## ABSTRACT

BROWN, DYLAN M. Statistical Optimization of Black Liquor-containing Media for Growth and Organic Acid Production by *Paenibacillus glucanolyticus* SLM1. (Under the direction of Dr. Joel Pawlak and Dr. Amy Grunden).

The pulp and paper industry is one of the largest global consumers of lignocellulose raw materials. Lignocellulose is plant material composed of cellulose, hemicellulose, and lignin. Wood is one of the most common and widely used lignocellulosic materials. These polysaccharide and phenolic polymer constituents may be hydrolyzed or degraded and used as a feedstock for biological upgrading. Black liquor is one stream that poses a production bottleneck in the pulp and paper industry and contains enough fermentable lignocellulose degradation products for conversion to value-added chemical products. Black liquor is produced during the wood digestion process from Kraft pulping methods. In the Kraft process, wood is digested with sodium hydroxide and sodium sulfide to separate cellulose fibers from hemicellulose and lignin. The resulting extracted lignin and hemicellulose is mixed with spent pulping chemicals, generating black liquor. The black liquor is then sent through a recovery process. The organic materials are burned for energy and the chemicals recovered to feed back into the Kraft process. The bottleneck in paper pulp processing occurs at the recovery boiler, which accounts for a large capital cost of pulping mills, and it is not economically feasible to replace if mills want to increase pulp output. Because of this, alternative forms of processing such as microbial fermentation, can remove some volume of black liquor from the process allowing for increased pulp production and the generation of value-added products that could improve the economics of the mill.

*Paenibacillus glucanolyticus* SLM1 is a bacterial species that has shown the ability to grow on black liquor and lignocellulose components under mildly alkaline conditions. Previous

studies of this organism have demonstrated production of value-added chemicals such as lactic, succinic, gallic, vanillic, and hexanoic acid, among others. This makes it an ideal candidate for black liquor fermentation applications. To extend these findings and develop early stage strategies for improving growth and organic acid production of *P. glucanolyticus* SLM1 on black liquor, statistical optimization approaches were used to formulate optimal aerobic growth media and optimal lactic acid producing media. Findings indicated that growth on black liquor was statistically improved with the addition of high amounts of nutrient (yeast extract) and buffer (potassium phosphate) along with other salts and trace metals. Optimized media for aerobic conditions reduced the lag phase of *P. glucanolyticus* SLM1, increased the generation time, and resulted in higher biomass accumulation. Optimal formulation for anaerobic production of lactic acid also improved lactic acid accumulation over a 14-day time period when compared to un-optimized media. These results present a step towards developing consolidated bioprocesses for upgrading black liquor as an alternative to burning the lignocellulose components.

# Statistical Optimization of Black Liquor-containing Media for Growth and Organic Acid Production by *Paenibacillus glucanolyticus* SLM1

by
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## DEDICATION

To my mother Katherine Brown, who has supported me through my entire education process. To my sister Lauren Brown, who has given positive encouragement during my time in school. To my best friends Jake and Keoni, who have stuck with me through many years of personal growth. To my undergraduate mentor Dr. David Whitten, who provided intensive research guidance, and continues to support my path in academia. Finally, to my Ronald E. McNair Scholars family, who have been a pivotal part in my graduate support system.

## BIOGRAPHY

Dylan M. Brown was born on December 18th, 1994 in Las Vegas, Nevada. He spent a few years of his childhood living in Ashland, Kentucky before moving to Albuquerque, New Mexico where he was raised. Dylan received his BS in Chemical Engineering from the University of New Mexico (UNM) in Albuquerque, NM as the first person in his family to obtain a college degree. Dylan participated in two research positions at the university, one in developmental biology and another studying polyelectrolyte photochemistry and antimicrobial activity. During his undergraduate career he held leadership roles in the American Institute of Chemical Engineers, participated heavily in the Society of Women Engineers, was a Ronald E. McNair Scholar, and helped develop a variety of mentorship programs for other students at UNM. Immediately after graduating, he enrolled in North Carolina State University's College of Natural Resources for a Master of Science Degree in Forest Biomaterials to expand his knowledge of bioprocessing and biotechnology under the guidance of Dr. Amy Grunden and Dr. Joel Pawlak.

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## Literature Review

## Bacterial Valorization of Pulp and Paper Industry Process Streams and Waste


### Abstract

The pulp and paper industry is a major source of lignocellulose containing streams. The components of lignocellulose material are lignin, hemicellulose, and cellulose which may be hydrolyzed into their smaller components and used as feedstocks for valorization efforts. Much of this material is contained in underutilized streams and waste products, such as black liquor, pulp and paper sludge, and wastewater. Bacterial fermentation strategies have suitable potential to upgrade lignocellulosic biomass contained in these streams to value-added chemicals. Bacterial conversion allows for a sustainable and economically feasible approach to valorizing these streams which can bolster and expand applications of the pulp and paper industry. This review discusses the composition of pulp and paper streams, bacterial isolates from process streams that can be used for lignocellulose biotransformations, and technological approaches for improving valorization efforts.


### 1.1 Introduction

Industrial waste streams containing fermentable carbon sources are produced in a variety of processes within the food, agriculture, fuel, pulp and paper, and waste/water treatment industries.(Fava et al. 2015; Alonso et al. 2015). Pulp and paper waste and side streams provide a particularly desirable source of carbohydrates and fermentable compounds because of the large proportion of lignocellulose material contained within these streams. Lignocellulosic biomass, the most abundant renewable resource on earth, is composed of the polysaccharides cellulose and hemicellulose, and the phenolic polymer lignin (Abdel-Hamid et al. 2013). The cellulose and

hemicellulose components of lignocellulose have been the predominant focus of valorization efforts. While lignin is considered detrimental to biological processes, it can be upgraded to value-added phenolic compounds (Alonso et al. 2013; Zeng et al. 2014; Ponnusamy et al. 2019).

Processing of pulp and paper waste and underutilized streams is desired because it can (1) reduce environmental impact of the waste, (2) add value to industrial facilities, and (3) provide a new direction for industries that are undergoing production platform changes (i.e. paper, sugar industries) (Farzad et al. 2017). Along with this, concern about the state of petroleum and fossil fuel derived commodity chemicals and need for alternative energy sources such as biogas and biofuel has sparked interest in processing these streams.

Biological processing is one approach that has been used for adding value to pulp and paper process streams, where lignocellulose materials can be hydrolyzed and processed through various microbial fermentation methods. Historically, the focus of industrial biological lignocellulose valorization and degradation has been dominated by wood rotting fungal studies because of their ability to effectively produce enzymes that break down lignin and polysaccharides (Sánchez 2009; Wan and Li 2012; Cragg et al. 2015). Problems with filamentous fungal degradation include relatively slow growth, loss of polysaccharides during lignin degradation (to cell growth), and use primarily for enzyme production and pretreatment as opposed to direct valorization (Singh and Singh 2014; Sharma and Arora 2015). Here, the use of lignocellulose metabolizing bacterial species may reduce some of the industrial barriers associated with other lignocellulose degrading microbes because of their ability to produce appropriate enzymes, and efficiently convert hydrolysates and degradation products.

## 1.2 Pulp and Paper Waste and Underutilized Stream Overview

Figure 1.1 shows the generalized Kraft pulping process, where wood is digested under alkaline conditions and sent through a series of washing and screening stages to separate brownstock and black liquor. Brownstock is bleached, while black liquor is burned for energy and chemical recovery. Wastewater from the entirety of the process is sent to the wastewater treatment facilities producing paper mill sludge. Many of the streams throughout the process contribute to effluent pollution, containing fibers, dissolved organic solids (DOS), salts, and chlorinated compounds. These streams also contain lignocellulose biomass components of varied compositions and concentrations.

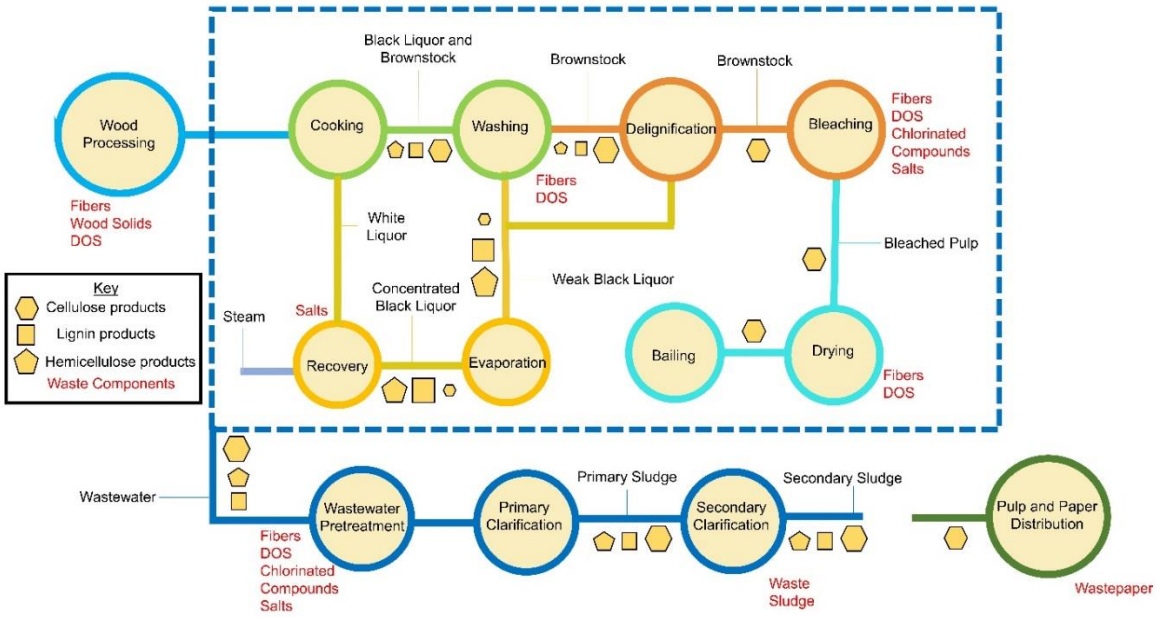


**Figure 1.1**. Schematic of the Kraft pulping process with indication of potential major carbon sources in the relevant streams. Red text indicates the summarized contaminants or waste products generated in major stages of the process. Stream content with the relative proportion of lignocellulose material to their respective stream is symbolized. Adapted from (Mathews et al. 2015; Cabrera 2017).

### 1.2.1 Paper Mill Sludge

Pulp and paper mill sludge (PPMS) is a solid organic waste produced by the pulp and paper industry during waste water treatment.(Guan et al. 2016a; Patrick Fauberta, Simon Barnabéb, Sylvie Boucharda, Richard Côtéa 2016). Paper mill sludge contains a variety of compounds, such as cellulose fibers, lignin, hemicellulose, and a number of fermentable sugars and fermentable sugar precursors. Additionally, sludge may be pelletized and burned for energy, or dried and taken to landfill where it is considered a waste a product. (North Carolina State University. Department of Wood and Paper Science. et al. 2016; Patrick Fauberta, Simon Barnabéb, Sylvie Boucharda, Richard Côtéa 2016). Wastewater from the pulp and paper industry contains many pollutants that are tracked in part by quantifying biochemical and chemical oxygen demand (BOD & COD), suspended solids (SS), toxicity of components, and color (Pokhrel and Viraraghavan 2004). During the wastewater treatment process, water from various areas within a mill is sent through a series of clarification stages which separate sludge from clarified water (Möbius and Helble 2004). During clarification, flocculant flows into an open clarifier where gravity separates sludge and clarified water. Sludge produced in this process is called primary sludge (PMPS). Clarified water is then treated biologically using aerobic or anaerobic treatments. This water is sent to a secondary clarification stage, where secondary pulp mill sludge (PMSS) is sent back to previous stages for retreatment or is processed elsewhere, and clarified water may be further treated for reuse in the process or sent back to a body of water.

The composition of mill sludge is highly dependent on the process and feedstock. A recent report by Soucy *et. al.* studied PMSS:PMPS ratios of 1:9 and 3:7 from thermomechanical (TMP), chemico-thermomechanical (CTMP), and Kraft paper processes and found that sludge from these processes contained 34-42% cellulose, 21-32% lignin, 17-48% ash, along with

extractives and nitrogen sources (Soucy et al. 2016). Other studies show broad ranges of cellulose and related materials between 14-77% (Liu et al.; Jes´ and Ochoa De Alda 2008; Veluchamy and Kalamdhad 2017).

Many management practices look to alternatives to landfilling the product. Currently, there is much research being done on both pyrolysis and carbonization routes to remediate waste and produce activated carbon (Khalili et al. 2000; Reckamp et al. 2014; Mäkelä et al. 2016; Fang et al. 2017). However, many of these studies ae focused on compositional analysis as opposed to valorization efforts. Fermentation provides a secondary route of processing (Gottumukkala et al. 2016). Because of the composition of fermentable sugars in mill sludge, there is much incentive to valorize the material as opposed to waste it. Due to the composition of PPMS and the process by which it is generated, it is a promising waste for both isolating microorganisms that can produce value added compounds as well as carbon source for other microorganisms to generate. One major problem using PPMS as a carbon source is the non-uniform composition, which can cause issues for reproducible and consistent product generation. To combat this in consolidated bioprocessing, it is important to control for feedstock composition in later stage processes.

### 1.2.2 Black Liquor

Black liquor is an underutilized byproduct of the Kraft pulping process used in the pulp and paper industry. In this process, pretreated wood is digested using sodium hydroxide (NaOH) and sodium sulfide ($Na_2S$) at a pH range of 13-14, which creates brown stock and black liquor (Fallavollita et al. 1987; Naqvi et al. 2010). These streams go through a multistage screening and washing process. Brown stock is used in final paper product and is composed predominantly of cellulose fibers which go through a bleaching process to remove residual lignin. Black liquor is removed during washing and sent to evaporation to concentrate the solution from 15 % solids to

65-80 % solids before being burned for energy in the recovery boiler (Cardoso et al. 2009; Bajpai 2018). In the recovery boiler, concentrated black liquor $Na_2S$ and $NaCO_3$ are produced as molten salts and recovered. The combustion of organic material also acts to generate steam which provides enough energy to drive the pulping facility. Modern processes can generate steam around 500 $^oC$ at pressures around 120 bar. The heating value, for an array of lignin containing biomass, ranges from near 0 MJ/kg dissolved solids (kgds) for black liquor solids under 20% to 10 MJ/kgds with concentrated solids nearing 80% (Vakkilainen 2017).

Black liquor is largely composed of sodium salt forms of acetic acid, saccharinic acids, carboxylic acids, hemicelluloses, methanol, lignin, along with a variety of sugars and many other organic components stemming from side reactions of the lignocellulosic biomass (Bajpai 2018). Additionally, many inorganic compounds are released from macromolecular complexes during the production of black liquor, including small amounts of elemental metals such as potassium, magnesium, and calcium. Weak black liquor is made up of approximately 10% organic content and 5% inorganic content on a weight basis (w/w % solids).

Though black liquor is converted to energy and recovered in the pulp and paper industry, the recovery boiler accounts for a high percentage of the capital cost and is not usually replaced because of this (Fallavollita et al. 1987). This provides a bottleneck in the process preventing increased productions due to volumetric limitations from the generated black liquor. The residual organic components in black liquor, like PPMS and SPMS, provide a potential platform for fermentation and a method for overcoming the bottleneck in the pulping process. Here, a percentage of black liquor can be partially treated by microbial fermentation, while generating value-added chemicals, and allowing for increased pulp and paper productions due to additional processing of black liquor that is separate from the recovery boiler. Problems with fermentation

of black liquor are due to cytotoxic components, compositional variability, and alkalinity which limits the set of microorganisms that can grow in media containing it (Kortekaas et al. 1998; Sandberg and Holby 2008). Additionally, neutralization of black liquor causes precipitation, and reaction of the components within the liquor and may affect availability of the fermentable components (Sun et al. 1999; García et al. 2009).

### 1.2.3 Wastepaper

Wastepaper, considered solid municipal waste, is generated by consumers post paper process, where products such as office paper and packaging, amongst others are used and discarded. These include, but are not limited to, old corrugated cardboard (OCC), old newspaper (ONP), high grade de-inked paper, waste office paper (WOP), and waste office cardboard (WOC) (US EPA; Rahman et al. 2014). Wastepaper is predominantly composed of cellulose and may contain small amounts of lignin and hemicellulose, with different residual percentages depending on the type of paper waste.

Often times paper waste is mixed with other house hold wastes including plastics, textiles, food, and electronics amongst others making up municipal solid wastes where paper products may account for 10-37 % of municipal solid waste, which may vary by country depending on waste management policies (Edjabou et al. 2015; Liikanen et al. 2016; Hietala et al. 2018). Many developed countries have specific policies to manage paper product waste where fermentation may account for some of the recyclability while adding value to recycled paper which is currently reused for other paper products. The European Union (EU) condones upwards of 65 % recycling for house-holds, while the United States of America (USA) achieves these rates of recycling with 67 % newspaper and mechanical paper recycling in 2013 (US EPA; Liikanen et al. 2016; Dijkgraaf and Gradus 2017).

Though waste paper, when compared to paper mill sludge and black liquor, is relatively consistent in material composition, especially between paper produced by the same facilities, it also may contain a variety of contaminating chemicals which may act as inhibitors in paper waste fermentation process (Pivnenko et al. 2016). Additionally, hydrolysis of paper waste requires technological advances and increased yield to become economically viable (Brummer et al. 2014). Paper waste hydrolysis and fermentation shows potential as a fermentable feedstock as cheaper technologies to carry out hydrolysis become available, such as advances in separate hydrolysis and fermentation processes, cationic polymer addition, and membrane separation (Guerfali et al. 2015; Rad et al. 2017; Yu et al. 2018).

### 1.2.4 Pulp and Paper Wastewater

Wastewater from the pulp and paper industry is generated at many points during the process and therefore has variable composition depending on the point from which it is released (Ashrafi et al. 2015). Emissions into wastewater during Kraft pulping are generated during wood handling, cooking, washing, screening, white liquor preparation, bleaching, drying and from tanks (Ashrafi et al. 2015; Hubbe et al. 2016). Each of these points introduces different types of contaminants including solids such as fibers, salts, chlorinated compounds, and DOS. The bleaching process contributes to a large amount of effluent. White water, water containing cellulose fibers, is generated during sheet making of the pulp or paper (Hubbe). Wastewater is usually treated in plants and generates PMS, but portions of wastewater, such as whitewater containing high amounts of cellulose fibers, could be potentially removed from the system and fermented.

### 1.3 Bacteria from Pulp and Paper Streams

There are many bacterial species that have been isolated from pulp and paper waste or side streams with the ability to ferment lignocellulosic material and potentially generate value-added chemicals, shown in Table 1.1. Isolates may stem from the environment, but many are obtained from pulp mill effluent streams themselves, including wastewater, sludge, and black liquor. Many of the isolated microorganisms are *Bacillus* sp., *Paenibacillu*s sp., *Klebsellia* sp., *Serratia* sp., and *Citrobacter* sp. These species have been demonstrated to degrade a variety of the pollutants such as COD and AOX compounds, which is a major focus of the studies. However, many studies also describe the formation of value-added chemicals after bacterial treatments.

**Table 1.1**. Bacterial species isolated from pulp and paper streams with value-added products

| Organism | Strain/Isolate Number | Lignocellulosic Substrates | Isolation Source | Value-added Products | Citation |
|---|---|---|---|---|---|
| *Aneurinibacillus aneurinilyticus* | AY856831 | Paper Mill Sludge | Paper Mill Sludge | - | (Chandra et al. 2007) |
| *Bacillus megaterium* | ETLB-1 | Lignin, black liquor | Soil contaminted from paper mill effluent | 4-hydroxy-benzoic acid, 3-hydroxy-4-methoxybenzaldehyde, ferulic acid, and t-cinnamic acid, etc. | (Paliwal et al. 2015) |
| *Bacillus sp.* | - | Lignin, black liquor, low molecular weight aryl compounds | Black liquor contaminated soil, activated sludge, liquid from bagasse heap | butanoic acid, pentanoic acid, propanoic acid, hexanoic acid, acetic acid, heptanoic acid, and butanedioic acid, etc. | (Sapapporn et al. 2019) |
| *Bacillus* sp. | IITRDVM-5 | Pulp Mill Effluent | Pulp Mill Effluent | Lignin degradation products, hydrocarbons | (Sonkar et al. 2019) |
| *Bacillus sp.* | AY952465 | Paper Mill Sludge | Paper Mill Sludge | - | (Chandra et al. 2007) |
| *Brevibacillus agri* | RJH-1 | Synthetic Wastewater | Pulp Mill Sludge | - | (Hooda et al. 2015) |
| *Brevibacillus parabrevis* | *MTCC 12105* | wastewater | Paper mill sludge | - | (Hooda et al. 2018) |

**Table 1.1.** (continued).

| | | | | | |
|---|---|---|---|---|---|
| *Brevundimonas spp.* | NAC1 | activated sludge and wastewater kraft, pulp, and cardboard | Cardboard waste industry | Polyhydoxylalkanoates | (Yang et al. 2010a) |
| *Citrobacter freundii* | FJ581026 | Synthetic dyes, lignin | Lab isolates | Butanoic acid, 2,4,6 tricholorphenol, dibutyl phthalate, hexadecenoic acid, octadecanoic acid,etc. | (Chandra and Abhishek 2011) |
| *Citrobacter freundii* | SU5 | Kraft Lignin | Paper mill sludge | Benzyl benzoate, glyoxylic acid, tetradecanoic acid, 1-Phenanthrene carboxylic acid, phthalates | (Abhishek et al2017) |
| *Citrobacter sp.* | IITRBL4 | Lignin, black liquor, monosaccharides | Century Pulp Mill Sludge | Formic acid hydrazide, 4-cyclohexane-1,2-dicarboxylic acid, carbamic acid, 1,2-benzenedicarboxylic acid, erythropentanoic acid | (Chandra et al. 2011) |
| *Citrobacter sp.* | FJ581023 | Synthetic dyes, lignin | Lab isolates | 2,3,4,5 tetrachlorophenol, pentachlorophenol, tetradecanoic acid, octadecanoic acid, etc. | (Chandra and Abhishek 2011) |
| *Clostridium acetylbutylicum* | ATCC -824tm | Kraft Pulp Mill Sludge | - | Acetone, Butanol, Ethanol (ABE) | (Guan et al. 2016b) |
| *Clostridium tyrobutyricum* | NRRL B-67062/RPT 4213☆ | Pulp Mill Sludge Hydrolyzate | Pulp Mill Sludge | Butyric acid | (Liu et al. 2018) |
| *Cupriavidus necator* | NCIMB 11599 | Waste Office Paper Hydrolyzate | - | poly-3-hydroxybutyrate | (Neelamegam et al. 2018) |
| *Dysgonomonas sp.* | *WJDL-Y1* | Kraft Lignin | Pulp Mill Sludge | Lactic acid, propanoic acid, lignin degradation organic acids, etc | (Duan et al. 2016) |
| *Enterococcus spp.* | NAP11 | activated sludge and wastewater kraft, pulp, and cardboard | Cardboard waste industry | Polyhydoxylalkanoates | (Ko et al. 2007b) |
| *Halomonas sp.* | 19-A, Y2 | guaiacol, vanillin, dibenzo-*p*-dioxin, biphenyl and fluorene | Black Liquor | Lactic acid, formic acid, acetic acid | (Yang et al. 2010a) |
| *Klebsiella pneumonia* | *PS4.2* | Synthetic Dyes, Pulp Mill Sludge, Black liquor, green liquor, white liquor | Pulp Mill Sludge | Polyhydoxylalkanoates | (Ghribi et al. 2016) |

**Table 1.1.** (continued).

| | | | | | |
|---|---|---|---|---|---|
| *Klebsiella pneumoniae* | IITRBL3 | Lignin, black liquor, monosaccharides | Century Pulp Mill Sludge | Formic acid hydrazide, 4-cyclohexane-1,2-dicarboxylic acid, carbamic acid, 1,2-benzenedicarboxylic acid, erythropentanoic acid | (Chandra et al. 2011) |
| *Paenibacillus campinasensis BL 11* | BL11 | Carboxymethylcellulose, xylan, hemicellulose and cellulose monosaccharides | Black Liquor | - | (Ko et al. 2007b) |
| *Paenibacillus glucanolyticus* | SLM1 | Lignin, black liquor, cellulose, hemicellulose | Black liquor | Lactic acid, gallic acid, vanillic acid, hexanoic acid, succinic acid, ethanol, etc. | (Mathews et al. 2014) |
| *Paenibacillus sp.* | AY952466 | Paper Mill Sludge | Paper Mill Sludge | - | (Chandra et al. 2007) |
| *Paenibacillus sp.* | PS12 | Synthetic Dyes, Pulp Mill Sludge, Black liquor, green liquor, white liquor | Pulp Mill Sludge | Polyhydoxylalkanoates | (Ghribi et al. 2016) |
| *Pseudomonas plecoglossicida* | ETLB-3 | Lignin, black liquor | Soil contaminted from paper mill effluent | 4-hydroxy-benzoic acid, 3-hydroxy-4-methoxybenzaldehyde, ferulic acid, and t-cinnamic acid, etc. | (Paliwal et al. 2015) |
| *Raoultella terrigena* | *MS9* | Synthetic Dyes, Pulp Mill Sludge, Black liquor, green liquor, white liquor | Pulp Mill Sludge | Polyhydoxylalkanoates | (Ghribi et al. 2016) |
| *Serratia marcescens* | IITRBL1 | Lignin, black liquor, monosaccharides | Century Pulp Mill Sludge | Formic acid hydrazide, 4-cyclohexane-1,2-dicarboxylic acid, carbamic acid, 1,2-benzenedicarboxylic acid, erythropentanoic acid | (Chandra et al. 2011) |
| *Serratia marcescens* | SU1 | Kraft Lignin | Paper mill sludge | Phenyl acetic acid, propanoic acid, benzyl benzoate, homovanillic acid, phthalates | (Abhishek et al2017) |

### 1.3.1. Bacteria from Pulp Mill Sludge

Pulp mill sludge is primarily treated with microorganisms, and many of the isolated bacterial species used for treatment are either native (in process) or synthetic consortia (in laboratory experiments). Because of this, pulp mill sludge provides a great source for the isolation of lignocellulose degrading bacteria that can be used in other pulp and paper valorization or remediation efforts such as growth on black liquor and waste treatment. Isolates from pulp mill sludge have been demonstrated to produce value added chemicals such as PHA, lactic acid, acetone, butanol, and ethanol from pretreated effluent streams from the pulp and paper process (Bhuwal et al. 2013; Shi et al. 2015a; Guan et al. 2016a; Ferreira et al. 2016). Additionally, many of these studies focus on lignin degradation, which is the major pollutant in pulp mill sludge.

In a study by Hooda *et. al.* sludge samples were placed in minimal salt media enriched with lignin resulting in the isolation of *Brevibacillus agri* strain RJH-1 (Hooda et al. 2015). The strain was further screened for lignin degradation using dye decolorization assays and assessing lignin degradation by growth in bioreactors. In another study by Ghribi *et. al.* microbial diversity of pulp mill sludge samples was assessed for the identification of industrially relevant enzymes (Ghribi et al. 2016). In this study, similar methods were used to isolate and screen for enzymes, where minimal media enriched with pulp mill sludge was used to grow samples. Twenty-four isolates were found to decolorize dyes and three isolates showed lignolytic activity. These isolates were *Paenibacillus sp. PS12*, *Klebsiella pneumonia PS4.2* and *Raoultella terrigena MS9*, which showed laccase, lignin peroxidase, and manganese peroxidase activities. Of isolated bacteria the dominant bacteria were *Bacilli*.

The methods used in these studies are common techniques for isolation. The decolorization of assorted dyes relates to enzyme functions that may break lignin-like bond structures and provides an initial step in determining if necessary enzymes are present. Bacterial species that show decolorization both in growth solutions and on agar plates containing dye can then be further assessed for lignin degradation using minimal salts growth media containing lignin. Studies such as these indicate a trend in using decolorization as a method to test lignin degradation or screen for potential lignin degrading bacteria.

### 1.3.2 Bacteria from Black Liquor

Multiple bacterial strains have been isolated from black liquor with the ability to metabolize many of the organic components including saccharides, polysaccharides, cellulose, hemicellulose, and lignin (Ko et al. 2007a; Yang et al. 2010b; Mathews et al. 2014; Mathews et al. 2016). Additionally, there are also strains that have been isolated elsewhere that can grow on and use carbon sources from black liquor (Yang et al. 2010b; Chandra et al. 2011; Chandra and Abhishek 2011). Because of this, black liquor from pulp and paper mills is an excellent stream to isolate lignocellulose degrading bacteria that can withstand, and produce industrially relevant enzymes that function in, high salt and alkali conditions. Black liquor decolorization is largely used to show degradation of lignocellulosic components, predominantly lignin which provides coloration to the liquor (Yang et al. 2010b; Chandra et al. 2011; Chandra and Abhishek 2011; Maki et al. 2012; Chai et al. 2014). In many of these studies, the decolorization and degradation of lignin components are enhanced with additional carbon sources such as glucose or need to be neutralized due to high pH. This indicates the inability for many bacterial species to remediate lignin when it is present as a sole carbon source.

Decolorization research trends towards indicating the degradation of lignocellulose biomass with few studies focusing on the complex fermentation profiles or using black liquor as a feedstock for value-added commodity chemicals. Fermentation profiles from black liquor show the production of industrially relevant products such as gallic acid, malonic acid, succinic acid, vanillic acid, formic acid, and many more (Mathews et al. 2016). Other studies have developed fermentation system using sugars hydrolyzed and recovered from black liquor as opposed to raw black liquor (Kudahettige-Nilsson et al. 2015). There are currently few efforts attempting to valorize black liquor by direct fermentation of the stream which stem from problems with fermentation inhibitors (lignosulphonates and phenolics) and pH (Kudahettige-Nilsson et al. 2015).

### 1.4 Lignocellulose Catabolism

Lignocellulose catabolism involves pathways for the use of monosaccharides such as xylose, glucose, glucomannans, and galactose, along with polysaccharide metabolic pathways for the breakdown of hemicelluloses and celluloses, and lignin degrading pathways that break down the phenolic subunits of lignin. The initiation of these pathways largely involves the breakdown of larger constituents, cellulose, hemicellulose, and lignin, by extracellular enzymes before transport into the cell can occur, though some intracellular breakdown of oligomers can occur. A list of lignocellulose degrading enzymes is provided in Table 1.2.

**Table 1.2.** Lignocellulose degrading enzymes and their substrates adapted from (Mathews et al. 2015; Li et al. 2019)

| Enzyme | Substrate | Product |
|---|---|---|
| Cellulose | | |
| Endo-1,4-β-glucanase | 1,4-β-Glucose linkages | Cellulose |

**Table 1.2.** (continued).

| | | |
|---|---|---|
| Exo-1,4-β-glucanase (cellobiohydrolase, glucohydrolase) | Terminal 1,4-β-glucose linkages | Cellobiose |
| β-Glucosidase | 1,4-β-Glucose linkages | Glucose |
| Lytic polysaccharide monooxygenases | 1,4-β-Glucose linkages, $2e^-$, $O_2$ | $H_2O$, $H_2O_2$ |
| Hemicellulose | | |
| Endo-1,4-β-xylanase | 1,4-β-Xylose linkages | Hemicellulose |
| β-Xylosidase | 1,4-β-Xylose linkages | Xylose |
| α-Glucuronidase | α-D-Glucan, $H_2O$ | Glucan, alcohol |
| α-L-Arabinofuranosidase | α-L-Arabinose residue | α-L-Arabinose |
| Acetylesterase | Acetic ester, $H_2O$ | Acetate, alcohol |
| Endo-1,4-β-mannanase | 1,4-β-Mannose linkages | Mannans |
| β-Mannosidase | Terminal 1,4-β-mannose linkages | Mannose |
| α-Galactosidase | Terminal α-D-galactose residue | Galactose |
| Lignin | | |
| Lignin peroxidase | Alkyl side chains, C–C, aromatic ring | Aldehydes |
| Manganese-dependent peroxidase | Aromatic structures, $H_2O_2$, $Mn^{2+}$ | Cleave Cα–Cβ bond |
| Laccase | Phenolic compounds, aromatic amines | Cleave C–C bond (vanillin) |
| Horseradish peroxidase | Phenolic compounds, aromatic amines | Cleave C–C bond |
| Protocatechuate 3,4-dioxygenase | 2,4-Dihydroxybenzoate, $O_2$ | 3-Carboxy-*cis*,*cis*-muconate |
| Catechol 1,2 dioxygenase | Catechol | *cis*,*cis*-muconate |
| Superoxide dismutase | $O_2^-$, $H^\cdot$ | $H_2O_2$, $O_2^\cdot$ |
| Aryl alcohol oxidase | Aromatic primary alcohol, $O_2$ | Aromatic primary aldehyde, $H_2O_2$ |

### 1.4.1 Cellulose Catabolism

Cellulose is a homopolymer composed of monomers of glucose polymerized by β-1,4 glycosidic linkages. A schematic of cellulose degradation is given in Figure 1.2. Cellulose is

hydrolyzed extracellularly by systems of secreted enzymes or self-assembled membrane bound enzyme complexes termed cellulosomes, for anaerobic degradation (Bhat and Bhat 1997; Mickae¨ et al. 2000; Artzi et al. 2017). Secreted enzymes are grouped into 3 major classes: β-glucosidases, endo-1,4-β-D-glucanases, and exo-1,4-β-D-glucanases which function on different cellulosic structures (Bhat and Bhat 1997; Gupta et al. 2012). Endogluconases cleave β-1,4 glycosidic linkages along amorphous regions of cellulose chains. Exoglucanases cleave non-reducible ends of crystalline cellulose, creating fibrils and releasing cellobiose. β-glucosidases then hydrolyze cellobiose into glucose monomers that can then be imported into cells (Singhania et al. 2010). Other oligomeric units generated during cellulose hydrolysis, such as cellodextrin and cellobiose, may also be imported by certain species and processed into glucose monomers within the cytosol (Mickae¨ et al. 2000; Desvaux 2008). Glucose hydrolyzed from cellulose can then be used for glycolysis.

Cellulosomes provide an alternative method for anaerobic cellulose degradation. Cellulosomes are rare adaptations and have been identified in a variety of bacterial strains isolated from waste sludges, composts, and mammalian and insect guts (Artzi et al. 2017). The cellulose structure is composed of scaffoldin systems, which are attached to cell membranes via anchoring proteins (Bayer et al. 1998; Doi and Kosugi 2004; Artzi et al. 2017). The scaffoldin proteins are composed of cohesion and dockerin domains which contain catalytic sites for cellulose degrading enzymes. Cellulosomes are most closely associated with plant biomass degrading bacteria. Anaerobic species with specialized cellulosomes may then act as a platform for biotechnological processes for lignocellulose valorization, with recent work seeking to address chemical conversion and waste management from these strains (Bayer et al. 2007; Xu et al. 2010; Moraïs et al. 2012; Osiro et al. 2017).

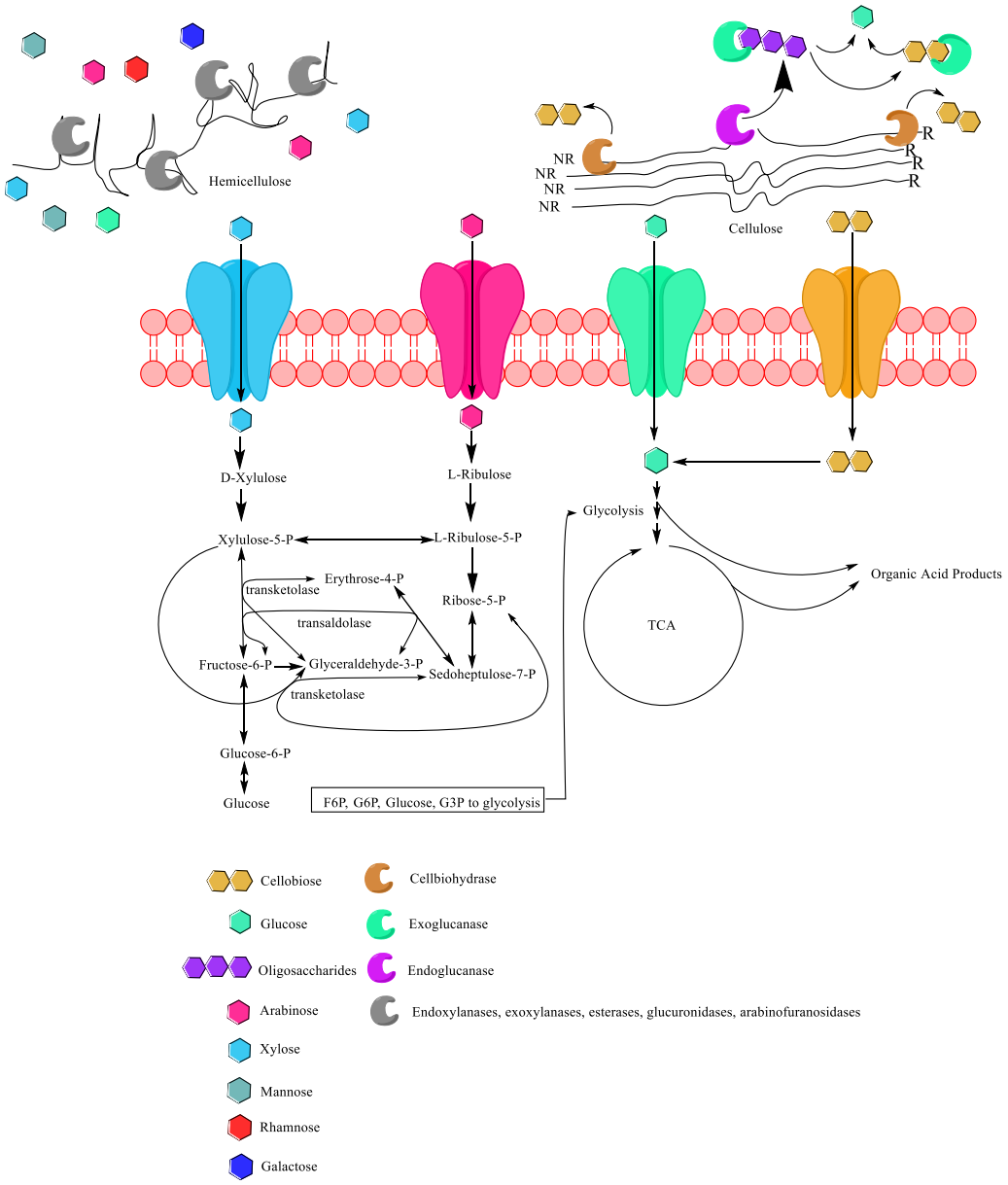


**Figure 1.2.** Simplified schematic of cellulose degradation with funneling into the TCA and hemicellulose degradation with the pentose phosphate pathway for xylose and arabinose metabolism.

### 1.4.2 Hemicellulose Catabolism

Hemicellulose components of lignocellulose have a higher complexity and require a larger set of enzymes to degrade each component. Hemicelluloses can be classed as xyloglucan,

mixed linkage β-glucan, glucuronoarabinoxylan, glucuronoxylan, galactomannan, and glucogalactomannan (Scheller and Ulvskov 2010). Much like in cellulose hydrolysis, hemicellulose must be hydrolyzed into oligosaccharides and monosaccharides before being transported into cells for further use which may be accomplished through complete extracellular hydrolysis or partial extracellular hydrolysis with additional intracellular hydrolysis by individual enzymes, or by complete hydrolysis using celullosomes and extracellular enzyme complexes (Gírio et al. 2010). Hydrolysis of hemicellulose requires a multitude of enzymes including endo-β-1,4-xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuonidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase, which are all involved in hydrolysis of the backbone or the multiple substitutions along the backbone (Saha 2003) .

Because of the higher complexity of hemicellulose, more enzymes are needed to carry out full hydrolysis of the polysaccharide. Endo-β-1,4-xylanase hydrolyzes the β-O,4 linkages along the hemicellulose backbone. B-xylosidases then hydrolyze xylooligosaccharides to xylose. α-L-arabinofuranosidase removes arabinose from the side chains of hemicellulose. α-glucuronidase removes 4-O-methyl glucuronic acid units from the side chains. Each esterase functions to cleave ester linkages of xylan and acetic acid or arabinose residues and phenolic acids found on hemicelluloses. Arabinose and xylose from hemicellulose hydrolysis may then enter the pentose phosphate pathway within the cell before entering glycolysis as glyceraldehyde-3-phosphate.

### 1.4.3 Lignin Catabolism

Lignin is initially degraded through biphenyl catabolic pathways or β-aryl ether pathways (Sainsbury et al. 2013). This upstream step is used to cleave dimeric structures from the larger 3-dimensional lignin structure. Lignin is initially degraded by extracellular oxidative enzymes such

as laccases and peroxidases in bacterial systems. Peroxidases (lignin peroxidases, manganese peroxidases, and dye decolorizing peroxidases) and laccases act by breaking lignin bonds into aromatic monomers (Heinfling et al. 1998; Van Bloois et al. 2010; Salvachúa et al. 2015; Janusz et al. 2017). Peroxidases may act nonspecifically on lignin subunits, with the most commonly studied linkage being β-O-4 which accounts for 50-60 % of lignin bonds from woody biomass (Wong 2009). Peroxidases act on lignin by the oxidative formation of radical cations, which can then promote side chain cleavage, demethylation, and addition and rearrangement reactions not carried out by peroxidase function. This results in the formation of a variety of aromatic monomers. Laccases generally act on benzenediols in the presence of oxygen to produce semiquinone monomers. Monomers produced from laccase and peroxidase activity may then enter cells to be further metabolized. Figure 1.3 shows the detailed metabolic pathways by which lignin degradation and upgrading can occur.

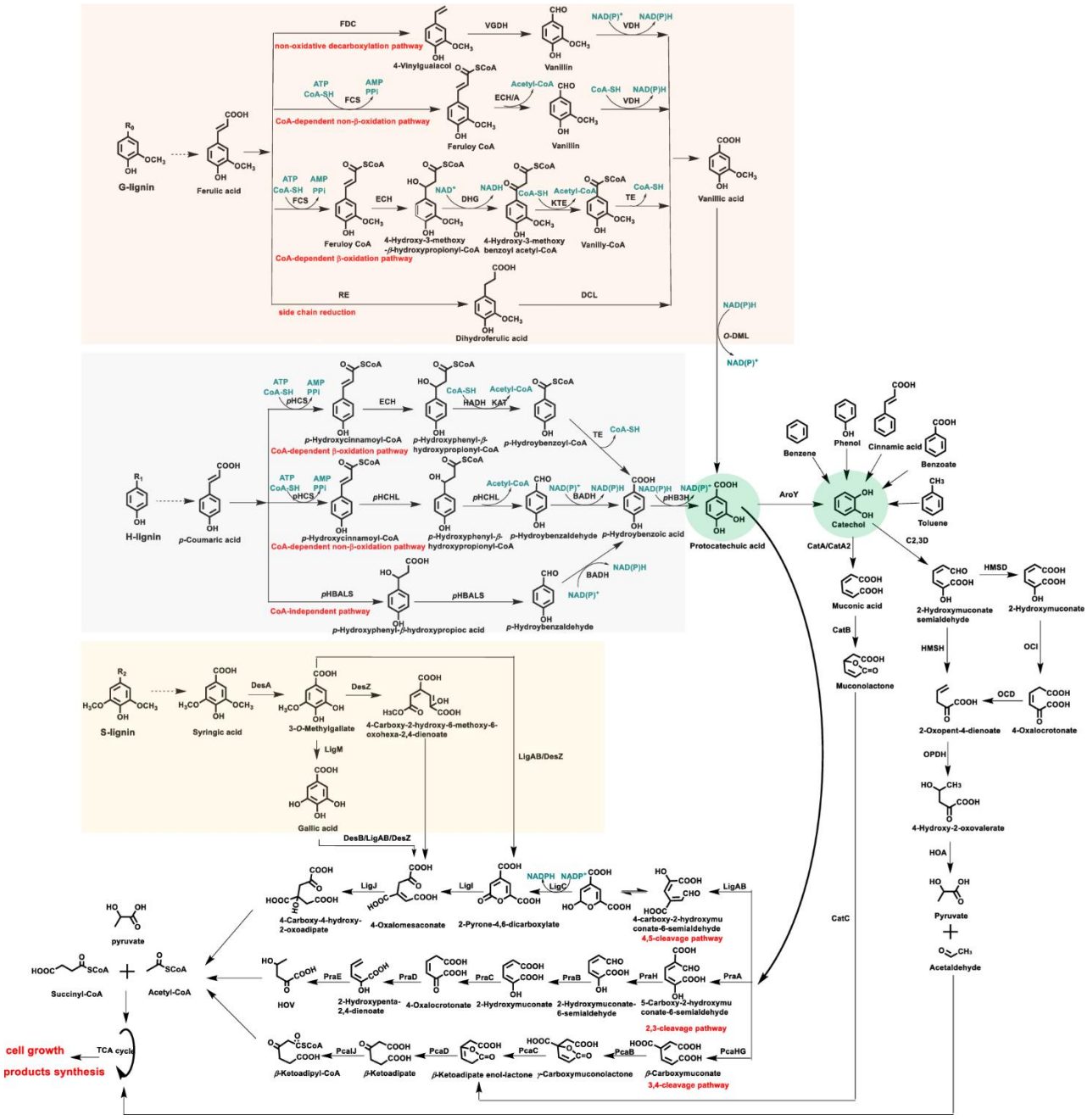


**Figure 1.3**. Lignin phenylpropane catabolic pathways, reprinted from (Xu et al.).

Bacterial lignin degradation can be segregated into 3 major groups: funneling pathways, O demethylation, and ring cleavages (Kamimura et al. 2019). Bacterial lignin degradation is best described for *Sphingobium* sp. SYK-6 in which it is thought that degradation occurs through a β-aryl ether pathway or biphenyl pathway. (Kamimura et al. 2019).

β-aryl ether compounds are initially transformed by LigD, where the α-carbon of β-O-4 linked lignin subunits is converted from a hydroxyl group to a ketone. LigE, LigF, and LigG then play a role in the cleavage of the two ring structures to their constitutive monomers which are then converted to vanillin (Masai et al. 2007). Biphenyl compounds follow a similar path, where cleaved dimers are linked with 5-5 linkages. LigX is capable of catalyzing demethylation of one methoxy group found in guaicyl units (Masai et al. 2007). Upon demethylation, LigZ performs a ring opening reaction on the demethylated phenylpropanoid group of the dimer resulting in ketone formation at the carbon 5 position and carboxylation at the $4^{th}$ carbon. LigY then releases the intact ring structure from the dimeric unit forming 5CVA, which is converted to the carboxylated form of vanillin, vanillate, by LigW/LigW2. LigM can further demethylate phenylpropanoids, while LigAB in conjunction with other enzymes is able to catalyze a ring opening reaction. In the gauiacyl pathway LigC and LigI function further in ring cleavage. LigJ and LigK catalyze reactions for compounds to enter the TCA cycle.

### 1.4.4 Value-added Metabolites

Metabolites produced by the presented pathways are of industrial interest. Some of these value-added metabolites include lactic acid and other low molecular weight organic acids, polyhydroxylalkanoates (PHA's), lignin degradation products such vanillic acid and cinnamic acid, as well as ABE fermentation products and biogas/biofuels. PHA production is one frequently researched topic for pulp and paper waste, where many regimes use wastewater or paper mill sludge as substrates for PHA accumulation by bacterial fermentation (Yan et al.; Jiang et al. 2011; Bhuwal et al. 2013; Queirós et al. 2014). PHAs are of value because of its use as a biodegradable polymer that extends beyond typical composting regimes (Bugnicourt et al. 2014; Rodriguez-Perez et al. 2018). Additionally, PHAs can be extracted from cells and processed

similar to industrial thermoplastics for applications in packaging, adhesives, non-wovens, as well as pharmaceuticals and therapeutics (Bugnicourt et al. 2014; Koller 2018; Rodriguez-Perez et al. 2018; Zhang et al. 2018). Current industrial processes for PHA production rely on expensive raw materials, and waste streams are of much interest as a feedstock replacement to remediate and valorize these streams.

Lactic acid is an organic acid product that can be derived from glycolysis and pentose phosphate pathway intermediates, like many other organic acid products of interest (Okano et al. 2009; Komesu et al. 2017). Lactic acid has been extensively researched because of its broad range of applications in areas of food and beverage, personal care, solvents and industrial uses, polymer synthesis, and pharmaceuticals (Komesu et al. 2017; Yang et al. 2018). The major interest in lactic acid production is the synthesis of poly lactic acid (PLA), which has similar applications as PHAs discussed above. PLA is both biodegradable and compostable, making it a candidate for replacing fossil fuel derived plastics (Garlotta 2001).

Lignin has perhaps been the most difficult component of waste streams to valorize because of its recalcitrant nature and difficulty to degrade. Though it is difficult to upgrade lignin, many of the aromatic degradation products, such as vanillic acid, cinnamic acid, and gallic acid have high value applications. Gallic acid has gained interest in therapeutics as a compound with antioxidant and antitumor properties (Yen et al. 2002; Punithavathi et al. 2011). Vanillic acid is a precursor to vanillin which is used widely in the food industry as a flavoring and preserving agent, and has pharmaceutical applications (Kaur and Chakraborty 2013). Many other value-added products are also produced during pulp and paper waste fermentations and other can be found in Table 1.1. For industrial applications, it will be important to tune production flux for specific compounds as many coexist in residual fermentation broth.

## 1.5. Approaches for Valorizing Pulp and Paper Waste

Muliple approaches are necessary to improve biomass valorization from pulp and paper waste products. These include 1) improving the production organism using gene editing, evolutionary, or metabolic engineering approaches 2) developing better engineered systems for valorization using different types of reactors or systems such as solid state fermentation, co-fermentation, and microbial consortia both native or synthetic and 3) statistical optimization and process parameters.

### 1.5.1 Biotechnological Approaches

There are a variety of biotechnological approaches that could be used for valorizing pulp and paper industry streams containing lignocellulosic materials. Some of these methods include enzyme discovery and screening for celluloytic and lignolytic function to increase the toolbox for enzymatic hydrolysis (Hess et al. 2011; Liu et al. 2013). Advances in synthetic biology and metabolic engineering approaches may be used to better design organisms to carry out these functions for either production of lignocellulose degrading enzymes or microbial fermentation (Chandel et al. 2013; Kricka et al. 2014; Chen and Dou 2016). Additionally, directed and adaptive laboratory evolution techniques of microbial strains can be used to create more robust organisms for use in waste valorization or remediation processes (Mohanram et al. 2013; Wang et al. 2014; Radek et al. 2017).

Rational design and directed evolution experiments provide one such approach for increasing the activity and understanding enzyme structure-function relationships of lignocellulose degrading enzymes, such as laccases, peroxidase, and dioxygenases (Beckham et al. 2016). Laccases, peroxidases, and dioxygenases have been investigated by both approaches extensively because of their broad industrial applications, such as a green catalyst for

biosynthesis of polyphenols, chlorine free bleaching in the pulp and paper industry, bioremediation, and removal of fermentation inhibitors from biomass feedstocks, amongst many other functions (Widsten and Kandelbauer 2008; Mate and Alcalde 2015; Su et al. 2018).

In a study by Festa et. al. mutant laccases were screened from a library of 1100 mutants, resulting in one mutant of improved function but lower stability from initial trials (Festa et al. 2008). The mutant enzyme was then put through error prone PCR resulting in another mutant with increased activity and increased stability. Additionally, much work is being done on improving screening assays for laccase activity. One recent approach use high through-put calorimetric screening to evaluate small improvements on evolved laccases, with many showing the ability to screen for phenolic and lignosulfonate compounds (Rodríguez-Escribano et al. 2017; Pardo and Camarero 2018) . Many directed evolution and rational design approaches seek to improve stability under a variety of process conditions including pH, temperature, and high solvent concentration. This approach allows enzymes to be developed that can withstand conditions of pulp and paper waste, such as alkaline black liquor or hydrolysis at higher temperatures which may desired in thermophilic and acidogenic/alkali fermentation processes (Prasetyo et al. 2011; Sharma and Kumar Bajaj 2017; Awasthi et al. 2018).

Genetic and metabolic engineering efforts for direct metabolism of pulp and paper waste streams has been under investigated for organisms that are directly isolated from these industrial streams, and particularly for lignin valorization efforts (Liu et al. 2019). Approaches such as -omics sciences, including transcriptomics, proteomics, metabolomics, and fluxomics will provide better insights into lignocellulose metabolic targets for engineering efforts (Poudel et al. 2017; Rosnow et al. 2017). Additionally, adaptive laboratory evolution may be a valuable way to

obtain organisms that can withstand the inhibiting environments of pulp and paper waste product streams, as opposed to strictly using enzyme evolutions approaches like those discussed above.

### 1.5.2 Bioprocessing Approaches

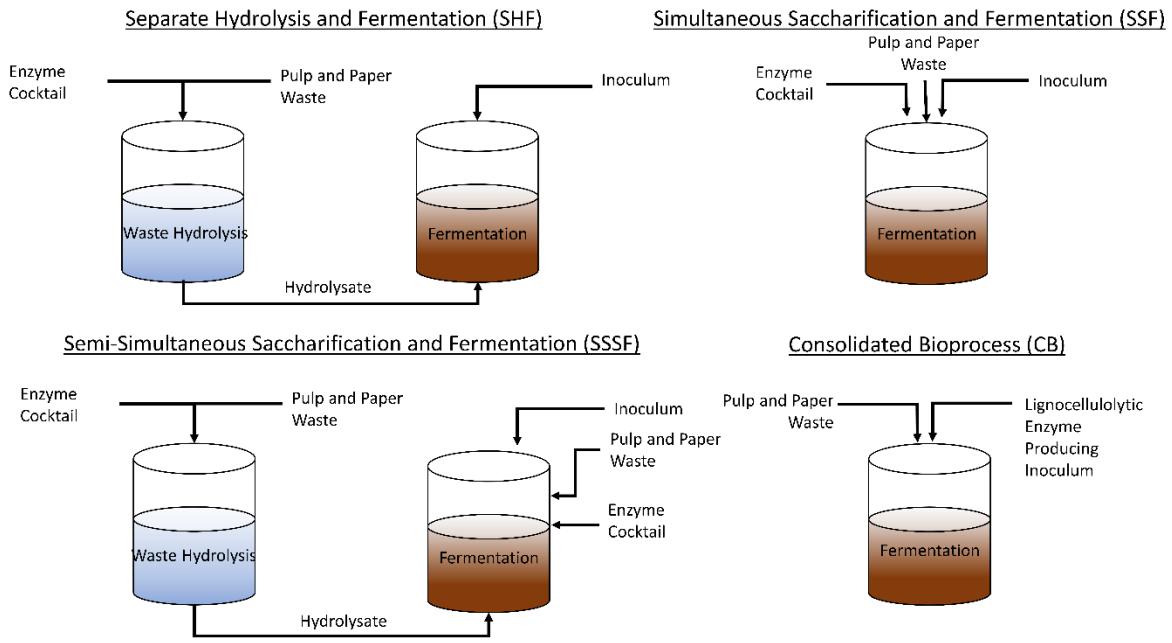


**Figure 1.4.** Simplified process diagrams for different fermentation regimes that can be used for pulp and paper waste.

The primary approach for valorization of pulp and paper waste is simultaneous saccharification and fermentation (SSF), where a microorganism is grown with lignocellulolytic enzymes and sludge to hydrolyze carbon within the material and produce products which have predominantly included acetone, butanol, ethanol products (ABE), as well as lactic acid which is a valuable precursor chemical (Figure 1.4) (Lee et al. 2004; Marques et al. 2008; Kang et al. 2010; Kang et al. 2011; Shi et al. 2015b; Guan et al. 2016c; Shi et al. 2018). In most SSF applications the process is run under anaerobic digestion conditions using native consortia since anaerobic digestion is considered one of the best methods for biogas production (Demichelis et

al. 2017; Moreno-Dávila et al. 2017; Solé-Bundó et al. 2019). SSF can also be extended to co-fermentation, where C5 and C6 sugars are hydrolyzed from biomass concurrently in the fermentation (SSCF) (Taherzadeh and Karimi 2007). The focus of SSCF is on the same products as SSF, with the added use of xylose, expanding the applications for this C5 sugar (Patel et al. 2005; Öhgren et al. 2006; Zhang and Lynd 2010; Morales-Rodriguez et al. 2011).

Pre-hydrolysis, hydrolysis of polysaccharides within pulp and paper waste streams before being fed for fermentation processes is also a common method employed for microbial fermentation of pulp and paper waste, also referred to as separate hydrolysis and fermentation (SHF) (Zhu et al.; Dubey et al. 2012). SHF methods may extend the fermentation time of pulp and paper waste compared to SSF while having similar or reduced yields (Marques et al. 2008; Drissen et al. 2009). Though enzymatic hydrolysis and fermentation can occur at their optimal reaction conditions, the extended time, potentially lower yield, and hydrolysis products inhibition of enzymes, make this technology less efficient than SSF (Lynd et al. 2002; Devarapalli and Atiyeh 2015).

Pre-hydrolyzed product may also be fed in to SSF (called semi-SSF, SSSF) processes to enhance hydrolysis of non-hydrolyzed materials during fermentation and increase product yield (Elliston et al. 2013; Gonçalves et al. 2014; Nishimura et al. 2017). SSSF consists of both SHF and SSF phases (Li et al. 2014). SSSF provides the benefits of both SHF and SSF, where initial hydrolysis of feedstock can be carried out at optimal conditions during initial phases, and then can be staged into SSF when inhibitory effects of SHF begin to occur to continue hydrolysis and begin fermentation (Shen and Agblevor 2011). For SSSF it is important to find optimal reaction conditions for pre-hydrolysis and SSF stages to achieve the best balance of yield and fermentation time.

Consolidated bioprocessing (CB) is also a promising approach for valorization of pulp and paper waste. As opposed to SHF, SSF, SSSF, and SSCF, CB is used to produce enzymes used for hydrolysis in the same pot as fermentation occurs using lignocellulolytic microbial species (Yamada et al. 2013). This acts as a potentially more cost effective replacement for SSF processes, which require high amounts of enzyme loading because of the non-optimal reaction environment (Hasunuma et al. 2013).

### 1.5.3 Optimization Approaches

Design of Experiments (DOE) approaches for lignocellulosic material fermentations are a popular method for optimizing product output. DOE methods often start with the use of a screening design, which describe designs that simultaneously test many process variables to identify variables that have the largest effect on the chosen system output (Vanaja and Rani 2007). A common screening design used to determine optimization variables is a Plackett-Burman Design. Plackett-Burman designs allow for orthogonal arrays to be developed that test for unbiased main effects of each variable in the smallest array achievable (Vanaja and Rani 2007). Plackett-Burman designs allow the screening of variables, such as carbon source, nitrogen source, other media components, and fermentation process parameters (Srinivas et al.; Naveena et al. 2005; Song et al. 2007; Liu and Tang 2010; Guo et al. 2010). In a paper by Das. et. al., researchers used a Plackett-Burman design to screen for both fermentation parameters and media components that had the largest effect on cellulolytic enzyme production for deinking office paper waste (Das et al. 2016). Researchers were able to screen for fermentation time, media pH, substrate ratio, substrate amount, inoculum size, moisture content, media buffer concentration, magnesium concentration, and salt concentration in a single experiment. Other screening designs may also be used to assess main effects of factors, such as two level factorial designs and

Taguchi Design, where Taguchi Design is popular in manufacturing (Emborg et al. 1989; Zhang et al. 2007; Knak Jepsen,' et al. 2593). Screening designs are used to choose variables for optimization designs which allow for quantification of the factors that have main effects on the system.

Two commonly used optimization designs are Box-Behnken and Central Composite Design (CCD), the results of which can be used for Response Surface Methodology (RSM). Box-Behnken designs are rotatable or semi-rotatable 3-level incomplete factorial designs that can be used to estimate the coefficients of second degree graduating polynomials which allows for optimization of the response variable (Box and Behnken 1960; Ferreira et al. 2007). CCD is a similar method for estimating coefficients of second degree polynomials however this design is contains five levels, where two of the five levels are axial points (Wang and Wan 2009). The polynomial equations derived from experimental results of both CCD and Box-Behnken can then be used to develop an empirical model called a response surface (Myers et al. 2016). Many DOE studies have been used for the production of lignocellulose degrading enzymes, biohydrogen from waste, ethanol production, and organic chemical production but few studies focus specifically on paper waste (Kim et al. 2008; Liu and Tang 2010; Sepahy et al. 2011; Peng and Chen 2011). This method could be further extended to pulp and paper waste fermentations and to allow for better designed systems with increased product yield.

### 1.6 Pulp and Paper Valorization Prospects

The pulp and paper industry can provide a major source of carbon for fermentation processes (Phillips et al. 2013; Branco et al. 2018). Understanding the composition and type of feedstock, organisms that are isolated from or screened to consume the materials, metabolism of lignocellulose, and the approaches to improve valorization of these feeds will allow for better

design of biological fermentation processes that can remediate the waste while generating value added chemicals. Figure 1.5 provides a framework for assessing and improving value-added chemical production from pulp and paper waste starting with feedstock choice and microbial isolation. Additionally, synthetic and systems biology approaches will likely play a more important role in valorizing biomass. By developing higher functioning organisms, better product yields, improved growth, and adapted tolerances to environmental pressures in fermentation reactions can be achieved. In understanding these aspects of paper waste fermentation, optimization can be carried out as a method of process improvement. Bacterial fermentation is less common compared to fungal degradation of lignocellulose material, however discovery of new bacterial species for fermentation or enzyme production can allow for cheaper and faster paper waste fermentations.

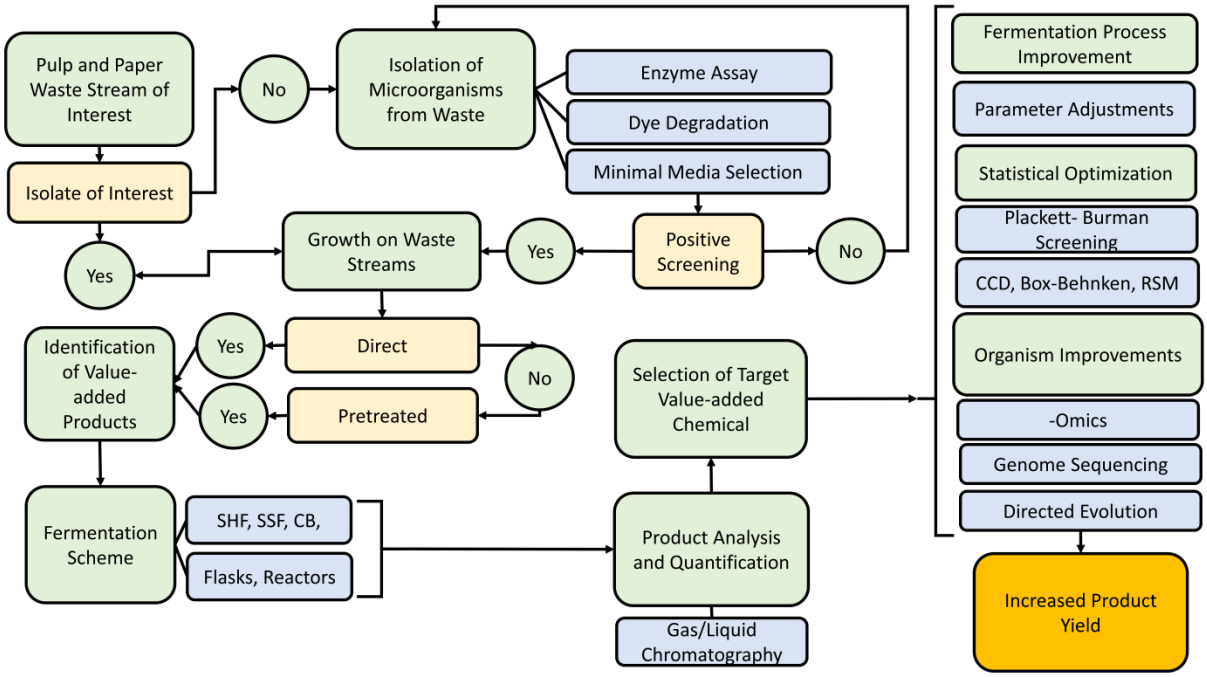


**Figure 1.5**. Flow diagram for approaches that increase product yield of pulp and paper waste bacterial fermentations. Green represents project phases, yellow represents milestone questions, blue represents methods that could be used in project phases.

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# CHAPTER 2

## Statistical Optimization of Black Liquor-containing Media for Growth and Lactic Acid Production by *Paenibacillus glucanolyticus* SLM1


### Abstract

*Paenibacillus glucanolyticus* SLM1 is a bacterial species isolated from black liquor that can metabolize lignocellulosic components such as cellulose, hemicellulose, lignin, and pentose sugars. To improve *P. glucanolyticus* SLM1 growth and metabolism of lignocellulose components contained in black liquor statistical optimization approaches were used to optimize media for aerobic growth and anaerobic production of lactic acid and vanillic acid. Generation time of *P. glucanolyticus* SLM1 was reduced from 4.10 h to 2.10 h in optimized media and biomass accumulation, measured as dry cell weight (DCW) in optimized media, was 3.6±0.39 g/L compared to 1.8±0.70 g/L in unoptimized media. Anaerobic formulation of optimized media showed increased production of lactic acid at 0.26±0.047 g/L. Additionally, low amounts of vanillic acid was produced from media used in the central composite design experiments. Optimization results indicate that increased nutrient, salt, and buffer content can reduce fermentation and growth inhibition of black liquor.


### 2.1. Introduction

Black liquor is a side stream produced during wood digestion in the Kraft or sulfate pulping processes within the pulp and paper industry. Black liquor, in its dilute form, contains approximately 10% organic solids composed of mainly hemicellulose and lignin degradation products (Vakkilainen 2017; Chutia et al. 2018). Black liquor is recovered during the pulping process through a series of evaporation stages, where the concentrated liquor is then burned for energy while molten salt components are extracted and fed back into the process (Cardoso et al. 2009; Bajpai 2018). Though this process recovers up to 100% of the generated black liquor in

modern systems, production of black liquor causes a process bottleneck at the recovery boiler and is considered a major pollutant in places without a recovery system (Paliwal et al. 2015; Yang et al. 2017; Chutia et al. 2018). The recovery boiler used to combust black liquor accounts for a large portion of process capital cost and cannot be replaced in an economically feasible manner (Uronen et al. 1978; Vakkilainen 2017; Kuparinen et al. 2019). This results in a need to remove excess black liquor from the system if increased production of cellulose pulp is desired. Additionally, black liquor contains many pollutants such chlorinated phenols, diphenyls, dioxins, and adsorbable (AOX) and extractable organic halogens (EOX) amongst others (Chaudhry and Paliwal 2018). Black liquor also causes black or brown coloration of bodies of water which are damaging to the ecosystem, reducing dissolved oxygen content in contaminated water, inhibiting photosynthesis, and preventing light penetration (Chaudhry and Paliwal 2018; Rivera-Hoyos et al. 2018).

Microbial fermentation of black liquor provides a platform for simultaneous remediation and production of value-added chemicals. This can be done using a consolidated bioprocess, which describes the simultaneous production of lignocellulose degrading enzymes for component hydrolysis and fermentation of the enzymatic degradation products (Fan 2014). Using microbial fermentation strategies, it is possible to 1) remove black liquor from the system reducing the bottleneck at the recovery boiler, 2) valorize the available hemicellulose and lignin components, and 3) potentially reduce the color of black liquor by degradation of lignin. To date, many studies focus on decolorization of black liquor, isolation of microbes from black liquor, and removal of sugars and polysaccharides from black liquor or alkali treated biomass for use as a carbon source, as opposed to using black liquor as a direct carbon source (Yang et al. 2010b;

Chandra et al. 2011; Chandra and Abhishek 2011; Mathews et al. 2014; Chai et al. 2014; Mathews et al. 2015).

*Paenibacillus glucanolyticus* SLM1 is one microorganism that has previously been demonstrated to grow directly on black liquor and degrade lignin, hemicellulose, and cellulose aerobically. (Mathews et al. 2014; Mathews et al. 2015). *P. glucanolyticus* SLM1 grows optimally at 37℃ and pH 9 but is capable of growth up to pH 11. Additionally, *P. glucanolyticus* SLM1 has been demonstrated to produce valuable organic acids such as lactic, succinic , hexanoic, gallic, and vanillic acid, amongst others (Mathews et al. 2016). This is unique in that common lignin degraders, such as wood rotting fungi, do not often metabolize degradation products to value-added products but are used as biological pretreatment methods for improving biomass quality (Singh and Singh 2014; Sharma and Arora 2015). Because of this, it is a strong candidate bacterium for biomass waste valorization efforts.

For this study, lactic acid was chosen as a target because of its use as a precursor for small compounds and larger acrylic polymers, such as poly lactic acid (PLA) (Garlotta 2001; Castillo Martinez et al. 2013). PLA is of specific industrial interest as an alternative plastic because of its ability to biodegrade and compost (Garlotta 2001). Vanillic acid is also a target of this study as it represents a lignin degradation product. Vanillic acid is a value-added phenolic organic acid that can be converted to vanillin, which can be used as a precursor for pharmaceuticals, as a flavoring agent, and preservative (Kaur and Chakraborty 2013).

This study aims to determine optimal media conditions for the growth and lactic acid production of *P. glucanolyticus* SLM1 using black liquor as a carbon source. To do this, Design of Experiments methods were used to formulate optimal growth media for aerobic growth, as well as optimal media for anaerobic production of lactic acid. Using central composite design

(CCD) and response surface methodology (RSM) allows an optimal balance between nutrient composition and black liquor concentration that can later be used for larger scale bioprocessing for aerobic growth regimes or anaerobic lactic acid production.

## 2.2. Materials and Methods

### 2.2.1 Microbial culture conditions and media components

*P. glucanolyticus* SLM1 was previously isolated from black liquor waste from North Carolina State Universities' Dept. of Forest Biomaterials wood pulping lab and regrown on pH 9 LB plates from 20% glycerol freezer stocks (Mathews et al. 2014; Mathews et al. 2016). Growth media used in this study contained varying amounts of 0.5% thiamine, 1M $MgSO_4$ solution, $Na_2HPO_4$, $KH_2PO_4$, $NH_4Cl$, NaCl, $CaCl_2$, tryptone, yeast extract, and black liquor collected from NCSU's Dept. of Forest Biomaterials wood pulping lab. Black liquor was a 50/50 % mix of hardwood and softwood liquors with unknown composition. Culture inocula were grown for 16-18 h at 37℃ in LB broth containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, which was sterilized by autoclave. All media was adjusted to pH 9 using NaOH and HCl. Media components were varied in accordance to Plackett-Burman and CCD RSM design matrix amounts. Optimally formulated aerobic media contains 6 g/L $Na_2HPO_4$, 6 g/L $KH_2PO_4$, 1 g/L $NH_4Cl$, 0.5 g/L NaCl, 0.15 g/L $CaCl_2$, 10mL/L 0.5% thiamine solution, 10 mM of $MgSO_4$, 10 g/L tryptone, 20 g/L yeast extract, and 15.45 v/v % black liquor in DI $H_2O$. Optimal lactic acid media contains 6 g/L $Na_2HPO_4$, 3.10 g/L $KH_2PO_4$, 1 g/L $NH_4Cl$, 0.5 g/L NaCl, 0.15 g/L $CaCl_2$, 10 mL/L 0.5% thiamine solution, 10 mM of $MgSO_4$, 10 g/L tryptone, 20 g/L yeast extract, and 20 v/v % black liquor in DI $H_2O$. Un-optimized media was LB containing 10% black liquor. Cell density of overnight cultures was counted using a Neubauer Improved Hemocytometer, and all experiment culture were inoculated with $1x10^7$ cells/ml.

### 2.2.2 Determination of Biomass Accumulation and Generation Time

To determine biomass accumulation and generation time, cultures were grown aerobically for 63 h during PB screening or 60 h for CCD and validation experiments in their respective media. Anaerobic cultures were used for metabolite analysis and contained the same media components as aerobic experiments. For anaerobic cultures, 1 $x10^7$ cells were inoculated aerobically in 200 ml serum flasks containing 50 ml of media then sealed. Fermentations were carried out for 14 days. Anaerobic and aerobic cultures were incubated at 37℃ with 200 rpm shaking.

For aerobic experiments $OD_{600}$ and cell count were taken at regular 3 h intervals during the day, with 12 h gap intervals, for 60 h (CCD) and 63 h (PB). $OD_{600}$ at 63 h was used as a response variable in the screening design. Cell count was used to generate growth curves for each condition in PB screening and CCD. Generation time was calculated from the linear region of the growth curves. Biomass accumulation for CCD and validation experiments was determined by dry cell weight (DCW). To determine DCW, 1 ml of culture for each duplicate flask were put in to three 1.5 ml Eppendorf tubes for a total of 3 samples per replicate. These were compared with uninoculated controls, which did not have large enough contribution to Eppendorf tube mass to be adjusted for in calculating DCW. Tubes were centrifuged for 3 minutes at 14,000 rpm to pellet the cells. Samples were washed with M9 minimal media two times. Washed cells were re-pelleted and placed in an incubator at 70℃ for 24 h. After 24 h samples were weighed, and the average of all replicate tubes per media was taken as DCW.

### 2.2.3 Fermentation Analysis by GC-FID

Fermentation analysis for lactic acid and vanillic acid was carried out using a Shimadzu 2014 GC-FID. 1 ml of anaerobic culture was centrifuged for 30 min at 14,000 rpm. 500 µl of

supernatant was placed in 1.5 ml tube and acidified to pH 1-3 using 50 µl of 37% HCl to precipitate residual lignin and clean the sample. 500 µl of ethyl acetate was then added to the Eppendorf tube and inverted 20 times. 400 µl of the organic phase was added to 1.5 ml Eppendorf tubes containing $MgSO_4$ to remove residual water. Samples were then centrifuged for 20 minutes at 14,000 rpm to separate $MgSO_4$ particles from the organic phase. 100 µl of supernatant was then added to glass GC vials with 100 µl of BSTFA-TMS and heated at 70℃ for 20 min to derivatize organic components.

A ZB-5HT inferno column (Phenomenex) was used for analysis on a Shimadzu GC. Helium was used as the carrier gas with a flow rate of 1 ml $min^{-1}$. One µl of sample was injected into the column at a split ratio of 1:50 after stabilization at 50℃. Inlet temperature was 300°C. For the oven method, the column is held at 50℃ for 5 min, then increased to 280℃ at a rate of 5℃ $min^{-1}$ and maintained at 280℃ for 20 min.

**2.2.4 Plackett-Burman Screening Design**

Plackett-Burman design was used to screen for media components with the largest effect. Table 2.1 shows the factors and level values used for the component screening before carrying out the CCD/RSM optimization.

**Table 2.1** Placket-Burman Design with factors and their representative media concentration for each coded level.

| Code | Factor | Level | |
|---|---|---|---|
| | | **+1** | **-1** |
| A | $Na_2HPO_4$ (g/L) | 1 | 13 |
| B | $KH_2PO_4$ (g/L) | 0.1 | 6 |
| C | $NH_4Cl$ (g/L) | 0.4 | 2 |
| D | NaCl (g/L) | 0.1 | 4 |
| E | $CaCl_2$ (g/L) | 0.0006 | 0.04 |
| F | 0.5%Thiamine (mL/L) | 0.06 | 0.14 |
| G | 1M $MgSO_4$ (mL/L) | 0.1 | 5 |
| H | Tryptone (g/L) | 1 | 30 |
| I | Yeast Extract (g/L) | 1 | 20 |
| J | Black Liquor (v/v%) | 5 | 20 |

Table 2.2 shows the design matrix for the Plackett-Burman screening design along with response variable values. The design was generated in R. Media sets were grown in duplicate and responses are the average of two flasks. Generation time was chosen as response variable 1 ($R_1$). Cell count and optical density were taken at regular intervals to monitor cell growth, and cell count was used to determine generation time. Maximum $OD_{600}$ was used as response variable 2 ($R_2$) to estimate biomass accumulation after 63 hours of growth in each media. Effects plots can be found in Appendix A. Analysis revealed no significant factors for both $R_1$ and $R_2$ when in combination with all 11 factors, despite this the components with the largest effects for each of the responses were chosen for the next stage of analysis.

**Table 2.2** Plackett-Burman Design matrix with responses for generation time and final $OD_{600}$

| Coded Plackett-Burman Variables | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Media** | **$Na_2HPO_4$** | **$KH_2PO_4$** | **$NH_4Cl$** | **NaCl** | **$CaCl_2$** | **Thiamine** | **$MgSO_4$** | **Tryptone** | **Yeast Extract** | **Black Liquor** | **Dummy** | **Generation Time (h)** | **Final $OD_{600}$** |
| 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | 3.2 | 11.77 |
| 2 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 2.7 | 8.78 |
| 3 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | 7.8 | 12.37 |
| 4 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | 1.7 | 15.39 |
| 5 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1.9 | 13.47 |
| 6 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 4.0 | 7.02 |
| 7 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1.9 | 4.70 |
| 8 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 3.8 | 6.41 |
| 9 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 2.4 | 3.34 |
| 10 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 4.2 | 2.00 |
| 11 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | 1.6 | 5.94 |
| 12 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 2.9 | 3.15 |

### 2.2.5 Central Composite Design and Response Surface Methodology.

Based on components that had the largest effect in the Plackett-Burman design, $KH_2PO_4$, yeast extract, and black liquor were chosen as for a 3-factor rotatable central composite design. The design and analysis were generated using JMP statistical software. The design is composed of 6 center points and five star points with an $\alpha$=1.682 corresponding to 3 factor rotatable design. The responses chosen for optimization were aerobic generation time and dry cell weight over a 60-hour period and anaerobic production of lactic acid and vanillic acid over a 14-day period.

## 2.3 Results and Discussion

### 2.3.1 Optimization of Growth Media for Biomass Accumulation and Generation Time

A 3 factor, rotatable, central composite design was developed with axial points values of $\alpha = \pm 1.682$. Table 2.3 shows details of the rotatable design with amounts of media component shown for each coded level. From the Plackett-Burman screening design, $KH_2PO_4$, yeast extract, and black liquor were chosen as the three factors to further investigate.

These components were chosen to strike a balance between the *P. glucanolyticus*' ability to cope with stressors present in black liquor by compensation from yeast extract and potassium phosphate, where yeast extract provides a nutrient source for better cell growth and potassium phosphate acts as a buffer in the solution. The experimental design in Table 2.3 was used for aerobic and anaerobic experiments.

**Table 2.3** Experimental design of CCD with media number, amount of media component, and the coded value of each media component.

| Media | $KH_2PO_4$ | | Yeast Extract | | Black Liquor | |
|---|---|---|---|---|---|---|
| | Actual (g/L) | Coded | Actual (g/L) | Coded | Actual (v/v%) | Coded |
| **1** | 3.05 | 0 | 10.05 | 0 | 12.5 | 0 |
| **2** | 3.05 | 0 | 10.05 | 0 | 12.5 | 0 |
| **3** | 6.00 | 1 | 1 | -1 | 20 | 1 |
| **4** | 0.1 | -1 | 20 | 1 | 5 | -1 |
| **5** | 0.1 | -1 | 1 | -1 | 20 | 1 |
| **6** | 6.00 | 1 | 1 | -1 | 5 | -1 |
| **7** | 3.05 | 0 | 10.05 | 0 | 12.5 | 0 |
| **8** | 0.1 | -1 | 20 | 1 | 20 | 1 |
| **9** | 6.00 | 1 | 20 | 1 | 5 | -1 |
| **10** | 0.1 | -1 | 1 | -1 | 5 | -1 |
| **11** | 6.00 | 1 | 20 | 1 | 20 | 1 |
| **12** | 3.05 | 0 | 10.05 | 0 | 12.5 | 0 |
| **13** | 3.05 | 0 | 10.05 | 0 | 12.5 | 0 |
| **14** | 3.05 | 0 | 26.5 | 1.682 | 12.5 | 0 |
| **15** | 8.00 | 1.682 | 10.05 | 0 | 12.5 | 0 |
| **16** | 3.05 | 0 | 10.05 | 0 | 12.5 | 0 |
| **17** | 0 | -1.682 | 10.05 | 0 | 12.5 | 0 |
| **18** | 3.05 | 0 | 10.05 | 0 | 0 | -1.682 |
| **19** | 3.05 | 0 | 10.05 | 0 | 25.12 | 1.682 |
| **20** | 3.05 | 0 | 0 | -1.682 | 12.5 | 0 |

Biomass accumulation and generation time were chosen as responses for aerobic experiments because they are relevant scalable parameters for technological applications. Biomass accumulation is to be maximized while generation time is to be reduced. A second order polynomial equation was used to fit the experimental results for each response variable.

Equation 1, for biomass accumulation, and Equation 2, for generation time, were generated using JMP software. The generated equations are:

$$Y_1 = 1.96 + 0.45PP + 0.53YE - 0.68BL + 0.26PP(YE) + 0.09PP(BL) + 0.34YE(BL) + 0.09PP^2 + 0.04YE^2 + 0.05BL^2 \quad \text{(Eq. 1)}$$

$$Y_2 = 1.3 - 1.3PP - 2.7YE + 2.0BL + 1.2PP(YE) - 1.4PP(BL) - 2.4YE(BL) + 0.3PP^2 + 1.5YE^2 + 0.6BL^2 \quad \text{(Eq. 2)}$$

Where $Y_1$ is generation time and $Y_2$ is biomass accumulation. PP refers to potassium phosphate, YE refers to yeast extract, and BL refers to black liquor.

The significant results are summarized in Table 2.4. $KH_2PO_4$, yeast extract, black liquor, along with the second order effects of black liquor with yeast extract and $KH_2PO_4$ and yeast extract with itself are significant effectors of generation time. This indicates that small variations in these components can lead to larger effects on generation time under aerobic conditions. The significant factors for biomass accumulation are yeast extract and black liquor, where black liquor has a negative effect on biomass accumulation and yeast extract has a positive effect. Potassium phosphate and the other second order effects that were significant on generation time did not have a significant effect on biomass accumulation, where medias with varying generation times may be able to achieve final similar cell densities but through a different time course.

**Table 2.4.** Statistical results for CCD of aerobic biomass accumulation and generation time.

| Factors | DCW (g/L) | | | Generation Time (h) | | |
|---|---|---|---|---|---|---|
| | **Coefficient** | **t-ratio** | **p-value** | **Coefficient** | **t-ratio** | **p-value** |
| Intercept | 1.96 | 6.32 | <0.0001 | 1.3 | 2.03 | 0.0701 |
| Potassium Phosphate | 0.45 | 2.18 | 0.0541 | -1.3 | -2.90 | 0.0158 |
| Yeast Extract | 0.53 | 2.58 | 0.0275 | -2.7 | -6.13 | 0.0001 |
| Black Liquor | -0.68 | -3.28 | 0.0083 | 2.0 | 4.66 | 0.0009 |
| (Potassium Phosphate)(Yeast Extract) | 0.26 | 0.98 | 0.3483 | 1.2 | 2.04 | 0.0687 |
| (Potassium Phosphate)(Black Liquor) | 0.09 | 0.34 | 0.7402 | -1.4 | -2.48 | 0.0326 |
| (Yeast Extract)(Black Liquor) | 0.34 | 1.25 | 0.2407 | -2.4 | -4.24 | 0.0017 |
| (Potassium Phosphate)(Potassium Phosphate) | 0.068 | 0.34 | 0.7414 | 0.3 | 0.68 | 0.5110 |
| (Yeast Extract)(Yeast Extract) | 0.02 | 0.10 | 0.9249 | 1.5 | 3.53 | 0.0055 |
| (Black Liquor)(Black Liquor) | 0.02 | 0.11 | 0.9135 | 0.6 | 1.33 | 0.2129 |

3-D surfaces with 2-D contours were developed based on the prediction equations for each of the response variables shown in Figure 2.1. The presented surfaces show combinations of the factor's effects on the response variable, given by the z-axis, while the third factor is held constant at the center point value. This is used to understand two variable interactions with the desired response variable and assess optimums of these variables for the desired response.

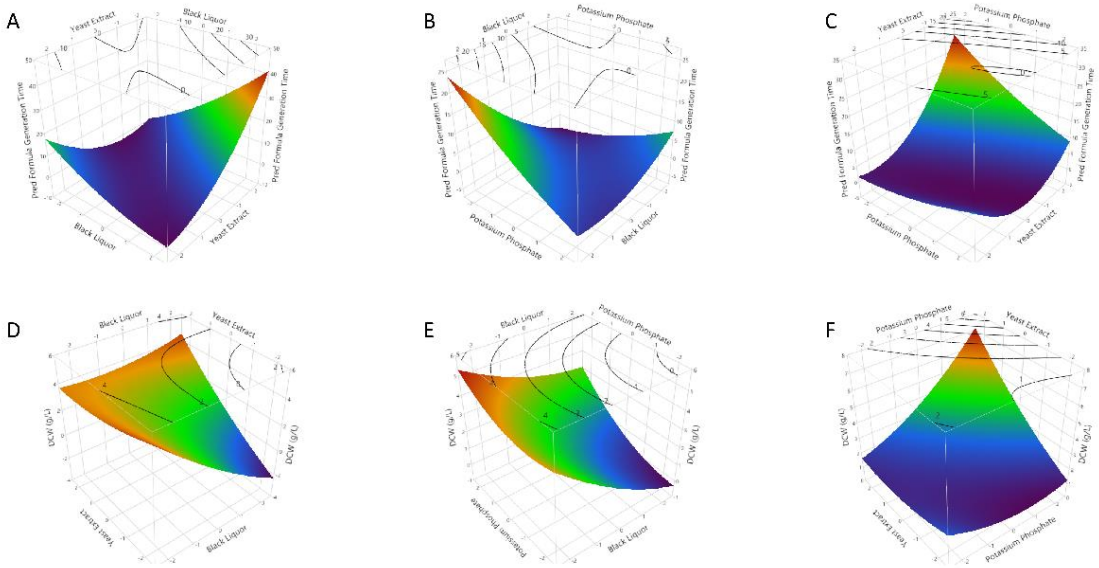


**Figure 2.1** RSM surface plots of dry cell weight (DCW) and generation time for aerobic growth media optimization. (A) Effect of yeast extract and black liquor on generation time; (B) effect of black liquor and potassium phosphate on generation time; (C) Effect of yeast extract and potassium phosphate on generation time, (D) effect of yeast extract and black liquor on DCW; (E) effect of black liquor and potassium phosphate on DCW; and (F) Effect of yeast extract and potassium phosphate on DCW.

For generation time, optimal conditions fall within a saddle point indicating that low levels of black liquor with low levels of $KH_2PO_4$ or yeast extract as well as high levels of black liquor with high levels of $KH_2PO_4$ or yeast extract provide desired reduction in generation time. This shows that high levels of buffer and nutrient can overcome stress factors (e.g. high pH and cytotoxic compounds) resulting from higher amounts of black liquor in the media, and that less buffer and nutrient are options if there is less stress introduced into the system. Biomass

accumulation conditions show similar patterns where the percentage of black liquor can be increased with increased amounts of yeast extract and $KH_2PO_4$. These results show the reliance on physiological buffer and nutrient source to cope with stressors found in black liquor, where higher nutrient content allows for more desired responses.

The best media formulation for growth was determined using a JMP software function to maximize desirability of the response when looking at generation time and biomass accumulation simultaneously. The formulation is 6 g/L $KH_2PO_4$, 20g/L yeast extract, and 15.45% black liquor. This represents an increase of 5% black liquor compared to the un-optimized media, which is LB with 10% BL.

### 2.3.2 Optimization of Growth Media for Organic Acid Production

The same design was used to optimize the production of lactic acid. The prediction equation for lactic acid production over the 14-day period is shown in Equation 3.

$$Y_3 = 0.40 + 0.07PP + 0.31YE + 0.19BL - 0.09PP(YE) + 0.02PP(BL) + 0.23YE(BL) - 0.10PP^2 + 0.06YE^2 + 0.04BL^2 \quad \text{(Eq. 3)}$$

Table 2.5 shows the results of anaerobic growth and lactic acid production by *P. glucanolyticus* SLM1. Black liquor and the second order effect of yeast extract with black liquor were significant factors for dry cell weight over the time frame. For lactic acid production, yeast extract, black liquor, and the second order effects of yeast extract and black liquor were significant at p=0.05.

**Table 2.5**. Statistical results of CCD for anaerobic production of lactic acid.

| Factors | Lactic Acid Production (g/L) | | |
|---|---|---|---|
| | **Coefficient** | **t-ratio** | **p-value** |
| Intercept | 0.40 | 3.45 | 0.0062 |
| Potassium Phosphate | 0.07 | 0.95 | 0.3665 |
| Yeast Extract | 0.31 | 4.00 | 0.0025 |
| Black Liquor | 0.19 | 2.46 | 0.0335 |
| (Potassium Phosphate)(Yeast Extract) | -0.09 | -0.90 | 0.3879 |
| (Potassium Phosphate)(Black Liquor) | 0.02 | 0.20 | 0.8476 |
| (Yeast Extract)(Black Liquor) | 0.23 | 2.30 | 0.0446 |
| (Potassium Phosphate)(Potassium Phosphate) | -0.10 | -1.29 | 0.2258 |
| (Yeast Extract)(Yeast Extract) | 0.06 | 0.80 | 0.4411 |
| (Black Liquor)(Black Liquor) | 0.04 | 0.58 | 0.5774 |

Figure 2.2 shows the response surfaces for lactic acid production. Figure 2.2 (A) shows that when potassium phosphate is at center point levels, increased levels of black liquor and yeast extract lead to desirable production of lactic acid. When yeast extract is held constant, higher levels of black liquor and near center point levels of potassium phosphate provide higher lactic acid production as shown in Figure 2.2 (B). Figure 2.2 (C) indicates that high levels of yeast extract produce high levels of lactic acid for ranges of potassium phosphate nearing center point levels to lower coded value levels. The surfaces in Figure 2.2 generally show the dependence of lactic acid production on yeast extract amount, which could also be tied to higher biomass accumulation seen in samples with increased nutrient content.

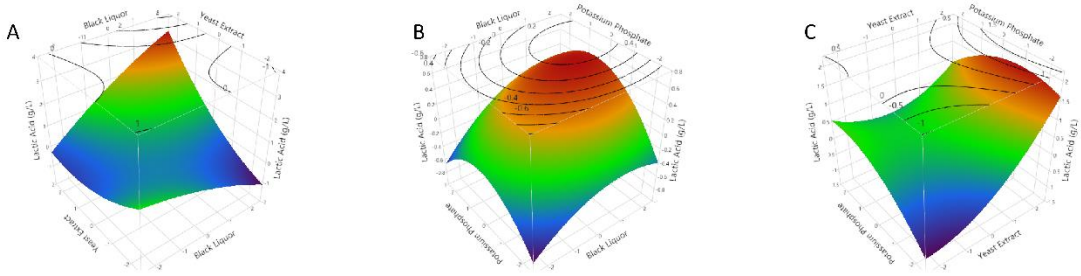


**Figure 2.2**. RSM surface plots of anaerobic lactic acid production. (A) effect of yeast extract and black liquor on lactic acid production, (B) effect of black liquor and potassium phosphate on lactic acid production; and (C) effect of yeast extract and potassium phosphate on lactic acid production

By maximizing desirability of the response in JMP, the optimal media formulation for production of lactic acid was determined to require 20 g/L yeast extract, 20% black liquor, and 3.10 g/L of potassium phosphate along with the additional minimal media components.

### 2.3.3 Validation of Optimized Media

To assess the optimized media formulation for aerobic growth on black liquor containing media, optimized media was compared to un-optimized media used in previous studies. The optimized media formulation for aerobic growth represents a 5.45% increase in the useable amount of black liquor for *P. glucanolyticus* growth regimes. Figure 2.3 shows growth curves over a 60 h period (A) as well as biomass accumulation by DCW (B).

In Figure 2.3 (A) a 15 h reduction in the lag phase is seen for cells grown on optimal media when compared to un-optimized media. This is likely due to potassium phosphate and addition of other M9 media components that help buffer the media and reduce cellular stress from components of the black liquor along with higher nutrient content. Additionally, the generation time of 2.04 h in optimized media is about two hours less than that for un-optimized media (4.1 h), indicating the ability of the cells to divide at a higher rate in optimized media. Cells in optimized media appeared to undergo slower growth from h 30 to 45 before reaching stationary phase, whereas un-optimized media did not show this prolonged growth pattern. DCW after growth can be seen in Figure 2.3 (B), with *P. glucanolyticus* grown in optimal media achieving a DCW of 3.6±0.39 g/L. Un-optimized media produced less biomass with 1.8±0.70 g/L DCW. The overall effects of media optimization were 3-fold, producing a reduced lag phase, faster generation time, and more biomass.

Lactic acid production in the respective optimized media was also compared to unoptimized media (Fig. 3C). To determine lactic acid production, uninoculated controls were analyzed to determine the amount of lactic acid already within each media. The amount of lactic acid in the media before fermentation was subtracted from the total amount after fermentation, and error was taken as the standard deviation of control and experimental samples added in quadrature. Optimized media showed increased amounts of lactic acid with 0.26±0.047 g/L attributed to bacterial fermentation. Unoptimized media after fermentation saw a reduction in lactic acid by 0.092±0.095 g/L, which indicates variability in production or consumption of lactic acid in media not defined nutrient content. This difference in production could be explained by inhibition of fermentation from components of black liquor, where higher yeast extract, minimal salts, and buffering capacity allow for positive flux through lactic acid producing pathways.

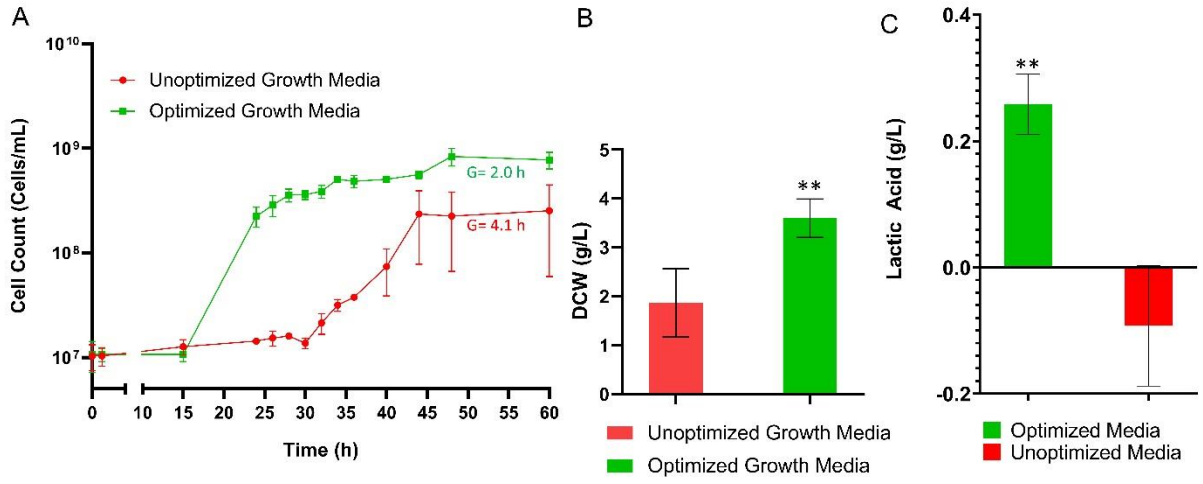


**Figure 2.3**. Optimized and unoptimized growth and biomass accumulation over a 60-hour period with lactic acid accumulation over a 14 h period. (A) Growth curve for optimized and un-optimized media with generation time indicated. Error is given as standard deviation. (B) DCW for optimized and un-optimized media after 60 h. Error is given as the 90% confidence interval. ** indicates significance at $\alpha=0.05$, $p=0.027$ (C) Lactic acid production after 14 h fermentation; error is given as the standard deviation added in quadrature. ** indicates significance at $\alpha=0.05$, $p=0.0026$

### 2.3.4 Production of Vanillic Acid in Fermentation Media

An additional goal of this study was to find an optimal media formulation to produce vanillic acid, which unlike lactic acid, is a direct byproduct of lignin degradation. Though vanillic acid was able to be quantified in the system, there were no significant factor effects distinguished in our analysis. Previous work showed the presence of vanillic acid after growth on black liquor, but the concentration was not determined (Mathews et al. 2016). Here, Figure 2.5 presents trends in vanillic acid production in select media sets.

The dotted line represents a center point media formulation for comparison with other sets. Media set 18 contains no black liquor and therefore no lignin. As expected, there was no detectable vanillic acid in this sample supporting the claim that lignin needs to be present for vanillic acid production to occur. Media 3 showed the highest production of vanillic acid at 0.035±0.0003 g/L, representing media containing 20% black liquor, low amounts of yeast extract at 1 g/L, and high amounts of potassium phosphate at 6 g/L. Media set 5 is similar, with slightly higher production than media set 1 representing a center point media formulation. Media 5 contains the same amount of black liquor and yeast extract as media 3 but with reduced potassium phosphate. This may indicate that 1) increased amounts of black liquor (higher lignin content) are necessary for higher levels of vanillic acid production and 2) higher levels of potassium phosphate are likely needed to cope with environmental stress stemming from black liquor leading to increased production of vanillic acid.

Additionally, media 17 contained no potassium phosphate, 10 g/L yeast extract, and 12.5% black liquor further supporting the necessity of phosphate in the system. Media 17 and 1 both contain 10 g/L of yeast extract and this increased nutrient content may also direct flux away from lignin metabolism resulting in lower vanillic acid production. More experimentation is necessary to parse the specific effects of media richness and potassium phosphate content on lignin degradation product generation.

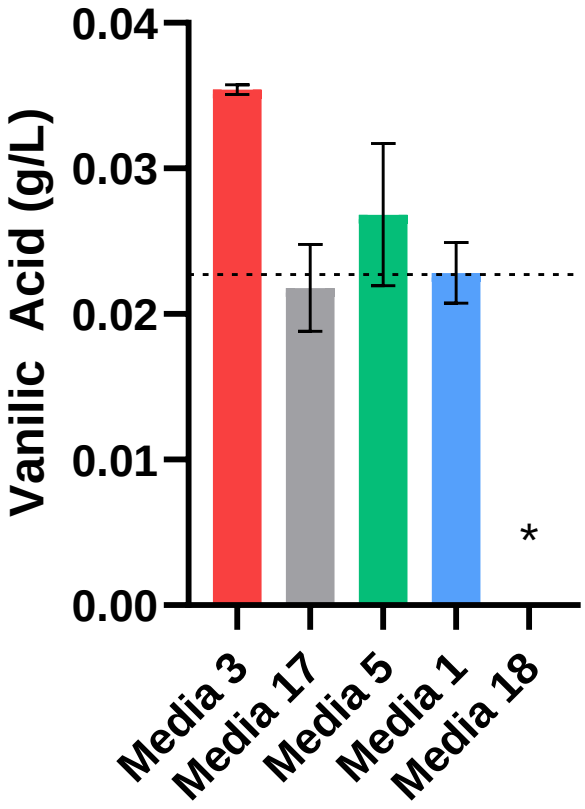


**Figure 2.5**. Vanillic acid produced in select media sets from RSM experimental design.

## 2.4 Conclusions

Optimized media for aerobic growth allowed a 5.45% increased use of black liquor while increasing growth rate and biomass accumulation compared to un-optimized media. Lactic acid production was also improved when compared to un-optimized media representing a push towards practical black liquor valorization processes that may improve pulp and paper industries, which are seeking revision and diversification. Vanillic acid concentrations were also determined for a variety of media, demonstrating that media formulation changes can improve commodity organic acid production by *P. glucanolyticus* cultured on the paper pulping side-stream black liquor.

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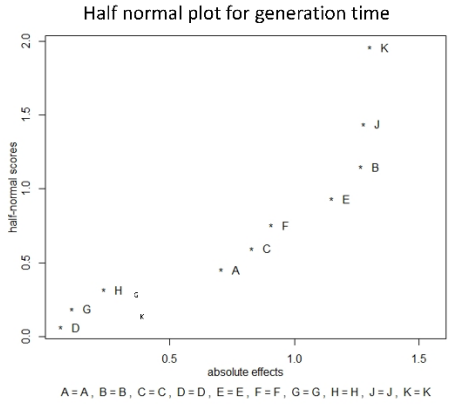
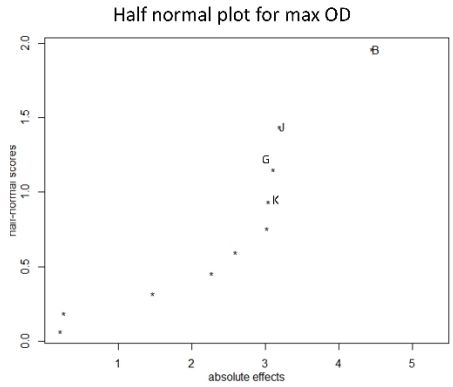
# APPENDICES

# Appendix A

## Outcomes of Plackett-Burman Screening Design

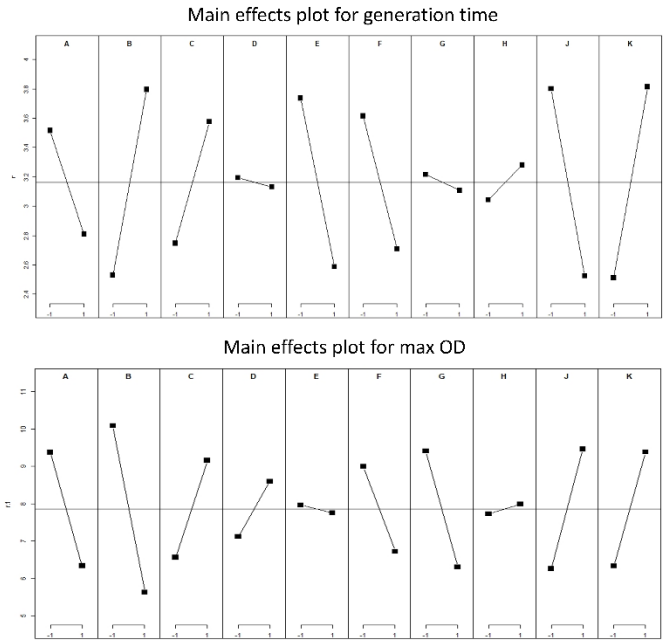
| Component |
|---|
| A (Na2HPO4) |
| B (KH2PO4) |
| C (NH4Cl) |
| D (NaCl) |
| E (CaCl2) |
| F (Thiamine) |
| G (MgSO4) |
| H (Tryptone) |
| J (Yeast Extract) |
| K (Black Liquor) |




Half normal plots from the Plackett-Burman design show the largest effects for each media component. Half normal plots were generated in R. Points further away from the origin represent variables that had the largest effect which was used to determine media components used in CCD.

| Component |
| --- |
| A (Na2HPO4) |
| B (KH2PO4) |
| C (NH4Cl) |
| D (NaCl) |
| E (CaCl2) |
| F (Thiamine) |
| G (MgSO4) |
| H (Tryptone) |
| J (Yeast Extract) |
| K (Black Liquor) |



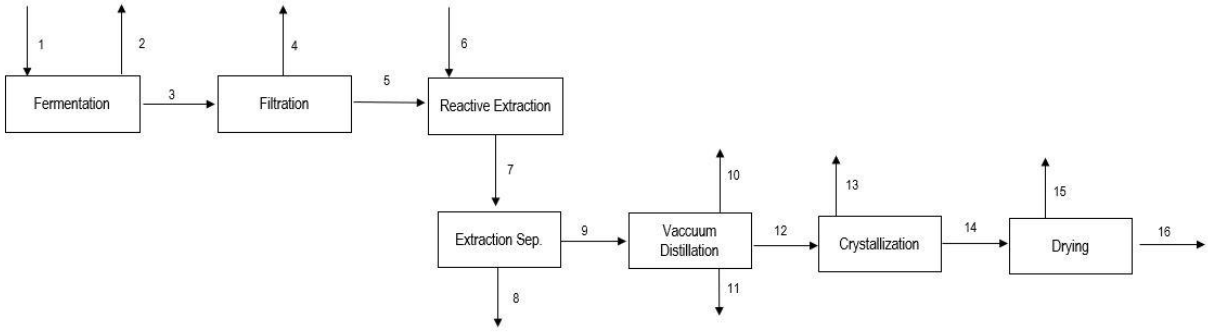

Main effects plots are another way to visualize effects of media components in a screening design. The variables with the largest slope represent larger effects. This was used in conjunction with the half normal plots for deciding factors for CCD. Factors were chosen for CCD based on largest effects from half normal plots, largest slopes in main effect plots, consistency between both response variables in the main effects plots.

## Experimental and Predicted Results of Central Composite Design

| Set | $KH_2PO_4$ | Yeast Extract | Black Liquor | Biomass Accumulation (g/L) | | Generation Time (h) | | Lactic Acid Production (g/L) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Actual | Predicted | Actual | Predicted | Actual | Predicted |
| 1 | 0 | 0 | 0 | 1.80 | 1.80 | 2.1 | 1.3 | 0.12 | 0.42 |
| 2 | 0 | 0 | 0 | 1.80 | 1.80 | 1.7 | 1.3 | 0.13 | 0.42 |
| 3 | +1 | -1 | +1 | 0.80 | 0.81 | 5.4 | 7.0 | 0.59 | 0.24 |
| 4 | -1 | +1 | -1 | 2.36 | 2.32 | 1.5 | 0.1 | 0.11 | 0.33 |
| 5 | -1 | -1 | +1 | 0.30 | 0.26 | 16.7 | 14.7 | -0.25 | -0.13 |
| 6 | +1 | -1 | -1 | 2.69 | 2.65 | 1.1 | 0.9 | 0.28 | 0.28 |
| 7 | 0 | 0 | 0 | 1.80 | 1.80 | 0.7 | 1.3 | 0.60 | 0.42 |
| 8 | -1 | +1 | +1 | 1.45 | 1.46 | 1.8 | 2.1 | 1.26 | 1.14 |
| 9 | +1 | +1 | -1 | 3.56 | 3.57 | 0.5 | 2.7 | 0.51 | 0.26 |
| 10 | -1 | -1 | -1 | 2.39 | 2.46 | 1.1 | 2.9 | 0.21 | 0.00 |
| 11 | +1 | +1 | +1 | 2.86 | 3.07 | 0.7 | -0.9 | 1.06 | 1.14 |
| 12 | 0 | 0 | 0 | 1.80 | 1.80 | 1.1 | 1.34 | 0.61 | 0.42 |
| 13 | 0 | 0 | 0 | 1.80 | 1.80 | 1.3 | 1.3 | 0.55 | 0.42 |
| 14 | 0 | +1.68 | 0 | 2.84 | 2.90 | 0.7 | 1.1 | 1.11 | 1.09 |
| 15 | +1.68 | 0 | 0 | 2.84 | 2.90 | 1.1 | 0.0 | 0.00 | 0.25 |
| 16 | 0 | 0 | 0 | 1.80 | 1.80 | 1.1 | 1.3 | 0.55 | 0.42 |
| 17 | -1.68 | 0 | 0 | 1.41 | 1.39 | 3.5 | 4.3 | 0.07 | 0.00 |
| 18 | 0 | 0 | -1.68 | 3.17 | 3.15 | 0.9 | -0.5 | 0.12 | 0.20 |
| 19 | 0 | 0 | +1.68 | 0.82 | 0.88 | 5.3 | 6.4 | 0.75 | 0.84 |
| 20 | 0 | -1.68 | 0 | 1.13 | 1.12 | 10.7 | 10.1 | -0.14 | 0.05 |
| Statistical outcomes | | | | $R^2$=0.71, p=0.064 | | $R^2$=0.92, p=0.0003 | | $R^2$=0.78, p=0.0203 | |

# Appendix B

## Preliminary Process Design for Techno-Economic Analysis




Simplified process flow diagram for the fermentation and separation of lactic acid. The process begins with a fermentation, where biomass is fermented with 20% black liquor and media composition corresponding to the optimum formulation from section 2.3.2. After fermentation, residual biomass is separated from the filtrate which then undergoes reactive extraction. After reactive extraction, the mixture is distilled to remove the organic phase and some water from the system leaving lactic acid and small amounts of other organic acid products. The lactic acid is then crystallized under vacuum and dried to achieve a 99% pure product.

| Component | Units | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Black liquor (sugar) | g | 4,000,000 | - | - | - |
| Black Liquor (inert) | g | 20,000,000 | - | 20,000,000 | - |
| Water | g | 1,940,000,000 | - | 1,940,000,000 | 94,146,293 |
| Nutrient (non-consumable) | g | 21,650,000 | - | 21,650,000 | - |
| Nutrient (consumable) | g | 60,000,100 | - | - | - |
| Biomass (Micro-organism) | g | 20,000 | - | 58,841,433 | 58,841,433 |
| Lactic acid | g | - | - | 500,000 | 100,000 |
| Other Organic Acids | g | - | - | 3,500,000 | 700,000 |
| CO2 | g | - | 1,178,667 | - | - |
| Solvent | g | - | - | - | - |
| **Total** | g | 2,045,670,100 | 1,178,667 | 2,044,491,433 | 153,787,727 |

Mass balances for streams one through four, describing fermentation and filtration processes. 2,021,690,900 g of material is put into the fermentation process. In stream 2 $CO_2$ is generated during the fermentation process and removed as exhaust. Stream three represents the fermentation broth where it is assumed that the consumable nutrient content is converted directly to biomass, except for the generation of $CO_2$ which is removed from biomass generation. All sugar content is converted to either lactic acid at a yield of 12.5%, and other organic acid products. Stream 4 is the filtered biomass at a ratio of 0.6:0.4 liquid to solid biomass.

| Component | Units | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|
| Black liquor (sugar) | g | - | - | - | - |
| Black Liquor (inert) | g | 20,000,000 | - | 20,000,000 | - |
| Water | g | 1,845,853,707 | - | 1,845,853,707 | - |
| Nutrient (non-consumable) | g | 21,650,000 | - | 21,650,000 | - |
| Nutrient (consumable) | g | - | - | - | - |
| Biomass (Micro-organism) | g | - | - | - | - |
| Lactic acid | g | 400,000 | - | 400,000 | 80,000 |
| Other Organic Acids | g | 2,800,000 | - | 2,800,000 | 2,240,000 |
| CO2 | g | - | - | - | - |
| Solvent | g | - | 94,535,185.33 | 94,535,185.33 | - |
| **Total** | g | - | - | - | - |

Mass balances for streams 5 through 8. These steps represent the reactive extraction of lactic acid from the fermentation broth. In stream 6, 5% solvent is added to the filtrate and mixed. After mixing, volatile compounds are vented in the extraction separation vessel causing the removal of some portion of organic acids (stream 8).

| Component | Units | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|
| Black liquor (sugar) | g | - | - | - | - |
| Black Liquor (inert) | g | - | - | - | - |
| Water | g | 1,845,853,707 | 369,170,741 | 1,107,512,224 | 369,170,741 |
| Nutrient (non-consumable) | g | 21,650,000 | - | 21,650,000 | - |
| Nutrient (consumable) | g | - | - | - | - |
| Biomass (Micro-organism) | g | - | - | - | - |
| Lactic acid | g | 320,000 | 64,000 | - | 256,000 |
| Other Organic Acids | g | 560,000 | 448,000 | - | 112,000 |
| CO2 | g | - | - | - | - |
| Solvent | g | 94,535,185.33 | 94,535,185.33 | - | - |
| **Total** | g | - | - | - | - |

Mass balances for stream 9 through 12. Stream 9 is sent through a distillation process, where lactic acid, mixed with water and some residual organic acids, are removed at a certain stage. During this process, non-consumed nutrients, solvent, and a large portion of water and other organics acids are removed. Stream 12 is then sent for crystallization.

| Component | Units | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|
| Black liquor (sugar) | g | | - | - | - |
| Black Liquor (inert) | g | | - | - | - |
| Water | g | 368,764,654 | 406,088 | 406,088 | - |
| Nutrient (non-consumable) | g | | - | - | - |
| Nutrient (consumable) | g | | - | - | - |
| Biomass (Micro-organism) | g | | - | - | - |
| Lactic acid | g | | 256,000 | - | 256,000 |
| Other Organic Acids | g | 112,000 | - | - | - |
| CO2 | g | | - | - | - |
| Solvent | g | - | - | - | - |
| **Total** | g | 368,876,654 | 662,088 | 406,088 | 256,000 |

Mass balances for stream 13 to 16. Stream 13 shows the removal of water and other organic acids that are present at this stage. The concentrated and crystallized lactic acid remains in the system and is further dried to remove excess water resulting in a pure product. The product yield from one batch is around 256 kg.

| Operation | T1 (C) | T2 (C) | Heat Capacity (kJ/kg C) | Mass (kg) | Heat (MJ) |
|---|---|---|---|---|---|
| Fermentation | 25 | 37 | 4.2 | 2,045,670 | 103,102 |
| Extraction Sep. | 37 | 37 | 4.2 | - | - |
| Distillation | 37 | 100 | 4.2 | 1,962,919 | 4,955,585 |
| Crystallization (cooling) | 100 | 50 | 4.2 | 369,539 | (77,603) |
| Crystallization (heating) | 50 | 100 | 4.2 | 369,539 | 912,761 |
| Drying | 50 | 100 | 4.2 | 662 | 1,635 |

Simplified energy balances for major unit operations.

| | | |
|---|---|---|
| | Required Heat from Natural Gas (MJ) | 6,194,481 |
| Energy Cost | $/MJ | 0.004 |
| Cost/Day to Run Distillation Process | $/Day | 24,777.93 |
| | Product | |
| Product at 12.5% conversion | Lactic Acid $/day | 256 |
| Product at 100% conversion | Lactic Acid $/day | 2048 |

Preliminary economic calculations. Distillation carries the highest energy requirement for the given process. The cost to run distillation for one day is orders of magnitude higher than the potential profit from produced lactic acid for both 12.5% and 100% conversion of sugars contained within weak black liquor diluted to 20% (w/w). This makes the process, as is, economically unviable. Potential improvements include the reduction of water used in the process (requires higher concentration of black liquor, limited by the organisms ability to grow in higher concentrations of BL), pretreatment and removal of sugars, or addition of sugars such as glucose for co-fermentation.