not using this compound in the same way that the *Sphingobium* sp. strain SYK-6 is.

Another bacterium that was reported to grow on acetovanillone is *Rhodococcus rhodochrous* GD02, and analysis of its genome sequence provided insights about its potential to encode for proteins involved in acetovanillone catabolism (Dexter et al. 2022). It is suggested that *Rhodococcus* is able to produce aromatic compounds that could be further used for the production of acrylamide (Hughes et al. 1998; Yam et al. 2010). It is also known that this bacterium can catabolize vanillate via protocatechuate using vanillate *O*-demethylase; vanillin via vanillate, with a vanillin dehydrogenase; and guaiacol via catechol, using *O*-demethylase. *Streptomyces* sp. S6, a bacterium shown to reduce the molecular weight of kraft lignin, was also reported to catabolize lignin-derived acetovanillone similar to *R. rhodochrous* GD02 (Riyadi et al. 2020). *Streptomyces.* sp. 2-6 does not appear to possess the enzymes for acetovanillone and vanillin metabolism encoded it its genome, and this is a likely explanation for why the depletion of vanillin is not observed.

Metabolic pathways analysis of *Streptomyces* sp. S6 and *S. thermocarboxydus* strain DF3-3 performed with the RAST subsystem and KEGG revealed genes responsible for the catechol branch of the ß-ketoadipate pathway, homogentisate pathways as well as salicylate and gentisate catabolism, which are pathways that interconnect lignin-derived compound degradation and central carbon metabolism (Riyadi et al. 2020; Tan et al. 2022). A similar analysis was performed for *Streptomyces* sp. 2-6 and genes encoding for 1) the metabolism of central aromatic intermediates (β-ketoadipate pathway), 2) resorcinol metabolism, 3) anthranilate degradation, 4)

homogentisate catabolism, 5) phenylacetate-CoA metabolism, 6) 2,3-dihydroxyphenylpropionate catabolism, and 7) additional putative genes involved in lignin degradation were identified and presented in **Supplemental Table 4-1** to **Table 4-7**, respectively.

Catechol and protocatechuate, and guaiacol and its derivatives are known lignin depolymerization products (Pérez-Pantoja et al. 2008; Hogancamp et al. 2018) that are generated through the β-ketoadipate pathway (Patrauchan et al. 2005). In this study, propenylguaiacol (23.4 min) was detected as a product in both the BCL and BL samples that had been treated with *Streptomyces* sp. 2-6 (**Table 4-5** and **Table 4-6**). Other black liquor utilization studies have been conducted to detect bacterial depletion of guaiacol and guaiacol-derivatives (guaiacol, 4-propanol guaiacol, 4-ethanol guaiacol) (Navas et al. 2021).

Metabolic routes that could be associated with the utilization or production of guaiacol are presented in **Supplemental Table 4-1.** It should be noted that *Streptomyces* sp. 2-6 only has two enzymes encoding for protocatechuate 3,4-dioxygenases that could metabolize intermediates of lignin through the β-ketoadipate pathway. However, this is similar to what was found in another *Streptomyces* sp., *S. thermocarboxydus* strain DF3-3, that was demonstrated to degrade lignin into low molecular weight compounds (Tan et al. 2022).

In Riyadi et al, 2020, the authors indicate that to achieve lignin depolymerization, the first step involves monomer production by breaking the bonds that connect phenyl propane units. Interestingly, there is a propane compound, 1-(4-methylbenzoyl)-2,3-diphenyl-cyclopropane (23.728 min), that was identified in the BCL control that was absent in the sample treated with *Streptomyces* sp. 2-6, which may be indicative of a lignin depolymerization process. Furthermore, the production of phenols such as phenol, 4-(1,1,3,3-tetramethylbutyl)- (20.759 min), Phenol 4,4’ (1-methylethylidene) bis (26.79 min) and 2-methoxy-4-(1-propenyl)-2 phenol

(4-propylguaiacol) (23.400 min) (**Table 4-5**) for the BioChoice lignin samples treated with *Streptomyces* sp. 2-6 is also commonly seen for microbial lignin depolymerization studies (Tan et al. 2022).

A benzoic acid compound, 2-(methylthio)-benzoic acid (24.374) was identified in the uninoculated sample of BCL but was absent in the *Streptomyces* sp. 2-6 inoculated sample, which suggests that it may have been used as carbon or energy source in cell production. Benzoic acid compounds can be produced by the steps of depolymerization of aromatic organic compounds that form additional smaller benzene compounds (Chen et al. 2012). In Tan et al., associated compounds such as 4-hydroxybenzoic acid was identified. Benzoic acid can also be utilized for the production of intermediate metabolites like acetyl-CoA, succinyl-CoA, and pyruvate in order to enter the TCA cycle (Fuchs et al. 2011). The depletion of benzoic acid was also observed in (Sapapporn et al. 2018).

The propionic acid (20.764 min) was detected in the bacterial treated BCL and BL samples. It is known that benzoic acid can enter the β-ketoadipate pathway or be converted to 4-hydroxy-2-oxovalerate for further degradation (Tan et al. 2022). The enzymes associated with this pathway can be found in the **Supplemental Table 4-1**, **Supplemental Figures 4-2,4-3**) and the majority are present in the both the *Streptomyces* sp. 2-6 and *S. thermocarboxydus* strain DF3-3 genomes, with the exception that *S. thermocarboxydus* strain DF3-3 possesses enzymes such as vanillate O-demetylase and dioxygenases that are not present in *Streptomyces* sp. 2-6. This might be an explain the differences observed in the products generated when the two strains were incubated with lignin.

The production of propionic acid can be achieved by acetate kinases (Genes 01018, 06660) or acetyl-CoA synthetases (Gene 01771, 02430, 05815) and then enter to the TCA cycle.

The process of producing propionic acid has been described in anaerobic bacterial strains such *as Propionilbacteria acidipropionici, P. shermanii, P.freudenreichii, Clostridia propionicum, Bacteroides fragilis, B. ruminicola, Veillonella parvula, V. alcalescens, Propionigenum modestum, Selenomonas ruminantium, S. sputigena, Megasphaera elsdenii, Salmonella typhimurium* (Gonzalez-Garcia et al. 2017). This pathway is also found in the genome of *Streptomyces* sp. 2-6 as part of the propanoate pathway **(Supplemental Table 4-3)**, and further exploration could be of interest in identifying a new bacterial route for propionic acid production.

The production of propanoic acid was observed in Black liquor, and it was absent in the control sample. However, the opposite was identified in a study of decolorization of black liquor. The metabolite was only identified, but no further discussion was included. In the same study, a type of pentanoic acid (erythropentanoic acid) was produced as a new metabolite from bacterial growth in black liquor. Here, pentanoic acid was produced in both bacterial treatment conditions (BCL and BL). The production of pentanoic acids by bacteria has been seen from wastewater solids, and has been found as a secondary metabolite produced by other *Streptomyces* spp. (Law et al. 2017).

Diethyl phthalate (27.154 min) and phthalic acid (35.031 min) were shown to be depleted and produced, respectively, (**Table 4-5)** in the Biochoice lignin samples treated with *Streptomyces* sp. S6. Similar compounds belonging to the phthalate family were identified in the *Streptomyces* sp. S6 lignin degradation study (Riyadi et al. 2020); however, these compounds were present in all the samples (uninoculated and inoculated). In the study by Tan et al. 2022, a phthalate derivative was present in the treated sample, which suggests the production of this compound. It is known that dibutyl phthalate, is generated in lignin samples treated with *S.*

thermocarboxydus strain DF3-3 (Tan et al. 2022) and it is a common degradation lignin intermediate (Raj et al. 2007). It can serve as a substrate to produce phthalic acid that can then be converted into syringyl, protocatechin and other phenolic compounds (Xiong et al. 2020).

Interestingly, diethyl phthalate a low molecular weight phthalate produced by bacteria, fungi, and plants and is used industrially as a plasticizer (Thiemann 2021). The process of diethyl phthalate biodegradation in nature is restricted to microbes and has been seen in multiple bacterial genera (Cartwright et al. 2000). It is reported that the hydrolysis of this compound is achieved by esterases (Eaton and Ribbons 1982), enzymes that are annotated in the genome of *Streptomyces* sp. 2-6 as previously discussed in Chapter 3.

In comparing identified products from BioChoice lignin and black liquor (**Tables 4-5** and **Tables 4-6**, respectively), there are fewer identified compounds in the black liquor samples. This may be the result less efficient metabolite extraction for the black liquor samples compared to the lignin samples. However, the more diverse compounds identified in the lignin samples treated with *Streptomyces* sp. 2-6, suggest that these are products generated from bacterial metabolism of primarily the low molecular weight lignin.

Table 4-5. Compounds identified by GC-MS from *Streptomyces* sp. 2-6 cultured in 0.2% BioChoice lignin. Listed compounds have a >40% match confidence to compounds included in the NIST library. Retention time is shown if present in the samples (min).

	Compounds	**Retention Times (min)**	**Control**	***S.* sp. 2-6**
1	Azobenzene	17.231	-	+
2	Benzeneamine	16.646	-	+
3	Malonamic Acid	19.061	+	-
4	Vanillin	21.933	+	+
5	DL-Malic Acid	18.651	-	+
6	Acetic acid	18.97	-	+
7	Phenol, 4-(1,1,3,3-tetramethylbutyl)-	20.759	-	+
8	2,3-dimethyl-5-oxohexanethioic acid, 5-t-butyl ester	21.472	+	-
9	L-Glutamine	21.943	-	+
10	2,5-Dimethoxy-4-(methylsulfonyl) amphetamine	22.184	-	+
11	L-leucine	22.251	+	-
12	Azoxybenzene	22.861	-	+
13	2-methoxy-4-(1-propenyl)-2 phenol (4-propylguaiacol)	23.400	-	+
14	1-(4-methylbenzoyl)-2,3-diphenyl-cyclopropane	23.728	+	-
15	1-Methyldodecylamine	24.066	+	-
16	2-(methylthio)-benzoic acid	24.374	+	-
17	Cyclobutanol	24.723	+	-
18	Pentanoic acid	25.902	-	+
19	1-adamantanemethylamine α-methyl	25.995	+	-
20	2-Azafluoroene	26.677	-	+
21	Phenol 4,4’ (1-methylethylidene) bis	26.79	-	+
22	Diethyl Phthalate	27.154	+	-
23	2,5-Dihydroacetophenone	27.349	+	-
24	Ala-Gly	28.026	-	+
25	2,6-Diisopropylnaphtalene	29.062	-	+
26	Monopalmitin	29.59	-	+
27	Creatine	33.154	+	-
28	1,3,2-Dioxarsenane, 2-butyl-	33.154	-	+
29	1,3-Dimethyl-5,7-di-n-propyladamantane	33.933	-	+
30	Formamide, *N*,*N*-dimethyl-	34.487	-	+
31	Phthalic acid	35.031	-	+
32	3,3-Dimethyl-4-methylamino-butan-2-one	41.051	+	-
33	1-Pentanol, 4-amino-	41.051	-	+
34	*N*-acetyl-2,5-dimethoxy-4-ethylamphetamine	44.272	+	+
35	Quinomethionate	44.59	+	-

Table 4-6. Compounds identified by GC-MS from *Streptomyces sp.* 2-6 cultured in 10% black liquor. Listed compounds have a >40% match confidence to compounds included in the NIST library. Retention time is shown if present in the samples (min).

	Compounds	Retention Times (min)	Control	*S.* sp. 2-6
1	3-Hydroxyanthranilic acid	13.379	-	+
2	3-[1,1-Dimethyl-2-trimethylsilyloxy ethyl) amino]-2-trimethylsilyloxypropane sulfonic acid, trimethylsilyl ester	19.964	-	+
3	Propanoic acid	20.764	-	+
4	Vanillin	21.933	+	+
5	L-Glutamine	21.943	+	-
6	4-Hydroxyphenylacetylketone	23.082	+	-
7	2-methoxy-4-(1-propenyl)-2 phenol / (4-propenylguaiacol)	23.400	-	+
8	1-Methyldodecylamine	24.046	+	-
9	Acetovanillone (Apocyanin)	24.374	+	+
10	Pentanoic acid	25.072	-	+
11	2-(methylthio) benzoic acid	26.825	-	+
12	Ala-Gly	22.928	+	-
13	Ethylene oxide	28.354	+	-
14	Dodecyl Acrylate	29.39	+	+

4.4 Conclusions

Streptomyces sp. 2-6 is able to use lignin present in black liquor as carbon sources and could be harnessed for development of biotechnological applications to produce value-added products from lignin or lignin-derived compounds. Though by itself, it does not represent a potential candidate for bioconversion of high molecular weight lignin, it does appear to have utility for metabolizing low molecular weight lignin, and further experiments are needed to identify specific lignin fractions that can be effectively metabolized by *Streptomyces* sp. 2-6.

Additional contribution of this study described herein, includes the protocol developed for lignin extraction from of black liquor-derived lignin that could be applied in other research

focused on understanding its utilization by microbial catalysts. Many of studies have focused in understanding growth on black liquor for valorization purposes (Mathews et al. 2015; Brown et al. 2021); however, to understand bacterial lignin utilization from black liquor, alternative methods are required since the yield of the lignin recovered from black liquor can vary from source (Jardim et al. 2020).

The presence of biodegradation products from compounds of environmental concern suggests that this microbe warrants exploration for the degradation xenobiotic compounds. However, toxicity studies should be also added to provide a better picture of the potential risk of any metabolic products generated. Additional analytical techniques such as Nuclear Magnetic Resonance (NMR), and derivatizations for GC-MS, and GC-FID should be applied to future microbial-catalyzed black liquor degradation studies to provide a better understanding of the utilization of alternative carbon sources known to be present in black liquor and their impacts on lignin degradation/metabolism.

ABBREVIATIONS

BCL: BioChoice Lignin

BL: Black liquor

GPC: Gel-Permeation Chromatography

GC-MS: Gas Chromatography – Mass Spectroscopy

THF: Tetrahydrofuran

TIC: Tentatively Identified Compounds

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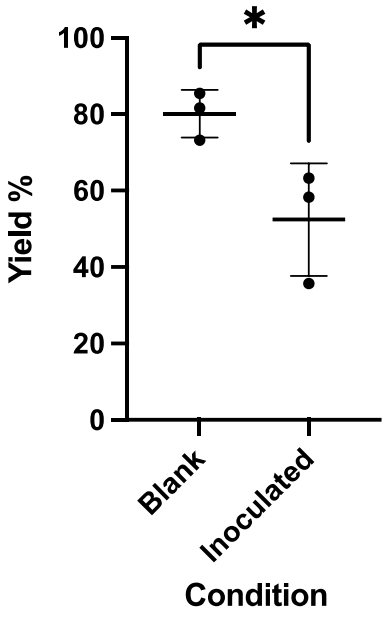


Supplemental Figure 4-1. Yield percent of recovered lignin from minimal media supplemented with Biochoice Lignin (Blank), and the sample post-growth of *Streptomyces.* sp. 2-6 on minimal media supplemented with BioChoice lignin. Paired T-Test nonparametric p-value (<0.05) = (*). Error bars represent the calculated standard deviation.

Supplemental Table 4-1. Putative genes of *Streptomyces* sp. 2-6 responsible for the metabolism of central aromatic intermediates (β-ketoadipate pathway)

Feature ID	Gene name	Encode protein	Closest match description	Species of reference	Accession no.	BLAST identity (%)	KEGG Pathway
000559	*pcaH*	Protocatechuate 3,4-dioxygenase subunit beta		*Streptomyces* sp. SID8499	WP_164394429.1	100	Polycyclic aromatic hydrocarbon degradation
000560	*pcaG*	Protocatechuate 3,4-dioxygenase subunit alpha		*Streptomyces* sp. SID9944	NED72399.1	100	Polycyclic aromatic hydrocarbon degradation
00428	*fadA*	Beta-ketoadipyl-coA thiolase	Thiolase family protein	*Streptomyces* sp. e14	WP_009191696.1	100	Benzoate, Ethylbenzene degradation
00558	*fadA*	Beta-ketoadipyl-coA thiolase	Thiolase family protein	*Streptomyces* sp. e14	WP_009191576.1	99.75	Benzoate, Ethylbenzene degradation
00561	*pcaB*	3-carboxy-cis	3-carboxy-cis, cis-muconate cycloisomerase	*Streptomyces sennicomposti*	MBY8866584.1	97.58	Benzoate degradation
03678	*pcaC*	Hypothetical protein	Carboxymuconolactone decarboxylase family protein	*Streptomyces* sp. SID8499	MYX45871.1	100	Benzoate degradation
04793	*pcaD*	Uncharacterized protein Rv2715	Alpha/beta hydrolase	*Streptomyces*	WP_009193129.1	100	Benzoate degradation
00562	*pcaL*	Putative aminoacrylate hydrolase RutD	3-oxoadipate enol-lactonase	*Streptomyces sennicomposti*	WP_223001447.1	99.21	Benzoate degradation
03496	*pcaL*	Probable nicotinate-nucleotide adenylyltransferase	Nicotinate-nucleotide adenylyltransferase	*Streptomyces*	WP_009188571.1	100	Benzoate degradation
04815	*pcaI*	3-oxoadipate CoA-transferase subunit A	3-oxoacid CoA-transferase subunit A	*Streptomyces*	WP_043262463.1	98.70	Benzoate degradation
04814	*pcaJ*	3-oxoadipate CoA-transferase subunit B	3-oxoacid-CoA-transferase subunit B	*Streptomyces* sp. SID8499	WP_164387730.1	99.09	Benzoate degradation
05080	*pobA*	4-hydroxybenzoate 3-monooxygenase (NAD(P)H)	4-hydroxybenzoate 3-monooxygenase	*Streptomyces* sp. e14	WP_009189015.1	99.49	Benzoate degradation

Supplemental Table 4-2. Putative genes of *Streptomyces sp. 2-6* responsible for pathways for resorcinol metabolism.

Feature ID	Gene name	Encode protein	Closest match description	Species of reference gene	Accession no.	BLAST identity (%)	KEGG Pathway
01633		Aryl-alcohol dehydrogenase	Aryl-alcohol dehydrogenase	*Streptomyces* sp. DvalAA-43	SCE57465.1	83.24	Xylene degradation, Toluene degradation
02641	*xylC*	Benzaldehyde dehydrogenase (NAD)	Benzaldehyde dehydrogenase	*Streptomyces* sp. SID8499	WP_164389149.1	100.00	Xylene degradation, Toluene degradation
00428	*fadA*	Beta-ketoadipyl-coA thiolase	Thiolase family protein	*Streptomyces* sp. e14	WP_009191696.1	100	Benzoate, Ethylbenzene degradation
00558	*fadA*	Beta-ketoadipyl-coA thiolase	Thiolase family protein	*Streptomyces* sp. e14	WP_009191576.1	99.75	Benzoate, Ethylbenzene degradation
00735		Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase	*Streptomyces* sp. SID89	MYX39722.1	100	
01556		Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase family protein	*Streptomyces*	WP_009188741.1	100.00	
01766	DCAA	Acyl-CoA dehydrogenase	Acyl-CoA/acyl-ACP dehydrogenase	*Streptomyces* sp. SID8499	WP_164387712.1	99.74	Caprolactam degradation
01770		Acyl-CoA dehydrogenase	Acyl-CoA/acyl-ACP dehydrogenase	Unclassified *Streptomyces*	WP_009192179.1	100.00	
02261		Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase	*Streptomyces*	WP_009187386.1	100.00	
04891	*gcdH*	Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase family protein	*Streptomyces* sp. SID8499	WP_164392843.1	100.00	Benzoate degradation
05404		Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase family protein	*Streptomyces*	WP_019983921.1	100.00	

Supplemental Table 4-2. (Continued)

06751		Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase family protein	*Streptomyces* sp. e14	WP_009192220.1	100.00	
06854		Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase family protein	*Streptomyces sennicomposti*	WP_223000362.1	100.00	

Supplemental Table 4-3. Genes encoding for anthranilate degradation pathway enzymes in the genome of *Streptomyces* 2-6

Feature ID	Gene name	Encode protein	Closest match description	Species of reference gene	Accession no.	BLAST identity (%)	KEGG Pathway
05715	*abmG*	Benzoate—CoA ligase	AMP-binding protein	*Streptomyces* sp. SID8499	MYX45642.1	99.07	Amonobenzoate degradation
06512	*TDO2, kynA*	Tryptophan 2	Tryptophan 2,3-dioxygenase family protein	*Streptomyces*	WP_009189767.1	100.00	Tryptophan metabolism
06513	*KYNU, kynU*	Kynureninase	Aminotransferase V-fold PLP-dependent enzyme	*Streptomyces* sp. SID8499	WP_239115577.1	96.36	Tryptophan Metabolism
02893	*trpE*	Anthranilate synthase component 1	Anthranilate synthase component I	*Streptomyces* sp. SID8499	NED75210.1	100.00	
03181	*trpD*	Anthranilate phosphoribosyltransferase	Anthranilate phosphoribosyltransferase	*Streptomyces*	WP_164392149.1	100.00	
02889	*trpC*	Indole-3-glycerol phosphate synthase	Indole-3-glycerol phosphate synthase phosphate synthase TrpC	Unclassified *Streptomyces*	WP_027758495.1	100.00	
05380		Tryptophan 2-monooxygenase	NAD(P)/FAD-dependent oxidoreductase	*Streptomyces*	WP_019983900.1	99.65	

Supplemental Table 4-4. Putative genes from *Streptomyces* sp. 2-6 encoding for homogentisate catabolic pathway enzymes

Feature ID	Gene name	Encode protein	Closest match description	Species of reference gene	Accession no.	BLAST identity (%)	KEGG Pathway
04851	*hmgA*	Homogentisate 1,2-dioxygenase		Unclassified *Streptomyces*	WP_164389689.1	100.00	Styrene degradation
04434	*hppD*	4-hydroxyphenylpyruvate dioxygenase		*Streptomyces*	WP_009188861.1	99.48	
03665	*pheT*	Phenylalanine—tRNA ligase alpha subunit		*Streptomyces*	WP_009187702.1	100	
03666	*pheS*	Phenylalanine—tRNA ligase alpha subunit		*Streptomyces sennicomposti*	WP_223000404.1	99.76	
04987	*hisC*	Putative phenylalanine aminotransferase	Histinidol-phosphate transaminase	*Streptomyces* sp. Amel2xE9	WP_019981352.1	100.00	
00079	*fahA*	Hypothetical protein	fumarylacetoacetase	Unclassified *Streptomyces*	WP_164394352.1	100.00	Styrene degradation
01634	*gabD*	Succinate-semialdehyde dehydrogenase [NADP (+)]	Aldehyde dehydrogenase family protein	*Streptomyces* sp. GMY	WP_199293388.1	98.17	
02256	*gabD*	Succinate-semialdehyde dehydrogenase [NADP(+)]1	NADP-dependent succinic semialdehyde dehydrogenase	*Streptomyces sennicomposti*	WP_223000480.1	99.78	
03770	*aroE*	Shikimate dehydrogenase (NADP(+))	Shikimate dehydrogenase	*Streptomyces* sp. GMY02	WP_169438081.1	99.64	
05265	*aroE*	Shikimate dehydrogenase (NADP(+))	Shikimate dehydrogenase	*Streptomyces*	WP_164390999.1	100	
05266*	*aroG*	Phospho-2-dehydro-3-deoxyheptonate aldolase AroG	3-deoxy-7-phosphoheptulonate synthase class II	*Streptomyces*	WP_009191185.1	100	

Supplemental Table 4-5. Putative genes of *Streptomyces* sp. 2-6 responsible for phenylacetate-CoA metabolism

Feature ID	Gene name	Encode protein	Closest match description	Species of reference gene	Accession no.	BLAST identity (%)	KEGG pathway
01807	*paaK*	Phenylacetate-coenzyme A ligase	Phenylacetate---CoA ligase family protein	*Streptomyces viridiviolaceus*	WP_189880117.1	91.96	
03649	*paaK*	Phenylacetate-coenzyme A ligase	Phenylacetate---CoA ligase	*Streptomyces* sp. SID8499	WP_164392265.1	100	
00382	*paaF, echA*	Enoyl-CoA-hydratase	Crotonase/enoyl-CoA hydratase family protein	Unidentified *Streptomyces*	WP_019985488.1	99.61	Benzoate, Aminobenzoate, Caprolactam degradation
00521		Enoyl-CoA-hydratase	Enoyl-CoA hydratase-related protein	*Streptomyces* sp. GMY02	WP_169438586.1	98.80	
00822	*paaF, echA*	Carnityl-CoA dehydratase	Enoyl-CoA hydratase/isomerase family protein	*Streptomyces* sp. SID8499	WP_164389881.1	98.89	Benzoate degradation
00824	*paaF, echA*	Propable enoyl-CoA hydratase echA8	Enoyl-CoA hydratase	*Streptomyces* sp. SID8499	WP_164389878.1	100.00	Benzoate, Aminobenzoate, Caprolactam degradation
01765	*paaF, echA*	Short-chain-enoyl-CoA hydratase	Enoyl-CoA hydratase/isomerase family protein	*Streptomyces* sp. SID8499	WP_164387713.1	100.00	Benzoate, Aminobenzoate, Caprolactam degradation
01983	*paaF, echA*	Enoyl-CoA-hydratase	Enoyl-CoA hydratase-related protein	*Streptomyces* sp. SID9944	NED71270.1	99.59	Benzoate degradation
05716	*paaX*	Transcriptional repressor	PaaX family transcriptional regulator	*Streptomyces*	WP_019984137.1	100	
00731	*paaH, hbd, fadB, mmgB*	3-hydroxybutyryl-CoA dehydrogenase	3-hydroxybutyryl-CoA dehydrogenase	*Streptomyces* sp. e14	EFF89302.1	99.31	Benzoate degradation
02704	*paaH, hbd, fadB, mmgB*	3-hydroxybutyryl-CoA dehydrogenase	3-hydroxybutyryl-CoA dehydrogenase NAD-binding domain-containing protein	*Streptomyces* sp. GMY02	WP_169437345.1	98.96	Benzoate degradation

Supplemental Table 4-5. (Continued)

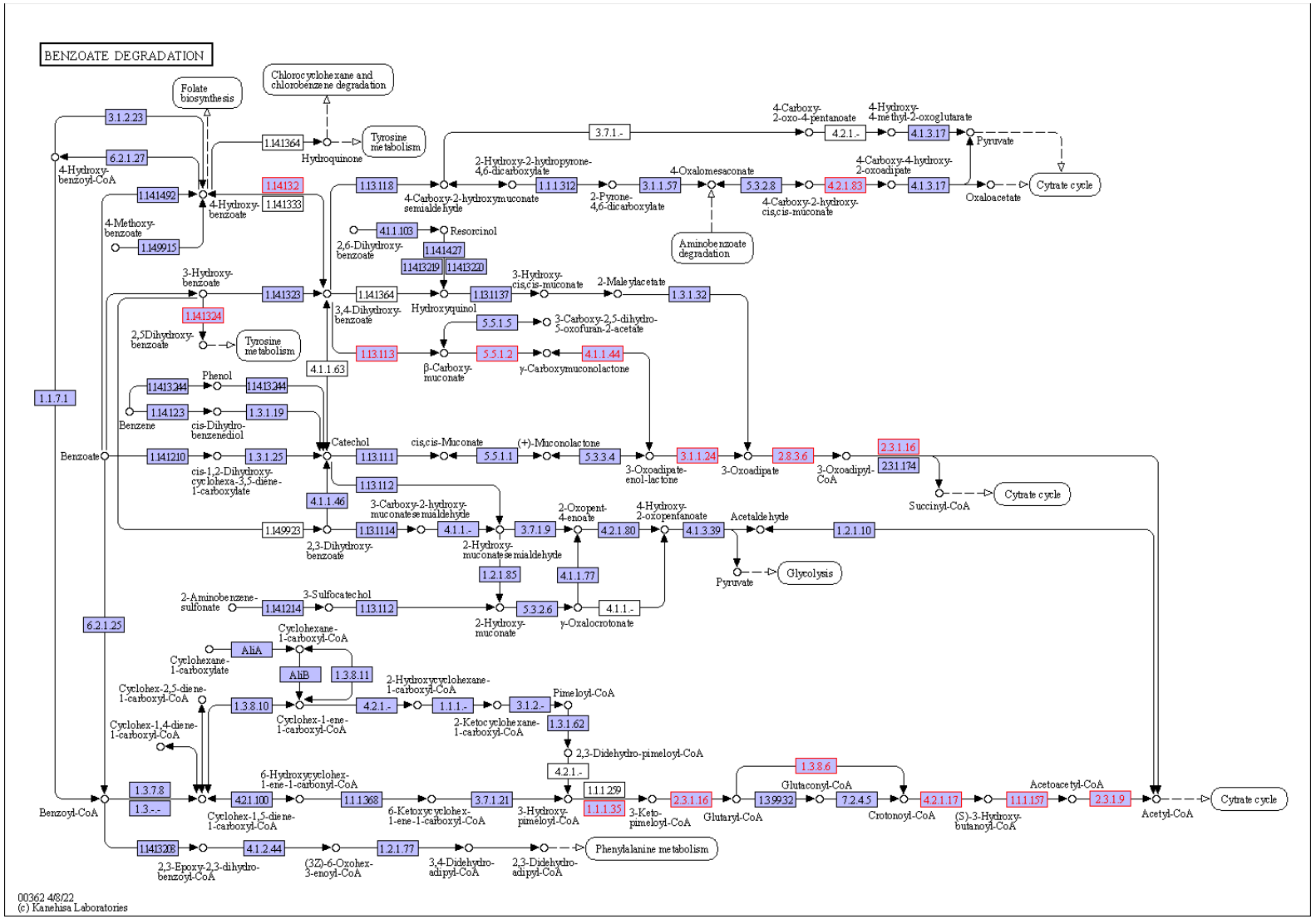
03670	*paaH, hbd, fadB, mmgB*	3-hydroxybutyryl-CoA dehydrogenase	3-hydroxyacyl-CoA dehydrogenase	*Streptomyces* sp. e14	EFF90117.1	100.00	Benzoate degradation
06191	*paaH, hbd, fadB, mmgB*	3-hydroxybutyryl-CoA dehydrogenase	3-hydroxyacyl-CoA dehydrogenase family protein	Unclassified *Streptomyces*	WP_009190651.1	100.00	Benzoate degradation

Supplementary Table 4.6. Putative genes encoded by *Streptomyces* sp. 2-6 involved in catabolic pathways for 2,3-dihydroxyphenylpropionate

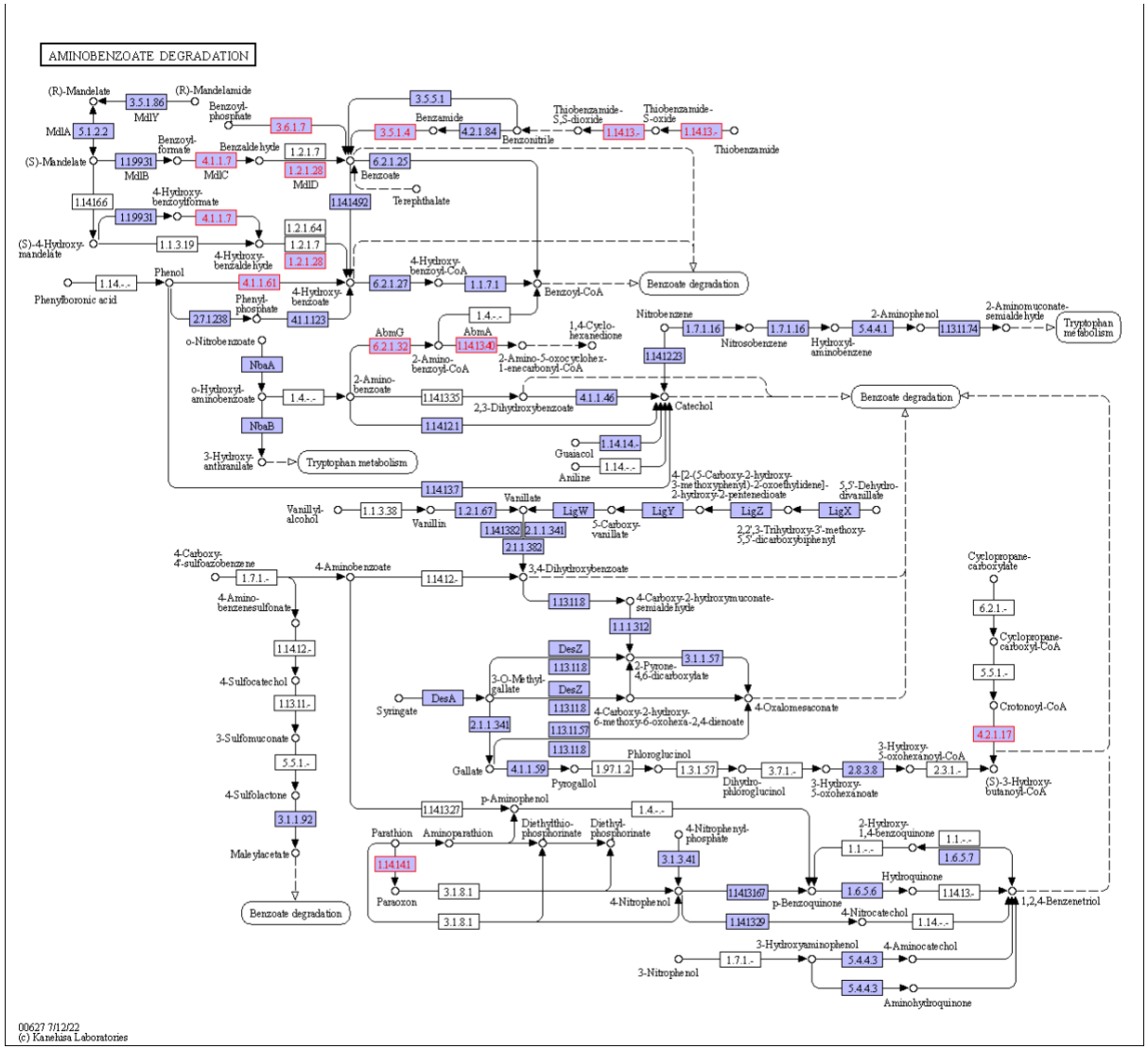
Feature ID	Gene name	Encode protein	Closest match description	Species of reference gene	Accession no.	BLAST identity (%)	KEGG pathway
03379		3-(3-hydroxy-phenyl)propionate	monooxygenase	*Streptomyces* sp. SID8499	NED33878.1	100.00	
05274		3-(3-hydroxy-phenyl)propionate	Bifunctional 3-(3-hydroxy-phenyl/3-hydroxycinnamic acid hydroxylase	*Streptomyces* sp. SID89	MYX44169.1	100.00	
04547		4-hydroxy-2-oxavalerate aldolase	Pyruvate carboxylase	*Streptomyces* sp. SID5998	MYS45061.1	99.91	
04789		Aldehyde dehydrogenase	Aldehyde dehydrogenase family protein	*Streptomyces* sp. e14	WP_009193131.1	99.79	

Supplemental Table 4-7. Additional putative genes involved in lignin degradation suggested by the RAST subsystem: Lignin degradation fragments

Feature ID	Gene name	Encode protein	Closest match description	Species of reference gene	Accession no.	BLAST identity (%)	KEGG pathway
04778	*ligJ, galB*	Hypothetical protein	Amidohydratase/ 4-oxalmesaconate hydratase	*Streptomyces* sp. SID9944 / *Streptomyces* sp. e14	NED73358.1 / EFF88532.1	100.00 / 99.71	Benzoate degradation
fig|6666666.729374.peg.2379		4-carboxy-4-hydroxy-2-oxoadipate aldolase (EC 4.1.3.17)					Benzoate degradation



Supplemental Figure 4-2. Benzoate degradation pathway predicted KEGG Annotation Server. Highlighted genes are the ones identified from *S.* sp. 2-6.



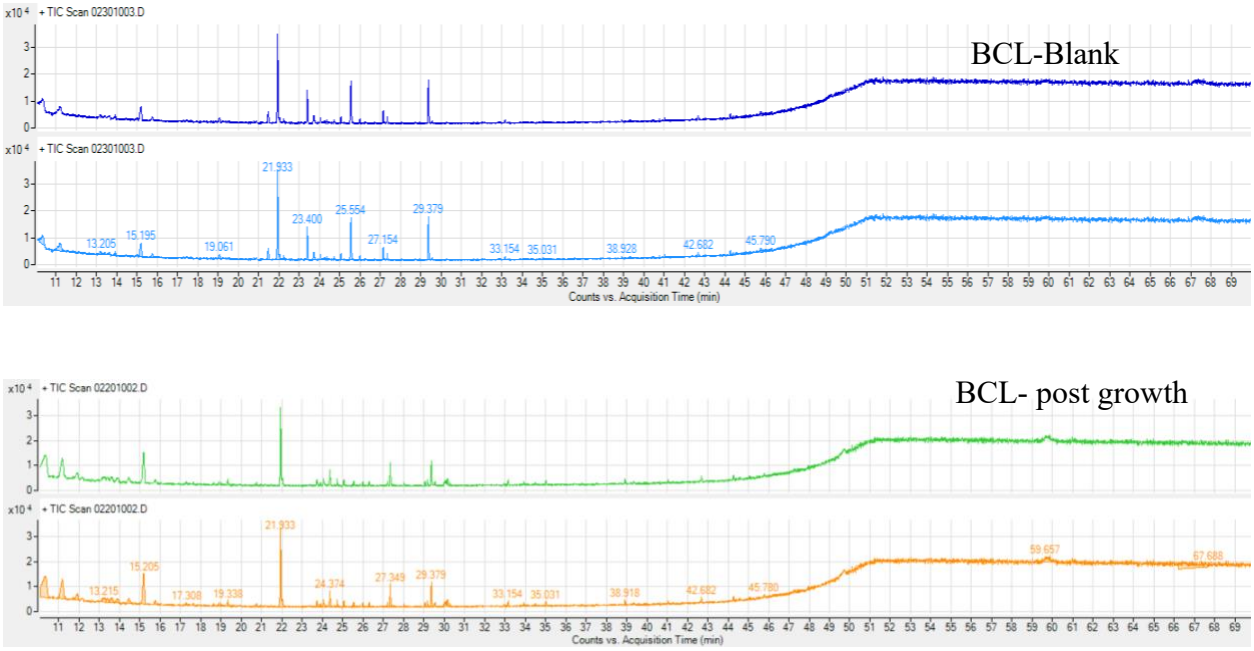
Supplemental Figure 4-3. Aminobenzoate degradation pathway predicted KEGG Annotation Server. Highlighted genes are the ones identified from *S.* sp. 2-6.

Compounds identified with >40% match of confidence compound from NIST library by GC-MS from *Streptomyces sp.* 2-6 with 0.2% BioChoice lignin and 10% Black liquor. Retention time is shown if present in the samples (min)

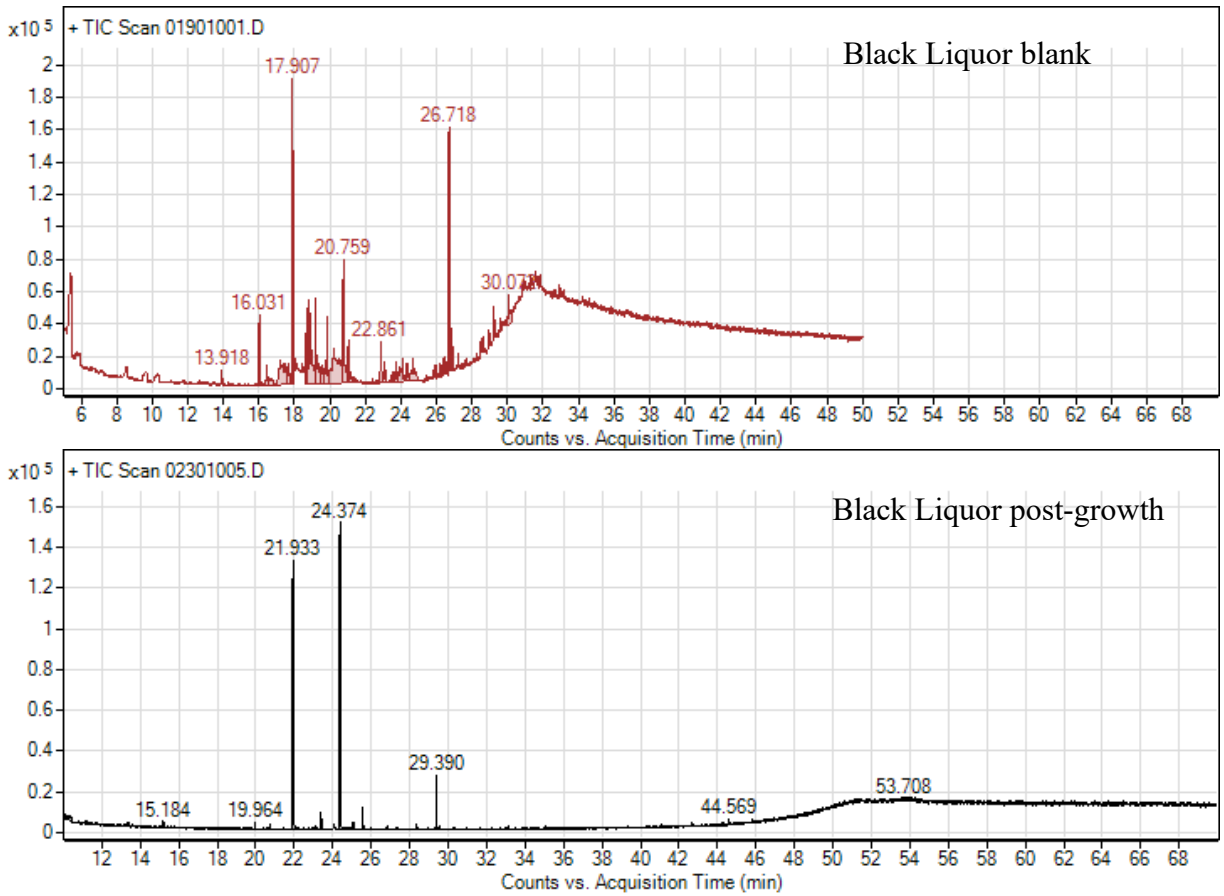
Compounds	Uninoculated (BioChoice Lignin)	Inoculated (BioChoice Lignin)	Uninoculated Black liquor	Inoculated Black liquor
3-Hydroxyanthranilic acid				13.379
3-[1,1-Dimethyl-2-trimethylsilyloxy ethyl) amino]-2-trimethylsilyloxypropane sulfonic acid, trimethylsilyl ester				19.964
Azobenzene		17.231		
Benzeneamine		16.646		
Propanoic acid				20.764
Malonamic Acid	19.061			
Vanillin	21.933	17.907	21.933	21.933
DL-Malic Acid		18.651		
Acetic acid		18.97		
Phenol, 4-(1,1,3,3-tetramethylbutyl)-		20.759		
2,3-dimethyl-5-oxohexanethioic acid, 5-t-butyl ester	21.472			
L-Glutamine		21.943	21.943	
2,5-Dimethoxy-4-(methylsulfonyl) amphetamine		22.184		
L-leucine	22.251			
Azoxybenzene		22.861		
4-Hydroxyphenylacetylketone			23.082	
2-methoxy-4-(1-propenyl)-2 phenol (4-propenylguaiacol)		23.4		23.4
trans- isoeugenol		24.4		
1-4-(methylbenzoyl)-2,3-diphenyl-cyclopropane	23.728			
1-Methyldodecylamine	24.066		24.046	
Apocynin (Acetovanillone)			24.374	24.374
2-(methylthio)-benzoic acid	24.374			
Cyclobutanol	24.723			
Pentanoic acid		25.902		25.072
1-adamantanemethylamine α-methyl	25.995			
2-Azafluoroene		26.677		
Phenol 4,4’ (1-methylethylidene) bis		26.79		

Supplemental Table 4-8. (Continued)

2-(methylthio) benzoic acid				26.825
Diethyl Pthalate	27.154			
2,5-Dihydroacetophenone	27.349			
Ala-Gly		28.026	22.928	
Ethylene oxide			28.354	
2,6-Diisopropylnaphtalene		29.062		
Dodecyl Acrylate			29.39	29.39
Monopalmitin		29.59		
Creatine	33.154	30.292		
1,3,2-Dioxarsenane, 2-butyl-		33.154		
1,3-Dimethyl-5,7-di-n-propyladamantane		33.933		
Formamide, *N*,*N*-dimethyl-		34.487		
2-Fluoro-1,3-dimethyl-1,3,diazaphosphole, 1-thioxide	35.031			
Phthalic acid		35.031		
3,3-Dimethyl-4-methylamino-butan-2-one	41.051			
1-Pentanol, 4-amino-		41.051		
N-acetyl-2,5-dimethoxy-4-ethylamphetamine	44.272			
Quinomethionate	44.59			



Supplemental Figure 4-4. Chromatograms of Tentatively Identified Compounds (TIC) of BioChoice Lignin and minimal media (Top), and BioChoice Lignin and minimal media after the growth of *Streptomyces* sp. 2-6.



Supplemental Figure 4-5. Chromatograms of Tentatively Identified Compounds (TIC) of Black liquor and minimal media (Top), and Black liquor and minimal media after the growth of *Streptomyces* sp. 2-6.

CHAPTER 5

Conclusions

Characterization of Bacterial Isolates from Carpenter Bees for Biodegradation of Lignocellulose Compounds

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5.1 Introduction

This research started with the hypothesis that solitary insects exposed to wood such as carpenter bees could be sources of microbes that can serve as platforms for recalcitrant polymeric deconstruction. For this task, polymers such as black liquor, Biochoice lignin, and dyes were used as substrates for screening these isolates. As it was described in previous chapters, polymers such as dyes and black liquor in the textile and paper industry can be potential hazards in the environment. Therefore, options for treatment and degradation or potential valorization are needed.

5.2. Main findings

The project started with three isolates (1-1, 2-6, and 2-10). These were identified to belong to the *Streptomyces* genus, and further analysis of their potential for degradation suggested *Streptomyces* sp. 2-6 as a good candidate organism for lignin degradation and *Streptomyces* sp. 2-10 as a candidate for cellulose deconstruction. The whole genomes of both strains were sequenced. Putative enzymes involved in the process of lignocellulose degradation were identified. *Streptomyces* sp. 2-6 was selected for experiments exploring lignin depolymerization as well as its ability to metabolize black liquor. GPC-data showed an increase in the recovered lignin molecular weight compared to the control, which suggest that this bacterial strain is mainly metabolizing low molecular weight lignin. GC-MS data was used to identify low molecular weight lignin related compounds or intermediates or products of pathways that could be involved in the lignin degradation. GC-MS analysis was also employed to show how *Streptomyces* sp. 2-6 metabolizes black liquor-derived lignin. Results of this research inform the use of potential microbial catalysts for industrial lignocellulose

bioconversion as an alternative renewable energy strategy and for waste degradation applications.

5.3. Applications

Technological solutions are needed to deal with the environmental and sustainability impacts of rapid urbanization. There is a need to achieve a truly circular economy, by recycling and producing value-added products from waste. Therefore, alternatives in the fields of biodegradation, bioconversion, bioprocessing mechanisms are critical. The genus *Streptomyces* spp. provides a platform for multiple biotechnological applications such as antibiotic production, but they also possess a repertoire of enzymes that could be exploited for bioremediation and bioproduction. These microbes should be further explored for microbial-based waste treatment.

This dissertation provided the following findings:

1) Carpenter bees can be sources of microorganisms with biotechnological potential.
2) *Streptomyces* sp. 2-6 is a novel species of this genus.
3) Methods for extraction of lignin from black liquor can be used for microbial based lignin treatment assessment.
4) *Streptomyces* sp. 2-6 can grow on lignin and black liquor and increase molecular weight of lignin under aerobic conditions.

5.4. Future work

A way to advance a systematic understanding of the underlying microbial metabolic processes could be through the implementation of transcriptomics and in conjunction with the waste compound degradation studies, and this approach could be applied to the analysis of pure

cultures as well as consortia performance. In particular, the following research directions merit further investigation:

1) Recombinant expression and characterization of enzymes from *Streptomyces* sp. 2-6 involved in lignin depolymerization.
2) Gene expression profiles to get insights about the transcriptional machinery involved in lignin degradation
3) Evaluate the effect of mediators in lignin degradation of *S.* sp. 2-6.
4) Quantification of metabolic products generated from the growth of *Streptomyces* sp. 2-6 on black liquor and lignin. This will provide a better understanding about the metabolic pathways and products that could be used for the valorization of these polymers.
5) Investigate the role of *Streptomyces* spp. as potential textile waste degraders.