Tools for improving and understanding microbial performance in biorenewable applications

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Tools for improving and understanding microbial performance in biorenewable applications

by

Kirsten Davis

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Program of Study Committee:
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

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DEDICATION

This dissertation is dedicated to Grandma Johnny. Grandma you followed your dreams but always kept a generous and fiery spirit.
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ABSTRACT

The production of biorenewable fuels and chemicals is attractive because biomass is diverse, cheap, and abundant. Chemicals and fuels derived from renewable feedstocks can contribute to mitigation of climate change and improved rural economies. To compete with the petroleum industry, the biological processes involved in biorenewable fuel and chemical production need to be efficient. Here we engineer microorganisms and their environment for improved performance in biorenewable applications. In Chapters 1-4, we focus on lignin valorization. Lignin is a component of biomass that is underutilized, but abundant in renewable aromatics. We developed an emulsion formulation composed of Tween®20, Span®80, and a lignin-rich fraction of pyrolyzed biomass which enables \textit{P. putida} KT2440 to grow in a lignin-rich fraction of pyrolytic bio-oil. This type of emulsion could be applied for microbial conversion of lignin-rich feedstocks to valuable products. To determine more about the specific compounds that are being utilized by the \textit{P. putida} KT2440 and other lignin utilizing microorganisms, we developed a unique disk diffusion assay (\textit{Diffusive Inhibition with Substrate Consumption}) which allowed for simultaneous quantification of inhibition and utilization. The DISC assay could be useful for quickly and easily screening lignin monomers or other monomers when little is known about their toxicity or microbial degradability. In Chapters 5, we focus on improving the robustness of biological membranes. Microorganism growth and production can be inhibited by both biomass feedstocks and the biorenewable products. One component of that inhibition is due to membrane damage. There is still little known about the biomolecular effects of alcohols which are attractive biorenewable chemicals. Here we utilized artificial biological membranes to characterize the membrane damage caused by alcohols. These experimental results are being used to inform
an *in silico* model of a yeast plasma membrane which can be used to inform design strategies for more robust membranes. We observed increased fluidity and leakage in artificial phospholipid bilayers when treated with alcohols. Also, altering just 5% of the phospholipid head groups from phosphocholine to phosphoethanoamine somewhat improved the leakiness and fluidity. Therefore, small alterations in the phospholipid composition could be helpful in creating more robust membranes.
CHAPTER 1. INTRODUCTION: PRODUCTION OF BIORENEWABLE CHEMICALS BY MICROORGANISMS

Biorenewable chemicals can be designed to have desirable and unique functionalities and they can help mitigate climate change. Competition with a currently cheap and abundant oil feedstock means that it is vital to find a niche in the market. Shanks and Keeling’s 2017 paper recommends that chemical precursors be strategically selected. Conversion of a biorenewable feedstock by engineered microorganisms allows for a staggering amount of possible chemical precursors. A good chemical precursor target can be converted via chemical catalysis into a myriad of chemicals with desirable functionalities that are difficult to produce from a petroleum-derived feedstock [1]. Here I have investigated two complementing strategies for competing with a cheap and abundant oil feedstock in the production of biorenewable chemicals.

The first strategy is to select an equally cheap, abundant feedstock, lignin. Lignin is a waste product of paper milling but is also a highly abundant source of renewable aromatics. The current technology on lignin upgrading is reviewed in Chapter 2. Chapters 3 and 4 describe the investigation into bacterial utilization of lignin-derived monomers from pyrolysis.

The second strategy is to design a robust microorganism which can thrive in the conditions required for industrial conversion to biorenewable chemicals. Many of the conditions such as pH, temperature, and chemical toxicity can negatively affect the microorganism. In particular, the membrane can be damaged. The relationship between phospholipid membrane composition and chemical membrane damage is still not well understood. Chapter 5 describes an investigation into S. cerevisiae membrane damage.
Strategy one and strategy two can inform each other because lignin pyrolysate is particularly complex and contains many chemicals that are toxic to the cell. Once we know more about how the phospholipid membrane composition related to the chemical membrane damage, we can develop engineering strategies to produce more robust biological membranes. These kinds of engineering strategies could be applied in biorenewable chemical production regardless of the feedstock.

**References**

CHAPTER 2. RECOVERY AND UTILIZATION OF LIGNIN MONOMERS AS PART OF THE BIOREFINERY APPROACH

Modified from a paper published in *Energies*

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Abstract

Lignin is a substantial component of lignocellulosic biomass but is under-utilized relative to the cellulose and hemicellulose components. Historically, lignin has been burned as a source of process heat, but this heat is usually in excess of the process energy demands. Current models indicate that development of an economically competitive biorefinery system requires adding value to lignin beyond process heat. This addition of value, also known as lignin valorization, requires economically viable processes for separating the lignin from the other biomass components, depolymerizing the lignin into monomeric subunits, and then upgrading these monomers to a value-added product. The fact that lignin’s biological role is to provide biomass with structural integrity means that this heteropolymer can be difficult to depolymerize. However, there are chemical and biological routes to upgrade lignin from its native form to compounds of industrial value. Here we review the historical background and current technology of (thermo) chemical depolymerization of lignin; the natural ability of microbial enzymes and pathways to utilize lignin, the current prospecting work to find novel microbial routes to lignin degradation, and some applications of these microbial enzymes and
pathways; and the current chemical and biological technologies to upgrade lignin-derived monomers.

**Keywords:** valorization; depolymerization; laccase; aromatic; pyrolysis; organosolv

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**Introduction**

Lignocellulosic biomass includes a wide variety of plant material, such as crops, agricultural residue, and wood. Humankind has utilized biomass throughout history to produce: heat for warmth and cooking; biochemicals, such as the ethanol and lactic acid produced by fermentation; and biofibers, such as those used in clothing and other textiles [1]. Present-day utilization of lignocellulosic biomass instead of petroleum in the production of chemicals and fibers could contribute to the improvement of environmental quality, national security, and rural economic development [1].

One component of lignocellulosic biomass, lignin, has long been viewed as a low-value or waste product in the wood pulping industry. The most common pulping process is the Kraft process, where lignin is dissolved in hot sodium hydroxide and sodium sulfide [2]. The top three pulping processes are the Kraft process, the sulfite process, and the soda lignin process. These three processes produce 60–100 Ktonnes of Kraft lignin, 1 Mtonne of lignosulfonates, and 5–10 Ktonnes of Sulfur-free soda lignin per year, respectively [3]. Typically, lignin is used as a fuel to fire pulping boilers [4]. However, the energy produced through lignin combustion is about sixty percent greater than the demand [5]. Traditionally, only 1%–2% of lignin was isolated from pulping liquors and used for specialty products, such as dispersants or binders [6]. It follows that lignin has also been combusted as an energy source in the conversion of biomass to ethanol [7].
There is a vast collection of literature on lignin processing, including improving the recovery of lignin from biomass, depolymerization of lignin into monomers by chemical and/or biological means, and upgrading of the depolymerized lignin monomers to industrially relevant chemicals, which have been described in several other recent reviews (Figure 1) [2,5,8,9]. The purpose of this review is to summarize strategies from each of these processing steps and to briefly describe their economic relevance.

**Lignin Structure and Abundance**

Lignin is a stable aromatic heteropolymer that accounts for 10–35 wt% of lignocellulosic biomass [8]. Table 1 details the variation of lignin content in various lignocellulosic biomass types. Lignin is the second most abundant terrestrial polymer after cellulose, and it is the only large-volume renewable source of aromatics [10,11]. In nature, lignin functions as a matrix that holds the plant together and provides protection from environmental factors. The properties of lignin that benefit the plant are also the properties that make lignin difficult to access and convert to industrially relevant products. Although the structure and composition of lignin vary from plant to plant, during lignin production, the three primary lignin monomers coniferyl alcohol, sinapyl alcohol, and \( p \)-coumaryl alcohol are subject to polymerization so that the resulting lignin polymer is comprised of three phenylpropanoid monomeric units guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) (Figure 2) [12,13].

**Challenges and Progress in Lignin Recovery**

Lignin is recalcitrant and has a heterogeneous structure. In addition, the separation of lignin from biomass can be energy intensive and sometimes requires harsh chemicals. The lignin isolation methods in Table 2 use combinations of acid/base chemistry, high temperatures and pressures, solvents, and catalysts.
Pulping Processes

Kraft pulping is the dominant pulping process, with about 90% share of the total global production capacity, while less than 10% of pulp is produced by sulfite pulping and less than 5% by sulfur free alkali pulping [2,21]. In the Kraft process, cellulose is isolated from hemicellulose and lignin using sodium hydroxide and sodium sulfide. The heating value of the hemicellulose and lignin in the by-product liquor is high: 14–16 MJ/kg on a dry basis [22]. A chemical produced from the lignin in the black liquor needs to be of sufficient value to compensate for this loss of possible heat energy or only excess lignin should be diverted from process heat production [2].

Organosolv pulping uses low-boiling, organic solvents (typically sulfur free) for delignification. Commonly used solvents for organosolv are ethanol, methanol, organic acids, and mixed organic solvent–non organic alkali. Organosolv pulping is more environmentally benign than Kraft and sulfite pulping, and it allows for almost complete separation of cellulose, hemicellulose, and lignin. Research activities on organosolv biomass fractionation are increasing, but there is not a full-scale process to date [2].

Thermochemical Depolymerization of Biomass

Pyrolysis is the heating of biomass in the absence of oxygen. Fast pyrolysis converts biomass to a liquid (bio-oil), gas, and solid (char) product at moderately high temperatures (up to 500 °C). Up to 75% of the pyrolysis product is bio-oil, which contains compounds of similar structure to the original molecules [2]. However, there are a lower number of methoxyl groups on the pyrolytic lignin compared to the native milled wood lignin which is likely caused by demethoxylation of guaiacyl and syringyl moieties to form methanol [31]. The carbohydrate-derived compounds in the bio-oil have a higher affinity for water than the lignin-derived compounds. Therefore, separation of the lignin component can be done with
water, controlled deposition, or solvent extraction [2,32–34]. Biomass pyrolysis also produces a solid, known as bio-char, that can be used as a soil amendment for carbon sequestration and to improve crop production [35,36].

**Dilute Acid Hydrolysis**

In the dilute acid hydrolysis process known as the Biofine process, shredded biomass is added to dilute sulfuric acid. Then the product is subject to two stages of dilute acid treatment at high temperatures to hydrolyze polysaccharides into their monomeric units. A solid called Biofine char is produced, which has a very high heating value of 26 MJ/kg and is mainly comprised of ligneous type components according to thermogravimetric-Fourier Transform infrared spectroscopy (TG-FTIR). The Biofine process is highly advanced in the processing of polysaccharides. The polysaccharides are converted into levulinic acid, formic acid, and furfural. However, the use of the Biofine char has limited applications because it is acid insoluble [2,27].

**Hydrothermal Fractionation**

Hydrothermal fractionation is the heating of wood in hot-compressed water (200 °C and moderate hydrogen pressure) in the presence of a hydrogenation catalyst [2,29]. The main products are the lignin-derived aromatic monomers propyl guaiacol, propyl syringol, guaiacol propanol, syringyl propanol, and also hydrolyzed hemicellulose, which all remain in the aqueous phase. The advantage of hydrothermal fractionation is good product selectivity. However, it can be difficult to separate the hydrogenation catalyst from the wood residue [2].

**Biphasic Fractionation**

Biphasic fractionation can be used to separate the cellulose, hemicellulose, and lignin from each other. Solvents that have been applied to the organic phase include phenol [37–39], cresol [40], lignin-derived phenolic mixtures [41], polyethylene glycol [42–45], and 2-
methyltetrahydrofuran [46]. The hemicellulose components can be extracted by the aqueous phase, the lignin components can be extracted by the organic phase, and the cellulose can precipitate as a solid. Although biphasic fractionation is advantageous because it can be carried out at lower temperatures and near atmospheric pressure, the toxicity of some of the solvents could pose a challenge [2].

**Modeling of Lignin Isolation**

There is no precise equation for the amount of lignin extracted relative to the “severity” of treatment [47]. In 1987, an equation for the severity was proposed that depended on two parameters: temperature and time [48]. However, the equation was intended to estimate the impact of the treatment on the hemicellulose fraction of the biomass and not the lignin fraction, and there was no direct correlation between extracted lignin and the severity factor. In addition, the equation was not applicable for temperatures lower than 100 °C and it had limitations for catalyst usage. In 1990 and 2007, the severity factor was modified to reflect the effect of acid and base respectively on the severity factor, but the equation had to be modified by a factor of n depending on whether an acid or base was being used [49,50]. A recent study proposed an improved model that is universal for both acid and base treatments, and shows good correlation for one- and two-shot steam explosion, hot alkali macerations, and Kraft pulping with different types of biomass [47].

**Lignin Utilization in Nature**

In nature, lignin is utilized by specialized microorganisms encoding metabolic pathways that can break down components of lignin. Microorganisms that can break down lignin are able to use it as a carbon and energy source for metabolite production and have an advantage over biological organisms that can only utilize the cellulose and hemicellulose components of lignocellulosic biomass. Throughout this review, the phrases model lignin and
lignin model compounds will be used. Researchers often use lignin model compounds when investigating what types of products can be produced using biological or chemical catalysis. Lignin model compounds have similarities to the lignin structure, such as common linkages or common structure seen in lignin. Zakzeski et al. categorize the most commonly researched lignin model compounds into β-O-4 linkage, carbon-carbon linkage, β-5 linkage, α-O-4 and 4-O-5 linkage, and p-coumaryl, coniferyl, and sinapyl alcohol [51].

**Lignin Degrading Enzymes**

Lignin degrading enzymes must have properties distinct from cellulose or hemicellulose degrading enzymes. Hydrolytic enzymes that can cleave other plant material cannot cleave lignin because of lignin’s heterogeneous C–C and C–O linkages [52]. The enzymes responsible for the initiation of lignin polymerization in plants, low potential oxidoreductases, cannot oxidize the non-phenolic aromatic components of lignin [5]. However, some fungal and bacterial species do express enzymes that can break down the bulky and heterogeneous structures of lignin and/or convert smaller lignin-derived molecules into carbon and energy (Table 3) [53,54].

There are four major types of ligninolytic peroxidases: ligninolytic peroxidase (LiP), manganese-dependent peroxidase (MnP), versatile peroxidase (VP), and dye-decolorizing peroxidase (DyP) [5,55]. LiP, originally isolated from *Phanerochaete chrysosporium*, can oxidize molecules with high redox potential, including the moderately activated non-phenolic aromatics that can make up to 90% of the lignin polymer [5,56,57]. Unlike LiP, MnP cannot oxidize non-phenolics, and it is dependent on Mn$^{2+}$ ions. However, MnP can oxidize phenolic model lignin compounds [5,58]. VP can oxidize both non-phenolic and phenolic compounds [5,59]. DyPs are the most recently discovered ligninolytic peroxidases. DyPs are unique because they can oxidize hydroxyl-free anthraquinone [55]. Many dyes are derived
from anthraquinone, and therefore, it is present in dye-contaminated wastewater [55]. Anthraquinone is also used in the pulping process as a redox catalyst in papermaking [2]. White-rot fungi produce aryl-alcohol oxidase and glyoxal oxidase, and these oxidases produce hydrogen peroxide for the peroxidases [60,61].

Laccases are another class of enzymes contributing to the degradation of lignin. These copper-containing oxidases are found in bacteria and fungi, reduce molecular oxygen to water, and oxidize a large range of compounds including polyphenols, methoxy-substituted phenols, and diamines [62]. However, laccases are bulky and have non-phenolic sub-units that prohibit direct action on the lignin polymer. Instead, laccases have been shown to depolymerize lignin and lignin-derived molecules by action on smaller mediator molecules such as 2,2’-azino-bis(3-ethylbenzothiazolinesulphonic acid (ABTS) and hydroxybenzotriazole (HBT) [54,63,64].

**Bacterial and Fungal Pathways of Lignin Utilization**

The bacteria Alpha-proteobacteria, gamma-proteobacteria, Firmicutes, and some actinomycetes have been shown to modify or degrade lignin. However, a bioinformatic analysis has shown a higher proportion of lignin-degrading genes in proteobacteria and actinobacteria than in Firmicutes [54,65]. The metabolic pathways for aromatic degradation depend on the microorganism and its environment, particularly its oxygen availability (Figure 3).

**Aerobic Degradation**

In aerobic degradation, aromatic compounds derived from lignin are normally attacked by oxygenases with the help of O$_2$ [9,66]. The aromatic compounds are funneled to a few key molecules known as central intermediates, which can then be more easily converted into elements of the tricarboxylic acid (TCA) cycle. Hydroxylated central
intermediates such as catechol (1,2-dihydroxybenzene), protocatechuate (3,4-
dihydroxybenzoate), and less frequently gentisate or homogentisate, are normally produced
from aromatic monomers with the help of bacterial and fungal oxygenases [67–72]. The
hydroxylated products are activated for oxidative ring cleavage because they have electron
rich functional groups in ortho and para positions. The central intermediates are then
converted by ring-cleaving enzymes [73–78].

The β-ketoadipate pathway is a classic example of oxygenation and ring-cleavage. Dioxygen aromatic cleavage can proceed in the ortho position between the two hydroxy
groups or in the meta position adjacent to the two hydroxyl groups [9].

Another route to cleaving the aromatic ring, which may be an adaptation of low or
fluctuating $O_2$ environments, is epoxidation of CoA thioesters. In this route, $O_2$ is used to
form a non-aromatic epoxide. Then the ring is cleaved by hydrolysis and the molecule is
converted to TCA cycle intermediates. The epoxidation route occurs in bacteria to degrade
benzoate, phenylacetate, or compounds that can be broken into these two molecules. The
epoxidation route requires monooxygenases in the class I di-iron protein pathway. In the case
of benzoate and phenylacetate degradation, the monooxygenases act as epoxidases to
catalyze ring epoxidation. The epoxidation of CoA thioesters to degrade benzoate and
phenylacetate occur either as the only pathway or as an additional pathway in low oxygen
conditions in about 5% and 16%, respectively, of all bacteria that have a sequenced genome
[79–89].

**Anaerobic Conditions**

In anoxic conditions, $O_2$ can no longer be used as a co-substrate, and the aromatic
ring must be reduced, which is a demanding reaction. Reduction of the aromatic ring requires
agents with redox potentials that are much more negative than a physiological electron donor
could provide. Therefore, the anaerobic pathways use central intermediates with substituents that have an electron withdrawing effect [9].

A common intermediate in the anaerobic breakdown of aromatic compounds is benzoyl-CoA, where the electron-withdrawing substituent is the carboxyl-thioester group. The benzoyl-CoA type molecules can then be reduced by ring-reducing enzymes [9,72,90,91].

Another group of intermediates in the anaerobic breakdown of aromatic compounds is those with two or more hydroxy groups in the meta position relative to each other. When the hydroxy groups are in the meta position relative to each other, they polarize the ring, which facilitates the reduction of the aromatic compound [9,92]. There have been two main anaerobic routes discovered that degrade aromatics. In the first anaerobic route, aromatic ring cleavage can occur via benzoyl-CoA reduction, driven by ATP hydrolysis and catalyzed by class I benzoyl-CoA reductases [9,93]. It is proposed that the ATP-independent class II benzoyl-CoA reductase recently discovered in *Geobacter metallireducens* and other similar systems could be used as an anaerobic ATP-independent route to aromatic degradation [9,94].

**Application Directed Studies of Lignin Degrading Microorganisms**

Specialized microorganisms that contain the enzymes and reaction pathways described above could be harnessed with the following applications in mind: microbial utilization of aromatic-containing waste streams and microbial production of industrially relevant fuels and chemicals from lignin-derived aromatic monomers. There is also an ongoing search for novel enzymes, pathways, and microorganisms, often isolated from unique environments that are suited for use in these applications.
Our knowledge of fungal lignin-degrading enzymes far exceeds our knowledge of bacterial lignin-degrading enzymes. However, fungal systems are typically difficult to manipulate and slow acting. There is a push for utilization of bacterial systems, which are simpler and faster. Tropical soils are depleted of oxygen, limiting fungal growth as well as the oxygen-dependent activities of traditional peroxidases. The unique tropical soil environment was hypothesized to harbor anaerobic lignin degrading bacteria. Enterobacter lignolyticus SCF1 was isolated by anaerobically culturing tropical forest soils on minimal media with lignin as the sole carbon source [95]. E. lignolyticus SCF1 degraded 56% (wt/vol) of the lignin in a lignin/xylose growth medium within 48 hours. The E. lignolyticus SCF1 enzymes up-regulated in the presence of lignin included: catalase/peroxidase, DyP-type peroxidase, and two glutathione S-transferases (GSTs) [96,97]. As mentioned earlier, peroxidases are a key component of lignin degradation. However, it is still unclear exactly how peroxidases are involved in anaerobic lignin degradation. The presence of GSTs is evidence of a possible β-aryl ether cleavage mechanism in lignin degradation [97].

Several bacteria with aromatic degradation capability have been isolated from termite guts and woodboring beetles [54]. There is some debate on the extent that the microorganisms degrade lignin in vivo compared to the extent that microorganisms degrade lignin in vitro. One metagenomics study of hindgut microflora did not find any lignin degradation genes [98]. However, microflora from the same termite were able to degrade lignin in vitro [99]. In another study on the microflora from Anoplophora glabripennis and Zootermopsis angusticollis, lignin was depolymerized, demethylated, and ring-hydroxylated. The aerobic reactions required for lignin depolymerization observed in A. glabripennis and Z.
*angusticollis* indicate that some of the lignin degradation occurs in the foregut rather than in the hindgut, which is mostly anaerobic [99,100].

In order to identify novel lignin degrading microorganisms, a fluorescent transcriptional reporter system was used as a biosensor [101]. This biosensor can respond to specific lignin degradation products such as vanillin, vanillic acid, and p-coumaric acid and was used to screen a DNA library prepared from metagenomes of coal beds. DNA fragments that were enriched were isolated, and the corresponding lignin transformation genes were identified. Recurring subsets of gene functions included: oxidoreductase activity, co-substrate generation (hydrogen peroxide generation), protein secretion, small molecule transport (multidrug efflux superfamily), motility (methyl-accepting chemotaxis proteins), and signal transduction [101]. Oxidoreductase activity, hydrogen peroxide generation, and protein secretion are associated with lignin degradation [54,102]. It was concluded that the small molecule transport systems had a role in regulating microbial responses when exposed to aromatic monomers. The cell motility was proposed to have a role in facilitating optimal positioning, which may be important in environments with microscale physicochemical gradients [101,103]. Signal transduction proteins could play a role in mediating lignin specificity in a microbial community [101].

A study of the structure and biochemistry of *Streptomyces* enzymes gave insight into unique laccase binding capability. SACTE_2871 is found in a *Streptomyces* species isolated from the Pinewood-boring wasp. SACTE_2871 can catalyze O$_2$-dependent ring opening of catechols. Catechols are often intermediates in the breakdown of lignin-derived molecules. SACTE_2871 can also directly bind to synthetic lignin polymers [5,104]. Similarly, small laccases found in *Streptomyces* species have been found to be able to bind directly to non-
phenolic model lignin compounds and rearrange non-phenolic compounds with the help of mediators. The small laccases can also oxidize phenolic β-O-4 linkages [5,105].

Studying the utilization of lignin-derived compounds in nature can be important in tracking the global carbon cycle and monitoring the degradation of pollutants. For instance, the lignin biphenyl component can account for up to 10% of the lignin structure. The biological fate of the lignin biphenyl component is therefore linked with the degradation of lignin. Bacterial biphenyl degradation is well documented in a number of genera and has been reviewed elsewhere [54]. The pollutants benzene, toluene, ethylbenzene, and xylenes (BTEX), naphthalene, and 2-methylnaphthalene are all aromatic in nature, have structural similarities to lignin-derived molecules, and can be degraded anaerobically [9,106].

The conversion or upgrading of lignin to a higher value molecule could contribute to a more cost-effective biomass processing scheme as discussed further in the economics section. Model lignin-derived compounds can converge via the downstream production of vanillin and vanillic acid before being converted to protocatechuic acid [7]. Extensive research on vanillin production via microorganisms, partially motivated by the global demands for vanillin (12,000 tons/year), has been reviewed elsewhere [107,108].

**Challenges and Progress in Depolymerization of Isolated Lignin**

Even when lignin is isolated, it often needs to be depolymerized into smaller molecules before it can be upgraded. There are five major methods of depolymerization (Table 4): pyrolysis of isolated lignin, catalytic hydrogenolysis, supercritical depolymerization, solvent depolymerization, and alkaline hydrolysis.

**Pyrolysis of Isolated Lignin**

Pyrolysis is a simple and fast process to depolymerize lignin. The bio-oils obtained from pyrolysis of isolated lignin are complex mixtures of hundreds of phenolic monomers.
and oligomers, with no specific compound making up more than 1% of the total product weight. It is known that the products of lignin pyrolysis differ by biomass type. Pyrolysis of hardwood lignin produces both syringol and guaiacol-type phenols, whereas pyrolysis of softwood lignin produces mostly guaiacol-type phenols. Pyrolysis of herbaceous lignin produces a mixture of syringol, guaiacol and phenol types of compounds [132,133]. An investigation of the thermal decomposition of lignin derived from both herbaceous (rice straw and rice husk) and woody (maple) biomass used TG-FTIR and pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS). There were three mass loss stages observed: the evaporation of water, the evolution of aromatic compounds, and the release of light gasses. It was found that more phenolic compounds, methanol, and methane evolved from maple lignin. Maple lignin was also the most thermally unstable because it formed phenolic compounds earlier than the herbaceous lignin. However, the formation of carbon dioxide was higher in herbaceous lignin than in maple lignin. Py-GC/MS analysis revealed that evolution of phenol-type and aromatic compounds increased with increased temperature due to more demethoxylation and dihydroxylation reactions [133]. Quantifiable phenolic monomers account for up to 17 wt% of the pyrolysis oil, [134] depending on the lignin feedstock. The majority of the compounds in the lignin-derived bio-oil are phenolic oligomers. It has been shown that during pyrolysis of lignin derived from corn stover by organosolv treatment, phenolic monomers and dimers are mainly produced. However, the reactive monomers can rapidly repolymerize [135]. Isolated lignin contains an increased amount of C-C bonds, which is more resistant to thermal depolymerization. Coupled with free radical initiated repolymerization during pyrolysis, isolated lignin could produce over
40% char [136,137]. Approaches to reduce char formation could significantly enhance lignin volatilization.

**Catalytic Pyrolysis of Isolated Lignin**

The addition of a catalyst to the pyrolysis reactor can improve product selectivity [118]. For instance, the use of a solid acid catalyst, such as HZSM-5 zeolite, can convert the wide range of phenolic compounds to a smaller number of aromatic hydrocarbons, such as benzene, toluene, and xylene (BTX). Other types of catalysts, such as HY zeolite [138], Al-MCM-41, (CoO/MoO$_3$) and Co/Mo/Al$_2$O$_3$ [109,110] have also been tested for lignin pyrolysis. However, these catalysts are less efficient in deoxygenating lignin compared to HZSM-5. Challenges with catalytic pyrolysis of lignin include coke deposits on the catalyst [2,109–113] and low product yield.

In catalytic hydropyrolysis, external hydrogen can help to stabilize reactive free radicals formed during lignin depolymerization and promote hydrodeoxygenation. Hydrocracking also lowers char and coke yields [139]. Under high partial pressure H$_2$ and in the presence of Ru/C catalyst, Alcell organosolv lignin was converted into cycloalkanes, alky-substituted cyclohexanols, cyclohexanol and linear alkanes [140]. A wide range of supported catalysts, Ru (C, Al$_2$O$_3$, and TiO$_2$), Pd (C, and Al$_2$O$_3$), and a Cu/ZrO$_2$, were also screened for catalytic hydrotreatment of Alcell lignin. It was found that Ru/TiO$_2$ outperforms other catalysts, yielding a mixture of alkylphenols, aromatics, and catechols [141]. The complex oil mixture formed during catalytic hydropyrolysis is analogous to the bio-oil formed during pyrolysis. However, there is a lower oxygen content in catalytic hydropyrolysis oil, which makes it more stable than pyrolysis bio-oil [114,115].

Incorporating transitional metals into HZSM-5 was beneficial because the bifunctional catalyst has both deoxygenation and hydrogenation abilities. Pyrolysis of steam-explosion
hybrid-poplar lignin using 1 wt% Pd/HZSM-5 at 1.7 MPa of H₂ produced 44% more aromatic hydrocarbons compared to HZSM-5 as the catalyst. Due to high partial pressure of hydrogen, saturation of the benzene ring occurred and cycloalkanes were found among the products [142].

**Supercritical Water**

In the supercritical and subcritical treatment of lignin, there is a lower concentration of lignin compared to catalytic hydropyrolysis of dry lignin, and therefore the probability of undesirable condensation reactions is lower. However, the process heat required for the production of supercritical water is high and the economic viability depends on process heat recovery. Alkali salts have been shown to improve oil production, however, the maximum theoretical yield of low molecular weight products is only one-third of the total lignin weight. The addition of phenol, butanol, and boric acid has been shown to help the depolymerization of lignin and to increase the selectivity of the desired oil product [119–123]. In the case of phenol, butanol, and boric acid addition, products will be biphenyl dimer structures, which can be used as a high boiling solvent. Alternatively, the dimers can be cracked into two aromatic monomers and be partially recycled into the process [2].

**Supercritical Solvents**

Supercritical solvents such as ethanol [124–127], methanol [128,129], CO₂/acetone/water [130], and butanol [122] have been used to dissolve isolated lignins at temperatures between 200 and 350 °C and high pressures. Mixtures of alcohols and water have also been utilized at milder pressures [127,143,144]. Lignin solvolysis can be categorized into either base-catalyzed depolymerization or hydrogenolysis. The hydrogen used for hydrogenolysis can come from a variety of sources, such as external hydrogen supply, a proton donor such as tetralin [144] and formic acid added to the solvent [145,146],
or partial reforming of the solvent in the presence of a metal catalyst [129]. When hydrogen donating solvents are used for depolymerization, the presence of a hydrogenation catalyst stabilizes lignin depolymerization products and therefore increases the yield of phenolic monomers. Conversion of birch wood lignin in alcohols (methanol, ethanol and ethylene glycol) using Ni-based catalyst resulted in a phenolic oil with the selectivity of propylguaiacol and propylsyringol higher than 90% [147]. Cyclic hydrocarbons (primarily monomeric substituted cyclohexyl derivatives) can be formed from supercritical solvolysis. The lower boiling point of the cyclic hydrocarbons allows for separation and purification at lower temperatures, and the lower temperatures help to prevent repolymerization reactions known to happen at higher temperatures [2].

**Base-Catalyzed Depolymerization**

Lignins can be used to produce low molecular weight compounds when subjected to high temperature and pressure in the presence of a base in aqueous or organic solution. This process is known as base-catalyzed depolymerization [131]. A study of the base-catalyzed depolymerization of three different organosolv lignins (acetosolv, acetosolv/formosolv, and formosolv) showed that a higher yield of desired oil product was achieved from base-catalyzed depolymerization of acetosolv and acetosolv/formosolv lignins. Undesired coke production was low in the acetosolv lignin but higher when formosolv was included or used by itself, indicating that formic acid decreased the effectiveness of the catalyst. However, the formosolv oil contained higher amounts of phenolic monomers because the formosolv lignin had the lowest molecular weight. Base-catalyzed depolymerization produces only about 20% (wt/wt) oil when compared to the total product that contains repolymerized lignin fragments formed from condensation fragments and a coke by-product [12]. In order to improve the yield of phenolic monomers, boric acid and phenol capping agents were compared in base-
catalyzed depolymerization of pruned olive tree branches. When phenol was used as a capping agent, the yields of the phenolic monomers were higher than with no capping agent or with a boric acid capping agent. Boric acid did prevent repolymerization, but the char production was higher compared to the phenolic capping [12].

Upgrading of Lignin Monomers

Depolymerized lignin monomers can be further upgraded into industrially relevant chemicals by biological or chemical processing. The chemical processing can be similar to lignin isolation and lignin depolymerization, and sometimes there are not clear distinctions between isolation, depolymerization, and upgrading (as discussed in Section 6.2).

Progress in Biological Utilization of Depolymerized Lignin Monomers and Lignin Model Compounds

There are two main approaches in the application of microbes to the upgrading of lignin. One approach is a biotransformation in which only a few catalytic steps are utilized from one target reactant to one target product. The other approach is to funnel a number of target reactants through the central metabolism of the microbe and tune the target product based off of industrial relevance. Chemicals produced from lignin-derived substrates or pure compounds known to be present in lignin are listed in Table 5.

Biotransformation

Vanillin can be produced by a number of specialized microorganisms from aromatic molecules such as eugenol [151–157], isoeugenol [157–168], ferulic acid [154,162,169–186], vanillic acid [172,174], and green coconut husk [187]. However, the low titers of vanillin and degradation of vanillin by the microorganisms are problematic [107].
Central Metabolism

Instead of looking at the capability of a microorganism to transform one lignin monomer into one product, microorganisms can be harnessed to utilize multiple substrates, addressing the challenge of the heterogeneous nature of lignin. This funnelling strategy was demonstrated by microbial utilization of alkaline pretreated liquor (APL), which contained 35% lignin derived molecules. Both low molecular weight lignins (200–400 Da) and high molecular weight lignins (as high as 30,000 Da) were present [53,148–150]. Fourteen taxonomically diverse microorganisms were tested for their ability to depolymerize lignin, uptake biomass-derived molecules such as aromatic monomers, produce extracellular oxidative enzymes, and accumulate carbon storage products from the lignin derived molecules when grown in APL. *Amycolatopsis* sp., *P. putida* KT2440, *P. putida* mt-2, and *Acinetobacter* sp. were the top lignin converters, demonstrating 15%–20% lignin conversion in nitrogen limiting conditions, and 22%–31% lignin conversion in nutrient rich conditions. These species were also able to utilize a wide molecular weight range of lignin. *R. jostii* could not depolymerize the high molecular weight lignin, but *R. jostii* did convert a high percentage of lignin overall by demonstrating 20% lignin conversion in nitrogen limiting conditions, and 26% lignin conversion in nutrient rich conditions.

It follows that the top lignin converting species consumed the major aromatic monomers in the APL and also produced laccase and peroxidase enzymes. The three Pseudomonads and *Cupriavidus necator* H16 produced high amounts of laccases, 3–6 mU/mL, in nutrient rich conditions. *P. putida* KT2440 produced the most laccase enzymes at day five of the seven-day incubation in nutrient-rich conditions, a total of 6 mU/mL. Although *C. necator* H16 was not a top lignin converter, it produced the most Mn$^{2+}$ independent peroxidases at 6 mU/mL by day two of the seven-day incubation. *Pseudomonas*
*fluorescens* Pf-5, *Rhodococcus erythropolis* U23A, and *P. putida* KT2440 all produced over 3 mU/mL of Mn$^{2+}$ peroxidases. The three Pseudomonads, *C. necator* H16, and *Enterobacter lignolyticus* SCF1 all produced over 2 mU/mL of Mn$^{2+}$ oxidizing enzymes in nitrogen limiting conditions. In nutrient-rich conditions, the three Pseudomonads, *C. necator* H16 all produced over 7 mU/mL of Mn$^{2+}$ oxidizing enzymes with *Pseudomonas putida* KT2440 producing 11 mU/mL.

Four of the five top lignin converters stored carbon as fatty acids or polyhydroxyalkanoates (PHAs) under nitrogen-limiting conditions. *Acinetobacter* sp. was the only top lignin converter that did not store carbon [149]. *P. putida* KT2440 stored 0.25 g/L medium chain length PHAs from APL. As a proof-of-concept, the medium chain length PHAs were subjected to thermal depolymerization and catalytic dehydrogenation to produce hydrocarbons [150].

Other target compounds can be produced by native lignin-utilizing microbes that have been subjected to additional metabolic engineering. For example, *P. putida* KT2440 was engineered to utilize both the protocatechuate and the catechol branches of the β-ketoadipate pathway to produce muconic acid. The engineered *P. putida* KT2440 produced muconic acid from a variety of model aromatic molecules including catechol, phenol, benzoate, protocatechuate, coniferyl alcohol, ferulate, vanillin, caffeate, *p*-coumarate, and 4-hydroxybenzoate. In fed-batch culture, the engineered *P. putida* KT2440 produced muconic acid at a titer of 13.5 g/L from *p*-coumarate in 78.5 hours. This muconic acid was purified and converted to adipic acid with a Pd/C catalyst. However, when APL was used as a substrate in shake flasks, only 0.7 g/L muconic acid was produced. While this production represented 67% yield of the two major aromatics detected in the APL (*p*-coumarate and
ferulic acid), the titer is much lower than that observed from pure substrates [148]. This is consistent with the use of biomass-derived sugars relative to pure substrate [36]. In this case, the APL contained both aromatic and non-aromatic compounds, and *P. putida* KT2440 did not convert all aromatics at the same efficiency.

Making changes to the catechol and protocatechuate pathways might improve production of target products, such as muconic acid, or change the target products altogether. The position where catechol or protocatechuate are cleaved affects the amount of succinate, acetyl-CoA, and pyruvate produced. For example, when the endogenous catechol ortho pathway in *P. putida* KT2440 was exchanged with the exogenous catechol meta pathway, the pyruvate yield increased from 23.9 ± 3.1 to 31.0 ± 0.9 percent. When the endogenous protocatechuate ortho pathway was replaced by the exogenous protocatechuate ortho pathway, the pyruvate yield increased almost five-fold [53].

**Progress in Chemical Utilization**

Lignin can undergo many chemical modifications including, but not limited to, alkylation, acylation, amination, carboxylation, halogenation, oxidation, reduction, nitration, and sulfonation [188].

Figure 4 shows the major thermochemical depolymerization processes in conjunction with the produced products [188]. Zakzeski describes three categories of catalytic lignin transformations: lignin catalytic cracking and hydrolysis, lignin reduction, and lignin oxidation. These processes have been employed with lignin substrates, lignin model compounds, and depolymerized lignin [51].

Liquefaction processes produce monophenolic compounds that can be converted to liquid fuels by hydrodeoxygenation [189]. Monomeric, aromatic-based compounds have also been obtained by steam treatment followed by base-depolymerization to generate two
fractions: a monomeric fraction and a dimeric and trimeric fraction [190]. The yield of the monomeric fraction was as great as 15 wt% of the initial lignin and included phenolic species such as vanillin, guaiacol, phenol, and catechol. Monomers provide an opportunity for green aromatic-based compounds [190].

Pyrolysis is viewed as one of the most promising thermochemical technologies for lignin utilization [191,192]. The main compounds produced from lignin during fast pyrolysis are gaseous hydrocarbons (i.e., CO\textsubscript{2}, CO), volatile liquids (methanol, acetone and acetaldehyde), monolignols, monophenols (phenol, guaiacol, syringol, and catechol) and other monosubstituted phenols [188].

Lignin is the key biorenewable source of aromatic compounds with phenolics, for example, vanillic acid, syringic acid, ferulic acid, syringol, guaiacol, and eugenol attracting the interest of polymer chemists [51,193–196]. They are also valuable building blocks for synthesis of bisphenols [194,197–199], aliphatic-aromatic polyesters [194,199–201], polyethylene terephthalate mimics [194,202], and epoxy resins [194,203–205]. Additionally, there is strong interest in the continued development of polyurethane precursors originating from renewable resources [194].

**Cracking and Hydrolysis of Depolymerized Lignin**

In lignin catalytic cracking, the $\beta$-O-4 linkage is cleaved, and the carbon-carbon bonds are relatively unstable [206]. The zeolite H-ZSM-5 has been used for catalytic cracking of pyrolytic lignin [109,207–210], pyrolytic oil [211], and model compounds obtained from flash pyrolyzed vegetable biomass [212]. Products obtained from catalytic cracking with H-ZSM-5 can include aromatic hydrocarbons, aliphatic hydrocarbons, alcohols, and undesired coke product [51]. Other catalysts such as Pt/Al\textsubscript{2}-SiO\textsubscript{2} [213],
supported or non-supported Pt-modified superacid catalysts, and metal-loaded large pore zeolites have also been successful in catalytic cracking of biomass derived substrates [109]. In the non-zeolite catalytic cracking, products can include aromatics and phenolic compounds [51].

**Reduction of Lignin Model Compounds and Depolymerized Lignin**

After the lignin is depolymerized using methods described previously in Section 5, the depolymerized lignin (oil) can be upgraded using similar catalysts. Initial hydrogenolysis or hydrocracking studies of phenol, o-cresol, anisole, catechol, syringol, and guaiacol revealed that removal of oxygen for the purpose of increased stability could be done under milder conditions than required for thermal fragmentation and deoxygenation [214–216]. Hydrodeoxygenation of guaiacol has yielded phenol or catechol, although phenol is the preferred product at higher temperatures [214,216]. Depending on the catalyst and temperature, anisole can yield phenol, o-cresol, and 2,6-dimethylphenol [214,216]. Further hydrodeoxygenation of the phenol (produced from guaiacol or anisole) can yield benzene and cyclohexane [216]. Excellent conversion of guaiacol and 77% selectivity of phenol was achieved at 598 K, 5 MPa H₂, with a Co-Mo/Al₂O₃ catalyst [216]. Catechol has been shown to be more reactive than phenol itself when subject to hydrodeoxygenation with a Ni-Mo/Al₂O₃ catalyst at 623 K [217]. A mixture of bio-oil model compounds has also been subject to hydrodeoxygenation with Co-Mo and Ni-Mo catalysts, and the catechol component of the bio-oil was converted to phenol [218].

Key conclusions were drawn from studies of hydrodeoxygenation of the lignin-derived phenolic model compounds. Higher temperatures caused rapid deactivation of the catalyst, which was attributed to large amounts of water release, coke formation, and loss of sulfur. However, below 523 K, the catalyst stayed active for 50 h [214]. In addition, the
alumina supports for catalysts have shown activity. In fact, when neutral supports such as carbon replaced the alumina, lower activity was observed. However, polycondensation products and coke formation are thought to be associated with the alumina support [218]. When an activated carbon supported Co-Mo catalyst was used instead, there was negligible coke production [219]. The range of lignin-derived model compounds was increased by the hydrotreatment of 4-methylguaiacol, 4-methylcatechol, eugenol, vanillin, o,o′-biphenol, o-hydroxydiphenylmethane, and phenyl ether using a Co-Mo/Al₂O₃ catalyst (523–598 K, 6.9 MPa). Substituted guaiacols and catechols could react to form thermally stable phenols at 573 K [220].

In exploring different iron and molybdenum catalysts on lignin-derived model compounds, it was found that the molybdenum catalysts significantly increased the aromatic bond cleavage, and the iron catalysts only slightly increased the aromatic bond cleavage. Therefore, molybdenum catalysts are better candidates for the production of monophenol and benzene in the hydrocracking process [221]. In order to study the effects of a promoter for the supported molybdenum catalyst, lignin-derived phenolic compounds were subject to hydrodeoxygenation over a Co-Mo/Al₂O₃ catalyst. It was found that 4-propylguaiacol was converted to phenol at temperatures lower than 573 K, but at temperatures greater than 673K, saturated and aromatic hydrocarbons were produced instead. A Ni-Mo catalyst with a more acidic support was shown to have higher dealkylation activity, which resulted in higher yields of cresols and phenol [222].

As mentioned before, the traditional hydrodeoxygenation catalysts discussed above encounter problems with deactivation by coke formation and poisoning by water [51]. With the common problems of traditional catalysts in mind, different metals and supports were
tested for hydrodeoxygenation of anisole. Zirconia and ceria supports were found to be the most effective, and in a comparison of a Ni-Cu/ZrO$_2$ and Ni-Cu/CeO$_2$, the former produced mostly aromatics from anisole, and the latter almost fully converted anisole to cyclohexane. In addition, rhodium catalysts performed well for the production of aromatics in some cases [223]. In the interest of using supported platinum-group catalysts, which are known to be more active than sulfided molybdenum catalysts and can be used at lower temperatures, Ru/C and Pd/C were tested for catalytic hydroprocessing of guaiacol. Substrate hydrogenation and loss of aromaticity were observed using both catalysts [224]. Similarly, Pd/C, Pt/C, or Ru/C combined with mineral acids were used to completely hydrogenate and deoxygenate phenols, guaiacols, and syringols to produce cycloalkanes and methanol [225]. Hydrotreatment of pyrolytic lignin with a Ru/C catalyst produced cycloalkanes, alkyl substituted cyclohexanols, cyclohexanol, and linear alkenes [140]. The catalyst types discussed above such as Ru/C are therefore too active for maintaining the aromaticity of the lignin model compounds or depolymerized lignin [51].

In order to try to maintain the aromaticity, guaiacol or catechol was subject to reductive deoxygenation in the presence of α-terpinene and a vanadium or alumina catalyst at atmospheric pressure. Phenol and methyl-substituted phenols were produced at high yield and selectivity [226].

Electrocatalysis has been researched as a possible route for efficient lignin degradation by hydrogenation [51]. Electrocatalysis of the model lignin compound 4-phenoxypheanol with Raney Ni and Pd supported on alumina and carbon showed high efficiencies of electrohydrogenolysis to phenol [227].
A few studies have been done with homogeneous catalysis of lignin-derived phenolic compounds. A di-μ-chlorobis (η⁴-1,5-hexadiene)-dirhodium(I) complex catalyzed the lignin-derived model compounds 4-propelphenol, eugenol, 1,2-dimethoxy-4-propylbenzene, and 2,6-dimethoxy-4-propylphenol. The temperature was 298 K and the medium was two-phase hexane/aqueous [228].

**Oxidation of Lignin Model Compounds and Depolymerized Lignin**

In the oxidation of lignin model compounds, the goal is to create more complex aromatic molecules, which could be industrially relevant. Although oxidation of lignin historically comes from the pulping industry, this review will focus on upgrading of monomers by oxidation. The Ng/MiO catalysts have been shown to oxidize phenolic, nonphenolic, monomeric, and dimeric lignin model compounds. Vanillyl and veratryl alcohol were oxidized to acids, aldehydes, and quinones (49% yield) and polymeric products [229,230]. In another oxidation study of lignin model compounds, methylrhenium trioxide was used to catalyze the oxidation of isoeugenol or *trans*-ferulic acid in the presence of hydrogen peroxide to produce vanillin [231]. Wet oxidation of ferulic acid was carried out by single metal, bimetal, multimetal, and multimetal oxide alumina or kaolin supported catalysts. Cu-Mn/Al₂O₃ was the most stable catalyst studied and it was the second most active catalyst [232]. An electrocatalysis study carried out the anodic oxidation of lignin model compounds in methanol, and it was shown that the Cα-Cβ bond was cleaved [233].

In the study of homogeneous catalysts for oxidation, the idea of biomimicry has been used [51]. Originally iron and manganese porphyrin catalysts were used to better understand the enzymatic degradation of lignin, and it was shown that the iron porphyrin catalysts cleave the Cα-Cβ and oxidize lignin model compounds [234].
Metalloporphyrin catalysts are well studied in the selective oxidation of hydrocarbons and therefore are of interest for selective oxidation of lignin and lignin model compounds and have been reviewed by Crestini and Tagliatesta [51,235]. High conversion (67%) was achieved in the oxidation of veratryl alcohol with free and ion-exchange resin-immobilized Fe(TPPS) and Mn(TPPS) complexes using KHSO$_5$ as an oxidant [236]. Several other metalloporphyrin or metalloporphyrin-like catalysts have been used to oxidize lignin model compounds including iron(III) and manganese(III) meso-tetraphenylporphyrin and phthalocyanine complexes [237], iron porphyrin catalysts [238], and trisodium tetra-4-sulfonatophthalocyanineiron(III) [239]. The incorporation of a variety of ring substituents, the incorporation of axial ligands, and the immobilization of the metalloporphyrin can improve stability, tenability, and recyclability of the catalyst [51].

Simple metal salt-based catalysts have been used for oxidation of lignin and lignin model compounds. Co(II) acetate and Mn(II) acetate were used as catalysts in the single-electron oxidation of a lignin model compound, and it was found that the oxidation occurred primarily by cleavage of the C$_\alpha$–C$_\beta$ bond [240].

A well-known example of adding value to lignin monomers involves the oxidative production of vanillin from spent sulfite liquor. A 227,000 kg/year facility was built for this purpose in Thorold, Ontario in 1945 and by 1981 was producing $3.4 \times 10^6$ kg/year, accounting for more than half of the world vanillin market [241]. However, the disposal of the waste generated by this process eventually led to this process falling out of favor, with the Thorold plant closing in 1987.

**Economic Analysis of Lignin Utilization Strategies**

The adage that “you can make anything from lignin except money” is well-known in the biofuels and pulp and paper industries. The technological advances reviewed here
regarding lignin recovery, depolymerization and upgrading are chipping away at this long-held belief. This establishment of lignin as a source of value appears to be critical to the economic viability of the biorefinery concept. An economic analysis of the utilization of lignocellulosic biomass relies on a number of factors including cost of the biomass feedstock, capital costs, operating costs, and the market size and selling price of the target product(s). The utilization of a lignin “waste” stream in an existing lignocellulosic biomass processing facility could provide an additional source of income for the facility. However, a detailed analysis is needed to determine if the additional income from selling lignin or a lignin-based product would exceed the required capital and operating costs for producing the purified lignin and/or lignin-based product, as highlighted by the example of vanillin production. Since existing lignocellulosic biomass processing facilities often utilize lignin for process heat and electricity, it would also be important to determine what fraction of the available lignin should be diverted to upgrading. The advantage of choosing a platform chemical as a target product is that it provides flexibility. Instead of targeting one product and one application, a platform chemical that can be converted into a variety of downstream products would help with marketability.

Multiple reports have concluded that selling lignin as a co-product contributes to the economic viability of biofuels. A comprehensive 2013 report by the US National Renewable Energy Laboratory (NREL) concluded that achievement of the target value of 3.00 US dollars per gallon of gasoline equivalent fuel required lignin valorization [242]. Kautto et al. modeled the organosolv-based production of ethanol from hardwood with lignin, furfural, and acetic acid as co-products [243]. Consistent with the NREL conclusion, the value of the lignin product was a strong determinant of the minimum ethanol selling price. Specifically, a
value of 1.00 US dollars per kg of lignin was required for the ethanol to be sold at market price. Analysis of the production of ethanol from corn stover using ionic liquids for biomass deconstruction concluded that if 65% of the lignin was recovered and sold, a lignin selling price of 2.62 US dollars per kg was sufficient to meet the market price for ethanol [244]. Finally, at least one technoeconomic analysis has included a specific upgrading method for the lignin. Chen and Fu modeled the production of ethanol from corn stover with lignin plastic composite and compressed natural gas as co-products, where the natural gas is produced from the spent fermentation media [245]. This analysis predicted that inclusion of these two co-product streams resulted in a 19% decrease in the ethanol production cost.

Studies have also compared how different lignin utilization strategies impact the process economics, though these have mainly compared the use of lignin to produce steam and electricity to the use of lignin as a soil amendment. Petrou et al. [246] compared corn stover-based ethanol processes in which lignin is burned to produce electricity and steam to processes in which lignin is modified to produce lignosulfonates and/or geomaterial. This study concluded that burning the lignin to produce steam and excess electricity was the top economic performer, but that the scenario in which lignin was used as a geomaterial was the best in terms of environmental performance [246]. Pourhashem et al. [247] analyzed the production of ethanol from agricultural residues, such as corn stover and barley straw, with the use of lignin as a soil amender, as a coal substitute to produce electricity or for the on-site production of electricity. The use of lignin as a soil amender was deemed the best in terms of both greenhouse gas intensity and capital cost.
As the technologies associated with lignin recovery and upgrading develop, future economic analyses can incorporate these processes and provide additional insight into which routes are the most promising for industrial use.

References


**Figures and Tables**

![Figure 1](image)

Figure 1 The lignin polymer can be processed via combustion, chemical processing, thermochemical processing, biological processing or a combination of these routes. This review covers chemical, thermochemical, and biological processing of depolymerized lignin to produce industrially relevant chemicals.
Table 1 Lignin content in lignocellulosic crops.

<table>
<thead>
<tr>
<th>Biomass Category</th>
<th>Biomass Type</th>
<th>Lignin Content (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Softwood</td>
<td>Pine</td>
<td>28 [14]</td>
</tr>
<tr>
<td></td>
<td>Poplar</td>
<td>21–27 [15]</td>
</tr>
<tr>
<td>Hardwood</td>
<td>Eucalyptus</td>
<td>29–32 [16]</td>
</tr>
<tr>
<td></td>
<td>Miscanthus</td>
<td>9–13 [17]</td>
</tr>
<tr>
<td></td>
<td>Switchgrass</td>
<td>17–18 [18]</td>
</tr>
<tr>
<td>Herbaceous</td>
<td>Corn Stover</td>
<td>18 [19]</td>
</tr>
<tr>
<td></td>
<td>Bagasse</td>
<td>20 [20]</td>
</tr>
</tbody>
</table>

Figure 2 Primary lignin monomers are hydroxycinnamyl alcohols which are known as monolignols. These primary lignin monomers are polymerized. The corresponding phenylpropanoid monomeric units in the lignin polymer are guaiacyl units (G), syringyl units (S), and p-hydroxyphenyl units (H), respectively, which can be polymerized at any of the wavy bond positions [12,13].
Table 2 Non-biological lignin recovery methods.

<table>
<thead>
<tr>
<th>Recovery Methods</th>
<th>Benefits</th>
<th>Challenges</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kraft [23] and sulfite pulping [21]</td>
<td>Well-developed</td>
<td>Harsh chemicals</td>
<td>Cellulose, hemicellulose/lignin</td>
</tr>
<tr>
<td>Sulfur free alkali (soda) pulping [24]</td>
<td>Sulfur-free</td>
<td>Lower lignin removal rate</td>
<td>Solid polysaccharides, lignin-rich liquid</td>
</tr>
<tr>
<td>Organosolv pulping [25]</td>
<td>Sulfur-free</td>
<td>Has not been adapted to production scale</td>
<td>Varies by process, some organosolv processes can essentially isolate cellulose, hemicellulose, and lignin</td>
</tr>
<tr>
<td>Fast pyrolysis [26]</td>
<td>Fast</td>
<td>Undesired char formation</td>
<td>Solid (bio-char), Liquid (bio-oil), and gas</td>
</tr>
<tr>
<td>Dilute acid hydrolysis [27]</td>
<td>Highly advanced</td>
<td>Solid product is acid insoluble</td>
<td>Monomeric sugars, Biofine ligneous char (high heating value)</td>
</tr>
<tr>
<td>Hydrothermal Fractionation [28,29]</td>
<td>High product selectivity, produces monomeric products</td>
<td>Separation of hydrogen catalyst from the wood residue is challenging</td>
<td>Aromatic monomers, hydrolyzed hemicellulose</td>
</tr>
<tr>
<td>Biphasic fractionation [30]</td>
<td>Lower temperatures, near atmospheric pressure</td>
<td>Toxic solvents used in some cases</td>
<td>Hemicellulose degradation products (such as Cs oligomers, furfural), Cellulose solid, and lignin fragments</td>
</tr>
</tbody>
</table>

Table 3 Major enzyme families involved in lignin degradation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligninolytic peroxidase (LiP)</td>
<td>Oxidizes molecules with high redox potential, including moderately activated non-phenolic aromatics (up to 90% of lignin polymer) [5,56,57]</td>
</tr>
<tr>
<td>Manganese-dependent peroxidase (MnP)</td>
<td>Oxidizes phenolic compounds [5,58]</td>
</tr>
<tr>
<td>Versatile peroxidase (VP)</td>
<td>Oxidizes both non-phenolic and phenolic compounds [5,59]</td>
</tr>
<tr>
<td>Dye-decolorizing peroxidase (DyP)</td>
<td>Oxidizes hydroxyl-free antraquinone and peroxidase substrates [55]</td>
</tr>
<tr>
<td>Lacasse</td>
<td>Oxidize aromatics and phenols, take action on smaller molecules in lignin such as ABTS and HBT in order to oxidize non-phenolic aromatics [54,62–64]</td>
</tr>
</tbody>
</table>
Figure 3 Aromatics can be degraded via aerobic routes (indicated by blue lines) or anaerobic routes (indicated by red lines). In the two far left routes, aromatics are converted to reactive intermediates and then converted to elements of the TCA cycle. In the third route, aromatics are first converted to reactive intermediates, then reactive intermediates are converted into non-aromatic epoxides. Next, the non-aromatic epoxides are converted to TCA cycle intermediates. In the far right route, aromatics are converted into reactive intermediates, then reduced, and finally converted into elements of the TCA cycle. This figure is an adaptation from Figure 2 in Fuchs et al. [9].
Table 4 Non-biological depolymerization methods for isolated lignin.

<table>
<thead>
<tr>
<th>Recovery Methods</th>
<th>Benefits</th>
<th>Challenges</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrolysis of isolated lignin [109–113]</td>
<td>Simple process</td>
<td>Selectivity for specific aromatic compounds is very low; char formation</td>
<td>Aromatic and non-aromatic molecules, char, and light gasses</td>
</tr>
<tr>
<td>Catalytic pyrolysis [114–118]</td>
<td>Products are less oxygenated and more</td>
<td>Coke deposits on catalyst</td>
<td>Aromatic hydrocarbon containing liquid, char, coke, light hydrocarbons,</td>
</tr>
<tr>
<td></td>
<td>stable</td>
<td></td>
<td>and oxygenate gasses</td>
</tr>
<tr>
<td>Supercritical water [119–123]</td>
<td>Lower concentration of lignin means</td>
<td>High cost for process heat; only one-third of lignin product is low molecular weight</td>
<td>Aromatic hydrocarbon containing liquid, char</td>
</tr>
<tr>
<td></td>
<td>lower chance of condensation reactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supercritical solvent [124–130]</td>
<td>Products have a lower boiling point</td>
<td>Mid-high pressure High temperature</td>
<td>Primary product is monomeric substituted cyclohexyl derivatives,</td>
</tr>
<tr>
<td></td>
<td>allowing for easier separation</td>
<td></td>
<td>negligible aromatics, little to no char</td>
</tr>
<tr>
<td>Base-catalyzed depolymerization [12,131]</td>
<td>Oil contains low molecular weight</td>
<td>Produces around 20% (wt/wt) desired oil product compared to the total weight of the products (oil, residual lignin, and</td>
<td>Coke (undesired), oil (desired)</td>
</tr>
<tr>
<td></td>
<td>species</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5 Biological lignin upgrading.

<table>
<thead>
<tr>
<th>Molecule Class</th>
<th>Demonstrated Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics</td>
<td>Vanillin [107]</td>
</tr>
<tr>
<td>Dicarboxylic acids</td>
<td>Muconic acid [148], Succinic acid [53]</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Fatty acid methyl esters (15–18) [149]</td>
</tr>
<tr>
<td>Polyhydroxyalkanoates (PHAs)</td>
<td>Short—medium chain length [149,150]</td>
</tr>
<tr>
<td>Alpha-hydroxy acids</td>
<td>Lactic acid [53]</td>
</tr>
</tbody>
</table>
Figure 4 Major thermochemical lignin processes used to depolymerize lignin and the resulting products, as shown by Macfarlane et al. [188].

**Acknowledgments**: Funding for this work was provided in part by CenUSA (USDA AFRI 2011-68005-30411) and Iowa State University’s Bioeconomy Institute.

**Conflicts of Interest**: The authors declare no conflict of interest.

**Abbreviations**

The following abbreviations are used in this manuscript:

- **TCA**: tricarboxylic acid cycle
- **TG-FTIR**: thermogravimetric-fourier transform infrared spectroscopy
- **Py-GC/MS**: pyrolysis-gas chromatography/mass spectrometry
- **PHA**: polyhydroxyalkanoates
CHAPTER 3. PROMOTING MICROBIAL UTILIZATION OF PHENOLIC SUBSTRATES FROM BIO-OIL

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Abstract

The economic viability of the biorefinery concept is limited by the valorization of lignin. One possible method of lignin valorization is biological upgrading with aromatic-catabolic microbes. In conjunction, lignin monomers can be produced by fast pyrolysis and fractionation. However, biological upgrading of these lignin monomers is limited by low water-solubility. Here, we address the problem of low water-solubility with an emulsifier blend containing approximately 70 wt\% Tween\textsuperscript{®} 20 and 30 wt\% Span\textsuperscript{®} 80. \textit{Pseudomonas putida} KT2440 grew to an optical density (OD$_{600}$) of 1.0±0.2 when supplied with 1.6 wt\% emulsified phenolic...
monomer-rich product produced by fast pyrolysis of red oak using an emulsifier dose was 0.076±0.002 g emulsifier blend per g of phenolic monomer-rich product. This approach partially mitigated the toxicity of the model phenolic monomer p-coumarate to the microbe, but not benzoate or vanillin. This study provides a proof of concept that processing of biomass-derived phenolics to increase aqueous availability can enhance microbial utilization.

**Keywords:** *Pseudomonas putida* KT2440; lignin; bio-oil; emulsion; phenols

**Introduction**

Recent models indicate that the economic viability of the biorefinery concept relies on the valorization of lignin [15,44]. Even though lignin is a highly abundant biopolymer, it is currently slated to be used primarily for heat and power as a low value by-product of pioneer biofuel production facilities [54]. Aromatic-catabolic soil microbes are a promising route to add value to the lignin fraction of biomass due to their ability to catabolize many lignin-derived phenolic monomers and convert these molecules into renewable fuels and chemicals [9,1]. Among others, *Pseudomonas putida* KT2440 has emerged as a promising platform organism for such applications [42,36,28,70]. For example, *P. putida* can utilize a broad range of aliphatic and aromatic compounds as a sole carbon and energy source [51,65].

The number of reports demonstrating native and engineered microbial biocatalysts for biological lignin conversion has increased substantially in recent years [9,65,31]. Exemplary products include vanillin [63], muconic acid [70,8,35,69], fatty acids [78,38], polyhydroxyalkanoates [36], aromatic dicarboxylic acids [41], 2-pyrone-4,6-dicarboxylic acid [40], and lactic and pyruvic acids [29]. Many of the studies to date that have reported high titers, rates, and yields, however, have utilized model compounds due to the difficulties associated with achieving high yields of bio-available aromatic monomers from lignin to date.
Even in model compound studies, substrate solubility in water is a substantial challenge [64].

Microbial modification of lignin requires some sort of processing to both separate the lignin from the other biomass fractions and then depolymerization of the lignin. A variety of methods exist for achieving these goals [49,14]. One approach is fast pyrolysis, which has the advantages of being rapid and low cost [55,73,23]. Fast pyrolysis of biomass produces several product streams: a heavy ends stream which is rich in sugars and water-insoluble phenolics, bio-char which is collected via cyclones and can be used as a soil additive, and lastly, a light ends aqueous stream rich in acetate (Fig. 1) [66,4,11]. The water-insoluble phenolic oil stream is mainly composed of lignin-derived products, including monophenols such as phenol and syringol, in addition to higher molecular weight phenolic oligomers [4], with its composition being impacted by the pyrolysis conditions [62]. The insolubles are separated from the unrefined sugars with a simple aqueous liquid-liquid extraction [61,72].

For the fast pyrolysis-based biorefinery, the addition of value to lignin requires upgrading the components of this phenolic oil. However, its low water solubility is problematic for traditional aqueous-phase bioconversions. The problem of low substrate solubility has previously been addressed through the use of emulsifiers to improve degradation of hydrocarbons, such as naphthalene and pyrene, by *Pseudomonas* species [30]. Emulsifiers increase the concentration and bioavailability of poorly soluble molecules by forming micelles in emulsions [37,17]. These compounds are adsorbed at the oil/water interface, lowering the interfacial surface tension [39], but because the resulting mixtures are non-equilibrium systems, they do eventually undergo breakdown processes [39,47]. Span® and Tween® emulsifiers, employed in this study, are amphipathic (i.e. contain both hydrophilic and hydrophobic functionality), non-ionic surface-active surfactants forming strong hydrogen
bonding with water [50]. Non-ionic surfactants are appealing for industrial processes because of their relatively simple production process, which can utilize a variety of feedstocks at a fairly low cost [2].

Emulsification and dispersion methods can be designed to tune the stability and rheology of the resulting mixtures [77]. Methods such as colloidal milling, homogenization, and ultrasonication, result in relatively stable mixtures [46]. Contrastingly, the use of vortex mixing produces mixtures containing isolated and sparse droplets with sustained release. For example, emulsions promoting the sustained release of adjuvants and antigens were consistently and reliably produced by vortexing [20]. Here, we have used vortex mixing to promote the slow release of the phenolic monomers into the aqueous cultivation medium.

The goal of this research is to investigate emulsification as a means of increasing the biological availability of aromatic monomers produced from lignin to microbial biocatalysts for the production of value-added products. Not only will this potentially enable the production of value-added products and specialty chemicals from lignin, this work also provides insight on how to mitigate the toxicity of these phenolic compounds to microbial biocatalysts.

**Experimental**

**Heavy Ends Bio-oil Production**

Red oak (*Quercus rubra*) was procured from Wood Residual Solutions of Montello, WS. As-received biomass was passed through a 60 hp hammer mill with a 3 mm screen, resulting in a particle size range of approximately 200 micron to 3 mm. The moisture content of the red oak was approximately 10 wt%. Bio-oil was produced in a fluidized bed reactor and collected in a bio-oil recovery system that separates the bio-oil into the heavy ends and light ends (Fig. 1). The heavy ends stage collects the viscous, high-boiling-point compounds referred to collectively here as heavy ends bio-oil, and was used in this research [61]. Details
of the pyrolyzer and recovery system can be found in Rover et al [62] and Pollard et al [45].

**Liquid-liquid Extractions**

The heavy ends bio-oil was subjected to an aqueous liquid-liquid extraction (Fig. 1) to separate the water-soluble components, such as sugars and acetate, from the water-insoluble phenolics, as detailed by Rover et al [62]. Briefly, a 1:1 w/w mixture of heavy ends bio-oil:water was mechanically stirred using a drill press equipped with a stainless steel open paddle for 10 – 15 min during bio-oil production, placed on a shaker table (MaxQ 2506, Thermo Scientific®, Hanover Park, IL) for 30 min at 250 motions min\(^{-1}\) and centrifuged (acuSpin™ 1R, Thermo Scientific®, Hanover Park, IL) at 2,561g force for 30 min. The water-soluble unrefined sugars were decanted from the water-insoluble phenolics. The water-insoluble phenolic fraction was centrifuged and decanted again to remove any remaining unrefined sugar solution.

Secondly, an extraction of lower molecular weight aromatics from the water-insoluble phenolic oil was accomplished by liquid-liquid extraction with toluene (Fig. 1) [3]. An equal mass of toluene was added to the water-insoluble phenolic oil and stirred with a hand drill equipped with a stainless steel open paddle for 10 min. The toluene-soluble extract was decanted from the toluene-insoluble fraction. The toluene was then recovered from the toluene-soluble extract via evaporation utilizing a Hei-Vap Precision rotary evaporator (Fisher Scientific, PA) at 77 mbar and 40 °C. The product from the toluene extraction after evaporation, referred to here as phenolic monomer-rich product, was used in the emulsions for this work.

A gas chromatograph with a flame ionization detector (GC-FID) was used for identification of components of the phenolic monomer-rich product using a 430 (Bruker Corporation, Bruker Daltonics, Inc. Fremont, CA) GC-FID. The column was a 1701 capillary,
60 m in length, 0.25 mm inner diameter, with a 0.25 mm film thickness (Phenomenex, Inc., Torrance, CA). Galaxie Chromatography Data System version 0.9.302.530 (Bruker Corporation, Bruker Daltonics, Inc., Fremont, CA) was the operating system. The carrier gas was helium (99.9995%) with a constant flow rate of 1.0 mL min$^{-1}$ and the helium make-up was 25 mL min$^{-1}$, with hydrogen flow at 30 mL and an air flow of 300 mL min$^{-1}$. The oven was programmed to be held for 4 min at 45 °C and ramped at 3 °C min$^{-1}$ to 235 °C and held for 10 min. The sample volume was 1 µL with a split ratio of 1:45. Peak identification was based on calibration standards purchased from Fisher Scientific (Thermo Scientific, Hanover Park, IL) [60].

**Phenolic Monomer-rich Product Emulsification**

Two emulsifiers were used in this study: Span® 80, a non-ionic surfactant, and Tween® 20, a non-ionic detergent. Span® 80 is widely used in food and pharmaceutical applications. Tween® 20 is a food additive.

To determine the required Hydrophile-Lipophile Balance (HLB) values, an expression of the size and strength of the hydrophilic (polar) and lipophilic (non-polar) groups of the emulsifier [24], test emulsions were made with different emulsifier blends. When two or more emulsifiers were blended, the HLB value was calculated using the weight percentage of each emulsifier multiplied by the HLB value of the neat emulsifier. The individual HLB values were then summed for all emulsifiers to calculate the HLB for the blend. Equation 1 [24] was used to calculate the HLB values.

\[
\text{Emulsifier #1: } (\text{wt}\% \text{ emulsifier #1}) \times (\text{neat HLB emulsifier #1}) = X \quad [\text{Eq. 1}]
\]

\[
\text{Emulsifier #2: } (\text{wt}\% \text{ emulsifier #2}) \times (\text{neat HLB emulsifier #2}) = Y
\]

\[
\text{HLB of the Blend} = X + Y
\]
The target HLB range for the blends can be determined either from known HLB values for the constituents of the material to be emulsified or from assessment of the amount of product emulsified, any indication of separation, and clarity of the resulting emulsion. The test emulsions were comprised of 1.0 g phenolic monomer-rich product, 10 g water and 0.15 g of the emulsion blend and were vortexed. Each emulsifier blend was evaluated in terms of the amount of phenolic monomer-rich product emulsified, any emulsion separation due to Oswald ripening, flocculation, coalescence, creaming, and sedimentation. Lastly, the clarity of the mixture was evaluated [12]. The control was 1.0 g of the phenolic monomer-rich product vortexed with 10 g water without emulsifiers.

The mixture for microbial utilization studies were prepared by first adding deionized (DI) water to a 50 mL centrifuge tube, followed by the phenolic monomer-rich product. The polar emulsifier (Tween® 20) was added, followed by the nonpolar Span® 80. The relative amount of emulsifiers, water and phenolic monomer-rich product varied according to experimental parameters. The mixture was vortexed for 5 minutes. The amount of emulsifier is reported as the grams of emulsifier blend per gram of phenolic monomer-rich product. The resulting liquid mixture was decanted away from the residual phenolic monomer-rich product that did not emulsify. This residual material was dried at 80 °C and weighed to determine the mass of phenolic monomer-rich product that was not emulsified.

**Total Phenolics Determination**

Modified micro-scale methodology for the Folin-Ciocalteu (FC) method was used to determine total water-soluble phenolic monomers present in the monomer-rich product using a procedure outlined by Rover and Brown [58]. Briefly, a 20 µL monomer-rich product sample (prepared with 0.15 - 0.30 g of monomer-rich product diluted with 5 mL deionized water then
diluted to 50 mL mark with deionized water and filtered with 0.45µm Corning syringe filter), a blank consisting of deionized water, and Gallic acid calibration standards were each placed in 2 mL polystyrene cuvettes. Deionized water (1.58 mL) was added, followed by 100 µL FC reagent. The solution in each cuvette was mixed thoroughly by pipetting, and each were incubated for 1-8 min. This was followed by the addition of 300 µL of sodium carbonate solution and incubation for 2 h at room temperature. The sample absorbance was measured at 765 nm with 1 cm cells and a 1.5 nm bandwidth with a Varian Cary 50 UV-visible Spectrophotometer (Agilent Technologies Inc., Santa Clara, CA) using Cary WinUV (Agilent Technologies Inc., Santa Clara, CA) Simple Reads module software. The sodium carbonate solution was made by dissolving 200 g anhydrous sodium carbonate in 800 mL water and brought to a boil. After cooling, a few crystals of sodium carbonate were added. The solution was stored for 24 h at room temperature and then filtered with a Whatman #42 filter paper and water was added to make 1.0 L. A minimum of five trials were performed for each monomer-rich product sample.

**Media Preparation**

Microbial growth media consisted of modified M9 medium, prepared according to Johnson et al., which contains no carbon source [29]. Pure monomers (benzoate or p-coumarate), in the emulsified or non-emulsified state, or emulsified phenolic monomer-rich product were added as carbon source. The carbon source concentration is reported as the weight percent of phenolic monomer in growth medium according to Equation 2. The estimated concentration of total emulsifier in growth medium is reported according to Equation 3. It was assumed that all of the emulsifier was recovered in the growth medium.
\[
\text{Mass}_{\text{phenolic monomer-rich product added}} - \text{Mass}_{\text{residual phenolic monomer-rich product}} \over \text{Total volume of growth medium} \times \text{Density of growth medium} \times 100\% \quad [\text{Eq. 2}]
\]

\[
\text{Mass}_{\text{emulsifier added}} \over \text{Total volume of growth medium} \times \text{Density of growth medium} \times 100\% \quad [\text{Eq. 3}]
\]

The density of the culture medium was assumed to be 1.0 g/mL. The resulting media was adjusted to a pH of 7.0 with sodium hydroxide and sterilized with a 0.2 µm pore size syringe filter.

**Microbial Cultures**

Bacterial cultures were grown in 20 mLs of growth medium with 250 mL shake flasks at 30 °C, 200 RPM, and initial pH of 7.0. Single colonies of *P. putida* KT2440 were pre-cultured overnight in 10 mL of Luria Broth. Pre-cultures were centrifuged (Thermo Fisher Scientific Sorvall Legend XTR) at a relative centrifugal force of 3,488 x g for two minutes and re-suspended in modified M9. These washed cells were diluted with growth media to an optical density of 0.05 at 600 nm.

Samples were taken regularly during growth until cells reached stationary phase. At each time point, samples were centrifuged (Eppendorf 5424 Microcentrifuge) at a relative centrifugal force of 21,130 x g for five minutes, the supernatant was removed, and the cells were re-suspended in DI water for measurement on a spectrophotometer at 600 nm (Thermo Scientific, Genesys20). Unless stated otherwise, p values were calculated using a pair-wise Student’s two-tailed, equal variance t-test.
Results and Discussion

Emulsion Studies

Selection of emulsifier blend. Microbial biocatalysts, such as *P. putida*, are an attractive method of upgrading the biomass-derived phenolic monomer-rich product to biorenewable fuels and chemicals. Unfortunately, this stream has a very low water solubility thus restricting the availability of the phenolic monomers in the cultivation media. Specifically, assessment of water-soluble phenolic monomers in the monomer-rich product were determined utilizing the modified FC method [57]. These measurements indicated that only 316±1 mg/L of the phenolic monomer-rich product was water-soluble. We proposed to address this low solubility by emulsifying the phenolic monomer-rich product, while also enabling the slow release of the phenolics from the emulsion for microbial utilization.

Emulsion stability is related to different parameters such as oil content, emulsifier content, pH, temperature, and HLB, a metric of the size and strength of the hydrophilic (polar) and lipophilic (non-polar) groups of the emulsifier [24]. While there is not a single parameter that can explain the stability of an emulsion [50], HLB values are a reliable predictor of how an emulsifier will behave in mixtures and can be used as a guide in selection of emulsifiers. Specifically, the HLB value of the emulsifier or emulsifier blend should be the same as the HLB value of the material to be emulsified [24]. The HLB value for the complex phenolic monomer-rich product is not known, but the phenolic monomers benzene, ethyl benzoate, and styrene all have HLB values between 13 and 15 [24]. Thus, we expect that the HLB value required for emulsification of the phenolic monomer rich product will be within the high end of the 8-18 range generally considered to be suitable for an oil-in-water emulsifier [2,21]. Tween® 20 (hydrophilic) and Span® 80 (lipophilic) were selected as model emulsifiers due to their known lack of biological toxicity, lower cost [2], and their HLB values of 16.7 and 4.3,
respectively [24].

Emulsification of 1.0 g of phenolic monomer-rich product in 10.0 g of DI water was assessed through the use of 0.15 g of pure Tween® 20, pure Span® 80, and five blends of Tween® 20 and Span® 80 (Fig. 2). The HLB values of the pure emulsifiers and the various blends ranged from 4.30 to 16.7. The test emulsion with an HLB value of 13.2 (sample 4) was chosen as the best candidate based on clarity. The use of pure Span® 80 (sample 8) was comparable to the no-emulsifier control (sample 1), in terms of the lack of solubilization of the phenolic monomer-rich product. Test emulsions with a calculated HLB values of 15.0 and greater (samples 2 and 3) and of 10.2 and lower (samples 5, 6 and 7) were deemed unsuitable due to the cloudy appearance.

While emulsification of the phenolic monomer-rich product was our primary criteria in selection of emulsifier blend, it was also important that the mixture would break down over time to release the monomers for microbial utilization. Stability was evaluated by visually judging the amount of sedimentation, flocculation, and creaming of the test emulsions over a several week time period. Emulsions 2 and 3 appeared to be too stable, as no phenolic monomer-rich product came out of the emulsion, while for samples 4 and 5 the phenolic monomer-rich product was partially released from the emulsion, as evidenced by collection on the bottom of the beakers. The remaining test emulsions, samples 6-8, did not have enough oil incorporated into the emulsions to assess stability. Because sample 4 (71.5 wt% Tween® 20, 28.5 wt% Span® 80) was acceptable both in terms of clarity and stability, it was chosen for further characterization.
Emulsified phenolic monomer-rich product contains monomers suitable for microbial utilization.

The goal of emulsification of the phenolic monomer-rich product is to increase the availability of the lignin-derived monomers in the aqueous cultivation media. A calibrated GC/FID was used to determine the concentration of compounds that were present in the emulsion generated using the 70:30 wt% Tween® 20, Span® 80 blend and to also identify compounds not detectable in the emulsion (Table 1, Fig. S1). Many of these compounds were also detected in the aqueous sugar wash solution produced in the same bio-oil recovery and processing framework [11].

The most abundant phenolic monomer was acetosyringone, which has been characterized as a signaling molecule in plant-pathogen interactions [5] and as an inhibitor of Saccharomyces cerevisiae, a commonly-used fermentation organism [5,34]. Microbial species such as Pseudomonas have been previously described to utilize many of the other monomers, such as phenol [19,7,68,35], cresol [7,35], styrene [43], xylene [67], ethylbenzene [67], and 3,4-dimethylphenol [68]. Other compounds, such as 2,6-dimethoxyphenol [74], 4-vinylguaiacol [32], and eugenol [48], have been reported to be subjected to biological modification, but it is not clear if the reaction products are funneled into central metabolic pathways. Thus, the emulsified phenolic monomer-rich product contains at least some species that can be utilized by microbial biocatalysts.

Microbial Studies

Microbial utilization of emulsified phenolic monomers.

The emulsified phenolic monomer rich product contains many phenolic monomers that have previously been demonstrated as substrates for microbial species, such as Pseudomonas. The microbial production of industrial fuels and chemicals from lignin-derived monomers
requires funneling of these monomers into central metabolism. Here, we used microbial growth as an indicator of utilization of these monomers through central metabolism. Specifically, utilization of the phenolic monomers by *P. putida* was assessed based on the maximum OD$_{600}$ observed over a 72-hour period (Fig. 3, Fig. S2). It should be noted that only a portion of the phenolic monomer rich product is emulsified, as accounted for with Equation 3. Thus, Fig. 3 reports the emulsifier dosage used for the emulsification process as well as the final concentrations of the emulsifier and the phenolic monomer rich product in the growth medium.

As shown in Fig. 3a, the 70:30 blend of Tween® 20 and Span® 80 with the phenolic monomers allowed *P. putida* to achieve an OD$_{600}$ of 0.87 ± 0.04, a 4-fold higher value ($p = 0.005$) than the maximum OD$_{600}$ observed when the growth medium contained only emulsifier and no phenolic monomers. This increased growth in the presence of the emulsified phenolic monomer indicates that the lignin-derived monomers are being used to produce the central metabolic intermediates needed for microbial growth. The concentration of each lignin-derived monomer in the growth medium will vary not only with the targeted concentration of phenolic monomer-rich product added to the medium, but also with the emulsification process. At an emulsifier dose of 0.205±0.008 g per g of monomer-rich product and 0.24 wt% phenolic monomer-rich product (Fig 3a bar 1), each lignin-derived monomer in the growth medium should be present at roughly 13% of the concentrations in the emulsion (Table 1a). For example, the growth medium should contain approximately 80 mg/L of acetosyringone. Our observed utilization of the emulsifiers as sole carbon source is consistent with previous reports involving Triton X-100 and Brij 30 [17]. The fact that there was no significant difference in maximum OD$_{600}$ in the media containing only the Tween® 20/Span® 80 mixture and the media containing only Tween® 20 suggests that *P. putida* is able to use the Tween® 20, but
not Span® 80, as a carbon source. Note that data are not shown for media in which Span® 80
is the only potential carbon source, because it was insoluble at the experimental concentration.

To assess the benefit of using an emulsifier blend, the phenolic monomer-rich product
was emulsified with just Tween® 20 or just Span® 80, similar to samples #2 and #8 in Fig. 2.
Microbial growth on these emulsions was much lower than the growth observed using the
70:30 blend (Fig. 3A), validating the tuning of the emulsifier blend described above.

**Microbial growth scales with emulsifier concentration.**

As shown above, emulsification of the phenolic monomer rich product with a 70:30
blend of Tween® 20 and Span® 80 promotes microbial growth on this stream. This growth is
a proxy for funneling of the phenolics into central metabolic intermediates. For the experiments
described above, 0.205±0.008 g of the emulsifier blend was used per g of phenolic monomer
rich product. Given that only a portion of the phenolic monomer rich product is solubilized in
the emulsion process (Table 2), the resulting concentration of emulsifier in the growth medium
(0.28 wt%) was roughly equal to the concentration of phenolic monomer in the growth medium
(0.24 wt%). However, since the emulsifier contributes to the process cost, the emulsifier should
be used as sparingly as possible.

Decreasing the amount of the emulsifier blend used resulted in a decrease in the amount
of phenolic monomer recovered in the emulsion (Table 2). The emulsified product was then
added to microbial growth medium such that the phenolic monomer concentration was
maintained at 0.24 wt%, resulting in varying concentrations of emulsifier in the growth
medium (Fig. 3b). Despite the constant concentration of emulsified phenolic monomer-rich
product, the maximum OD$_{600}$ reached by *P. putida* decreased as the emulsifier dosage
decreased (Fig. 3b). Note that emulsions made with lower doses of emulsifier still promoted
growth, although the maximum OD$_{600}$ was significantly decreased (p < 0.05). As described
above, *P. putida* is able to utilize the emulsifier itself as sole carbon source (Fig 3a), and thus the decrease in maximum \( OD_{600} \) could be due in part to the decrease in total carbon available to the *P. putida*. Decreasing the amount of emulsifier used may also change the nature of the emulsion, leading to stability issues over time. For the rest of the studies described here, the emulsifier blend was dosed at 0.08 g of emulsifier blend per g of phenolic monomer-rich product.

**Monomer emulsification can provide protection from growth inhibition.**

Phenolic monomers and other organic molecules produced during biomass degradation are known to inhibit the growth of bacteria and yeast [13,27]. Therefore, the effect of the concentration of the phenolic monomer-rich product on cell growth was investigated (Fig. 4, Fig. S3). When the concentration of phenolic monomer-rich product in the growth medium was increased from 0.24 wt% to 0.80 wt%, there was a significant increase of over 4.5-fold in the maximum \( OD_{600} \). In the range of 0.4-1.6 wt% phenolic monomer-rich product, no significant differences in maximum \( OD_{600} \) were observed, though the maximum \( OD_{600} \) did show a downward trend as the concentration of phenolic monomer was increased from 0.80 to 1.60. The concentration of phenolic monomer-rich product was not increased past 1.6 wt% due to the amount of phenolic monomer-rich product that can be emulsified.

Because the emulsifier dosage was maintained at a constant 0.08 g/g for these experiments, the amount of emulsifier in the media ranged from 0.03 - 0.82 wt%. Although *P. putida* was observed to use the emulsifier blend as sole carbon source (Fig. 3a), the maximum \( OD_{600} \) was significantly higher in the presence of emulsified monomer-rich product relative to the corresponding emulsifier-only control within the range of 0.24 – 1.06 wt% monomer-rich product. These results support the conclusion that the phenolic monomers are being used to support biomass production.
The primary goal of emulsifying the phenolic monomer-rich product is to make these compounds available to the biocatalyst in the aqueous phase. However, it is possible that the emulsification also helps to protect *P. putida* from inhibition by these monomers. It is difficult to test the effect of the emulsion against a non-emulsified control because the phenolic monomer-rich product is not soluble in water (Fig. 2). Coumarate and benzoate were selected here as model water-soluble aromatic monomers known to be utilized by *P. putida* KT2440, though they are unlikely to be present in the pyrolysis-derived material. Vanillin is a methoxylated aromatic aldehyde that has also been reported to be utilized by *P. putida* KT2440 [70] and has been detected in lignin depolymerized by fast pyrolysis [71].

During growth on non-emulsified *p*-coumarate, the maximum OD$_{600}$ dramatically decreased from 1.06-1.60 wt%, indicating growth inhibition due to *p*-coumarate toxicity (Fig. 5a). However, a maximum OD$_{600}$ of 3.39±0.02 was observed when cultures contained 1.60 wt% emulsified *p*-coumarate, indicating that the emulsified *p*-coumarate is not as inhibitory as the non-emulsified form. At 0.8 wt% *p*-coumarate, there was also an increase in maximum OD$_{600}$ for the emulsified *p*-coumarate relative to the non-emulsified *p*-coumarate. Although some of the improvement in the emulsion trials could possibly be attributed to the additional carbon provided by the emulsifiers, this benefit should only result in, if any, a small increase in maximum OD$_{600}$. As shown in Fig. 4, the same combination of Tween® 20 and Span® 80, but a higher total emulsifier concentration relative to the *p*-coumarate trials, resulted in a maximum OD$_{600}$ of only 1, much lower than the values observed during growth in the presence of *p*-coumarate. Therefore, the difference of more than 3 OD$_{600}$ units is unlikely to be due to utilization of the emulsifiers.

*P. putida* KT2440 growth on varying concentrations of the non-emulsified pure
phenolic monomer p-coumarate can be compared to previous studies. A report on tolerance mechanisms to p-coumarate indicated that the growth rate of *P. putida* KT2440 was negatively impacted at concentrations of 0.49 wt% p-coumarate and higher when supplemented with 0.2% glucose [10]. However, in our study, the maximum OD<sub>600</sub> was greatest at 1.1 wt% p-coumarate, but decreased to almost 0 at 1.6 wt% p-coumarate. The slightly increased tolerance to p-coumarate in the current study may be due to the difference in carbon source availability.

Unlike the clear improvement in growth on p-coumarate, the maximum OD<sub>600</sub> was not significantly different when cells were provided with 0.1 – 1.1 wt% emulsified benzoate relative to non-emulsified benzoate. Although a significant difference in the maximum OD<sub>600</sub> values during growth with 1.6 wt% benzoate was observed, both of the values were quite low, indicating that this concentration of benzoate is toxic to the cells. When we varied the concentration of non-emulsified pure benzoate to determine tolerance, growth was consistent with a previous study on benzoate stress response [53]. In the previous study, *P. putida* KT2440 was cultivated on pure benzoate as a sole carbon source. The growth rate was negatively impacted when benzoate concentrations were at or above 0.86 wt% [53]. Similarly, in our study, the maximum optical density was negatively impacted at concentrations of 1.06 wt% benzoate and higher (Fig. 5b).

Surprisingly, emulsification of vanillin was observed to significantly decrease the maximum OD<sub>600</sub> at low vanillin concentrations and did not lead to any significant differences in maximum OD<sub>600</sub> at higher concentrations (Fig. 5c). At 0.24 wt% vanillin, there was substantial variability between technical replicates, particularly in regards to lag time. However, extension of the observation period to 150 hrs still leads to the conclusion that there
was no significant difference between maximum OD\textsubscript{600} values when cells were provided with emulsified or non-emulsified vanillin. The low growth in the presence of 0.24 wt\% non-emulsified vanillin is consistent with previously reported inhibitory vanillin concentrations for commonly-used fermentation organisms [25].

Thus, substantially different outcomes were observed regarding the impact of emulsification of three model phenolic monomers on utilization of these monomers by \textit{P. putida}. Emulsification promoted growth in the presence of p-coumarate, had no impact on growth in the presence of benzoate, and had a negative impact on growth in the presence of vanillin. It has been previously reported that emulsification of the cyclic terpene D-limonene, the aromatic unsaturated aldehyde cinnamaldehyde, and the monoterpenoid phenol carvacrol with Tween® 20 and glycerol mono-oleate actually increased the toxicity of these compounds to \textit{Escherichia coli}, \textit{Lactobacillus delbrueckii}, and \textit{S. cerevisiae} [16]. Thus, emulsification is not a universal strategy to provide protection to microbes from inhibitory compounds present in the growth media.

These three representative monomers differ substantially in their toxicity to \textit{P. putida} (Fig. 5). For example, substantial growth was observed in the presence of 1.06 wt\% (65 mM) \textit{p}-coumarate but not 1.06 wt\% (74 mM) benzoate. Vanillin toxicity was much more severe, with very little growth observed in the presence of 0.24 wt\% (16 mM). Some studies characterizing the growth of \textit{Pseudomonas} species on these compounds use a monomer concentration of approximately 5 mM [52], well below the toxicity limits observed here. The differing toxicity of these molecules is similar to previous observations that aldehydes, such as vanillin, tend to inhibit microbial growth at lower concentrations relative to organic acids [75,76]. However, assessment of these three compounds as inhibitors of the osmophilic yeast
Zygosaccharomyces rouxii concluded that \( p \)-coumaric acid was less inhibitory than sodium benzoate and vanillin, but observed roughly equal effects of benzoate and vanillin [56]. The relative toxicity of these types of molecules is often attributed to differences in hydrophobicity [6,75,76], non-polar surface area [6] and positioning of electron-withdrawing and electron-donating functional groups [22].

**Process Feasibility**

Here, we used a blend of the emulsifiers Tween® 20 and Span® 80 to disperse the phenolic monomer-rich product into the aqueous microbial growth medium. Characterization of other emulsifier blends or other emulsification techniques may identify emulsification methods that are more effective and/or have a lower cost. It is possible that the emulsifiers could be recycled, but this has not been investigated here. The utilization of Tween® 20 by the microbial biocatalyst is concerning in the context of process cost and emulsifier recycling, further characterization may identify a more suitable replacement.

Regardless of the substrate type, a relatively low substrate concentration in a cultivation process will inherently lead to low concentrations of the metabolic product, resulting in a relative increase in product separation costs. This problem of substrate toxicity, and the associated increase in product separation costs, is widespread in the biorenewables field [26]. It is demonstrated above that the emulsified phenolic monomer-rich stream can be provided at concentrations up to 1.60 wt% without negatively impacting microbial growth (Fig. 4). Higher concentrations were not tested, but could possibly be achieved with changes to the emulsification method.

The biomass depolymerization and fractionation process used here involves two liquid-liquid extraction steps (Fig. 1). First, water is used to separate the water-soluble sugars from the phenolic-insoluble phenolic oil. There are a variety of potential applications for this bulk
phenolic oil, including coal replacement [59], production of gasoline and diesel [18,60], and use in resins [33]. Here, we performed a second extraction on the bulk phenolic oil, using toluene as the solvent, to separate the high-molecular weight phenolics from the phenolic monomer-rich product. The toluene was then removed via distillation with 99.8% recovery (data not shown). Other methods of removing the high-molecule weight phenolics from the bulk phenolic oil may be more effective, have lower cost or use less toxic solvents; these have not been investigated here. However, our demonstration that emulsification of this stream of depolymerized biomass promotes microbial utilization should be applicable across a wide array of biomass processing strategies. Technoeconomic analysis of this process, which is not presented here, could guide these types of decisions.

**Conclusions**

This work demonstrates that microbial utilization of the phenolic monomer-rich product of biomass fast pyrolysis is promoted by processing of this stream to increase the concentration of the monomers in the aqueous growth medium. Specifically, we were able to provide microbial cultures with more than 1.0 wt% emulsified material without negatively impacting growth. The emulsifier blend used here seems to not only increase the aqueous concentration of a variety of phenolic monomers (Table 1), but may also provide protection against the inhibitory effect of some of these monomers.

Here, we used *P. putida* KT2440 as a model microbial biocatalyst, but this approach can be generalized to other microbial species. The optimum emulsion formulation consists of 70:30 (by mass) Tween® 20 to Span® 80. The amount of emulsifier blend added to the phenolic monomer-rich product had a direct impact on the amount of phenolic monomer-rich product emulsified and on the microbial growth, and we did observe evidence of microbial utilization of the emulsifiers, particularly Span® 80. Additional emulsion work, identification
of specific compounds in pyrolysis phenolic oil utilized by \textit{P. putida} KT2440, and other suitable microbes are obviously needed before this approach could be used in an economically viable process. However, these results provide valuable proof of concept towards the microbial utilization of depolymerized lignin in a biorefinery concept and contribute to a better understanding of the influence of emulsions to enhance biological availability of bio-oil water insoluble constituents for fermentation.

**Conflicts of interest**

There are no conflicts of interest.

**Acknowledgments**

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57. Rover MR (2013) Analysis of sugars and phenolic compounds in bio-oil. Iowa State University, Ames

temperatures. Industrial Crops and Products 122:465-472. doi:10.1016/j.indcrop.2018.06.038


Fig. 1. Schematic of the fast pyrolysis reactor and the heavy and light ends of bio-oil recovery. The heavy ends is subjected to liquid-liquid extraction using water as the solvent during bio-oil production [45,62]. The products from the liquid-liquid extraction are unrefined sugars and water-insoluble phenolic oil. The cyclones collect the biochar while the light ends contain the water and is rich in acetate. The heavy ends water-insoluble phenolics are subjected to liquid-liquid extraction utilizing toluene as the solvent. Following removal of the toluene, the phenolic monomer-rich product is subjected to microbial utilization.
Fig. 2 Selection of a blend of approximately 70:30 wt% of Tween® 20 and Span® 80 (sample number 4) as optimum for emulsification of the phenolic monomer-rich stream. Each emulsion consisted of 1.0 g phenolic monomer-rich product and 10.0 g DI water. For samples 2 - 8, 0.15 g of total emulsifier was added. HLB values were calculated according to Eq 1. Each blend was assessed on the basis of clarity immediately after mixing. Stability of the mixture over the course of several weeks was assessed for some blends, as indicated.
Fig. 3. Emulsification enables microbial utilization of the phenolic monomer-rich product.

a). Phenolic monomer-rich product emulsions made with an approximately 70:30 combination of Tween® 20 and Span® 80 promoted higher cell growth than those without phenolic monomer-rich product. Emulsions with just Span® 80 were not assessed due to low solubility. Phenolic monomer-rich product emulsions made with only Tween® 20 or only Span® 80 supported significantly lower growth than the 70:30 blend. The letters above the bars indicate statistically significant groupings of the maximum OD$_{600}$ values (p<0.05), as shown in the bar graph. As not all of the phenolic-monomer rich product is incorporated into the emulsion, the relative abundance of emulsifier and phenolic monomer in the growth medium differs from the emulsifier dose. Growth curves are provided as Fig. S2.
Fig. 3. (continued) b). Growth on phenolic monomer-rich product emulsions is directly related to total emulsifier concentration. All emulsions were made using a emulsifier blend containing 66.3-73.9 Tween® 20 : 26.1-33.7 Span® 80. The amount of emulsified product added to the growth medium was varied so that samples contained 0.24 wt% monomer-rich product. P. putida KT2440 was grown at 30 °C and 200 RPM and initial pH of 7.0. Values are the average of at least two biological replicates with the error bars representing one standard deviation. The reported value for the concentration of monomer-rich product in the growth medium reflects the fact that not all of the monomer-rich product is solubilized (Table 2).

<table>
<thead>
<tr>
<th>wt% of phenolic monomer in growth medium</th>
<th>0.10</th>
<th>0.24</th>
<th>0</th>
<th>0.40</th>
<th>0</th>
<th>0.80</th>
<th>0</th>
<th>1.06</th>
<th>0</th>
<th>1.60</th>
<th>0</th>
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</thead>
<tbody>
<tr>
<td>estimated wt% of surfactant in growth medium</td>
<td>0.03</td>
<td>0.17</td>
<td>0.29</td>
<td>0.36</td>
<td>0.49</td>
<td>0.82</td>
<td>0.87</td>
<td></td>
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</table>

Fig. 4. Emulsified phenolic monomer-rich is utilized at concentrations of at least 1 wt%. P. putida KT2440 was grown for 70 hours at 30 °C and 200 RPM and initial pH of 7.0 and a emulsifier dosage of 0.08 g of emulsifier blend per g of phenolic monomer-rich product. Values are the average of two biological replicates with error bars representing one standard deviation. The letters next to the bars indicate statistically significant (p < 0.05) groupings of the maximum OD600 values such that any bars not sharing a common letter significantly differ. Asterisks indicate significance relative to the corresponding emulsifier-only control culture. The stars indicate p-values of ≤ 0.05 (*) and ≤0.0001 (**). Growth curves are provided as Fig. S3.
Fig. 5. Emulsification of model phenolic monomers (a) p-coumarate, (b) benzoate and (c) vanillin impacts growth. *P. putida* KT2440 was grown for 70 hours at 30 °C and 200 RPM and initial pH of 7.0, and 0.08 g of emulsifier blend per g of monomer was used. Values are the average of two biological replicates with the error bars representing one standard deviation. The stars indicate p-values calculated by pair-wise Student’s two-tailed, equal variance t-tests. The stars indicate p-values of ≤0.05 (*), ≤0.001 (**), and ≤0.0001 (****).
Supplemental Figure 1. GC/FID analysis of the emulsified monomer-rich product in modified M9 immediately after preparation.

The experimental conditions include the microbial growth media consisting of the modified M9 medium with no carbon source and the emulsified monomer-rich product at 0 hour. The sample was placed on the GC/FID with no further dilution following the previously described methodology. This data is presented as Table 1 in the main text.
Supplemental Figure 2. Growth curves of \textit{P. putida} KT2440 on the emulsified phenolic monomer rich product and corresponding controls. The maximum OD values are show in Figure 3a of the main text.
Supplemental Figure 3. Growth curves of *P. putida* KT2440 at 30 °C and 200 RPM and initial pH of 7.0 and a surfactant dosage of 0.07 g of surfactant blend per g of phenolic monomer-rich product. Values are the average of two biological replicates with error bars representing one standard deviation. The maximum OD values are presented in Figure 4 of the main text.
Table 1. Composition analysis of emulsified phenolic monomer-rich product. 1.03 g of the phenolic monomer-rich product was emulsified in 10.0 g DI water with 0.204 g of the 70:30 blend of Tween® 20 and Span® 80 and immediately characterized by GC/FID. A full chromatogram is provided as Fig. S1.

(a) Calibrated compounds detected in the emulsion. Values are the average concentration with the associated standard deviation. Monomers are ordered by concentration.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS #</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3',5'-dimethoxy-4'-hydroxyacetophenone (acetosyringone)</td>
<td>2478-38-8</td>
<td>610±10</td>
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<tr>
<td>2,6-dimethoxyphenol</td>
<td>91-10-1</td>
<td>394.0±0.9</td>
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<td>2,5-dimethoxybenzyl alcohol</td>
<td>33524-31-1</td>
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<td>95-48-7</td>
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<td>2-methylanisole</td>
<td>578-58-5</td>
<td>114.0±0.2</td>
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<tr>
<td>2,6-dimethylphenol</td>
<td>576-26-1</td>
<td>99.0±0.1</td>
</tr>
<tr>
<td>3,4-dimethylphenol</td>
<td>95-65-8</td>
<td>85.00±0.06</td>
</tr>
<tr>
<td>1,2,3-trimethoxybenzene</td>
<td>634-36-6</td>
<td>78.0±0.5</td>
</tr>
<tr>
<td>3,5-dimethylphenol</td>
<td>108-68-9</td>
<td>26.0±0.3</td>
</tr>
<tr>
<td>3,4-dimethoxytoluene</td>
<td>494-99-5</td>
<td>23.0±0.3</td>
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<tr>
<td>4-ethyl-2-methoxyphenol (4-ethylguaiacol)</td>
<td>2785-89-9</td>
<td>15.0±0.3</td>
</tr>
<tr>
<td>styrene</td>
<td>100-42-5</td>
<td>13.00±0.06</td>
</tr>
<tr>
<td>3-methylanisole</td>
<td>100-84-5</td>
<td>5.0±0.2</td>
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Table 1. (continued) (b) Calibrated compounds not detected in the emulsion.

<table>
<thead>
<tr>
<th>CAS #</th>
<th>CAS #</th>
<th>CAS #</th>
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<tr>
<td>furan</td>
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<td>guaiacyl acetone 2503-46-0</td>
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<td>indene</td>
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<td>p-xylene</td>
<td>106-42-3</td>
<td>2,4-dimethylphenol 105-67-9</td>
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<td>isoeugenol</td>
<td>97-54-1</td>
<td>1,2-benzenedimethanol 612-14-6</td>
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<td>91-20-3</td>
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<td>levoglucosan</td>
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<td>hydroquinone</td>
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<td>sinapaldehyde</td>
<td>4206-58-0</td>
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<td>2-methylfuran</td>
<td>534-22-5</td>
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<tr>
<td>4-methylanisole</td>
<td>104-93-8</td>
<td></td>
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</table>
Table 2. Phenolic monomer recovery is directly related to the amount of emulsifier used.

Emulsions were made at room temperature with 70:30 Tween® 20 and Span® 80 blend by vortexing for 5 minutes. Monomer recovery was assessed by the difference in the initial mass of phenolic monomer-rich product and the mass of material remaining after emulsification. Values reported are the averages of at least two replicates ± one standard deviation.

<table>
<thead>
<tr>
<th>Emulsifier dosage (g of emulsifier blend per g of phenolic monomer-rich product)</th>
<th>0.0136±0.0005</th>
<th>0.055±0.008</th>
<th>0.076±0.002</th>
<th>0.205±0.008</th>
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</thead>
<tbody>
<tr>
<td>Recovery of phenolic monomer-rich product in emulsion (wt%)</td>
<td>7.400±0.003</td>
<td>10.400±0.005</td>
<td>14.80±0.03</td>
<td>20.700±0.009</td>
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CHAPTER 4. CHEAP, FAST, and QUANTIFIABLE MICROBIAL ASSAY for LIGNIN UTILIZATION

Kirsten Davis, Aleeza Gerstein, Laura Jarboe, Zhiyou Wen, Robert C. Brown, X.L. Bai
To be submitted

Abstract

Lignin is recalcitrant, but its valorization is critical to the economic viability of the biorefinery model. One approach for lignin valorization is to use microbes to upgrade lignin-derived monomers. Certain bacteria are known to utilize monomers present in depolymerized lignin, especially aromatic monomers with acid and aldehyde moieties. However, depolymerized lignin is a complex mixture of aromatic and phenolic monomers, with the distribution varying based on biomass type and composition and the treatment method. Only a small fraction of these monomers have been tested for utilization by microbes. Since phenolic monomers can be sparingly soluble in aqueous systems and toxic to microorganisms, it can be difficult to characterize their utilization in liquid culture. Here we were inspired by the quick screening capabilities of the Kirby-Bauer test for quantifying antibiotic resistance. The diffusive inhibition with substrate consumption (DISC) assay described here identifies microbial candidates which can utilize phenolic monomers, and also assesses resistance to and scavenging of the monomers. In the DISC assay, the only carbon source is the phenolic monomer of interest, which diffuses in an agar plate creating a concentration gradient. The microbe only grows if it can resist and utilize the phenolic monomer, and the growth is easily visualized on the plate. The diskImageR platform for quantitative analysis of antimicrobial disk diffusion assays was modified for use with the DISC assay. We used the DISC assay to characterize seven microbial species and nine representative phenolic monomers, demonstrating that this approach not only identifies
microbial utilization of the monomer, but also provides quantitative measurements that are significantly correlated with assessment of monomer resistance and monomer scavenging in liquid culture. This work demonstrates not only a method that can be used in improve microbial valorization of lignin, but also the benefit of biorenewables researchers using methods developed for characterizing pathogenic microbes.

Introduction

Lignin’s untapped potential

Lignin is a heterogeneous polymer which protects and gives structure to plants, though is composition varies depending on biomass type [1]. Lignin has traditionally been viewed as a waste stream. For example, three billion m³ of wastewater containing 11-25 g/L of lignins and 17-800 mg/L of phenols are produced annually by the pulp and paper industry [2]. However, there is a growing interest in upgrading the lignin, motivated in part by a variety of analyses that have indicated that the addition of value to lignin is essential to the economic viability of the biorefinery model [3]. Given that interest in adding value to lignin is relatively new, strategies for improving microbial utilization of the carbohydrate portion of biomass is far more advanced than the technology for the utilization of the lignin component.

Current technology in lignin recovery

There are a number of technologies that recover lignin from biomass. In each, the biomass is subjected to some combination of mechanical treatment, chemicals, heat, and/or light. Lignin can be depolymerized during the recovery process or after isolation with a number of methods including pyrolysis, acid or base catalysis, and supercritical fluid treatment, as previously reviewed [4–7].
Biological upgrading of lignin-derived monomers and oligomers

Some microorganisms are naturally able to consume a variety of aromatic and phenolic molecules and use them for biomass and energy production [8,9]. Aromatic acids present in alkaline pretreated liquor including p-coumaric, ferulic, vanillic, and 4-hydroxybenzoic acids have been extensively studied for microbial utilization. *Pseudomonas putida* KT2440, *Amycolatopsis*, *Rhodococcus jostii* RHA1, *Rhodococcus erythropolis* U23A, and *Pseudomonas fluorescens* pf-5 have varying abilities to utilize aromatic acids [8,10,11]. These organisms provide vital design strategies for the production of microbial cell factories that can utilization a wide variety of lignin-derived molecules for the production of fuels and chemicals. For example, *P. putida* has been engineered to produce muconic acid, a polymer precursor, from p-coumaric acid [12,13]. Less is known about microbial utilization of some other phenolic monomers. For example, pyrolytic lignin is relatively lacking in aromatic acids and only a few of the most abundant monomers, such as vanillin, phenol, and 2-methoxyphenol, have been extensively tested for microbial utilization. There are still many questions about which particular aromatics can be utilized. In addition to the questions about utilization, another problem is that aromatics can inhibit the growth of microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, and *Rhodococcus opacus* PD630 [14–16]. *Pseudomonas putida* KT2440 is also inhibited by aromatics such as benzoate and p-coumarate [17,18]. To select organisms which will perform well for lignin valorization, it is important to understand both the utilization and the toxicity of the lignin-derived monomers. Organisms that can scavenge monomers at low concentrations are attractive for utilization of depolymerized lignin since it is complex mixture of many monomers.
Antibiotic diffusion assay

We propose a disk diffusion assay as a method to screen aromatic hydrocarbon utilization and inhibition simultaneously. Such assays allow researchers to test a range of antibiotic concentrations on one plate and are commonly used to assess microbial antibiotic resistance [19]. Briefly, a disk containing an antimicrobial drug is placed in the center of a plate which contains agar and a carbon source such as glucose plate that is freshly seeded with a lawn of the microorganism. The drug diffuses from the disk towards the plate edge, creating a concentration gradient. The microorganism is only able to grow in the region of the plate where the concentration of antibiotic is below the minimum inhibitory concentration of drug. The result is a visible zone of inhibition around the disk where there is little to no cell growth. A smaller zone of inhibition indicates that the microorganism is more resistant to the antibiotic.

Here, aromatic hydrocarbons act like antimicrobial drugs in the sense that they can inhibit microorganism growth in a concentration-dependent manner. However, in contrast to antimicrobial drugs, some aromatic hydrocarbons can also be used as a source of carbon and energy. In our phenolic DISC assay, inhibition and utilization are both expected to occur. Thus, in addition to the region of inhibition similar to the antibiotic assay, we also anticipate a region of resource limitation near the edges of the plate.

The zone of inhibition in a disk diffusion assay was traditionally determined by measuring the diameter across the observed area of growth inhibition either with a physical ruler or virtually in image analysis software such as ImageJ/FIJI. The open-access R package `diskImageR` improved upon this method by providing researchers with a fully-automated, computational pipeline that quantifies the zone of inhibition around the antimicrobial disk (see methods, [19]). Here we show that an extension of this framework can also be used to
quantify inhibition and utilization of different lignin monomers by a diversity of bacterial taxa in a high throughput fashion.

**Materials and Methods**

**Materials**

Precultures were grown in LB media which contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride at pH 7.0. The disk diffusion plates were made with modified M9 medium: 13.56 g/L disodium phosphate, 6.0 g/L monopotassium phosphate, 1.0 g/L sodium chloride, 2.0 g/L ammonium chloride, 2mM magnesium sulfate, 100µM calcium chloride, 18 µM iron sulfate, and 15.0 g/L bacto agar [12]. The pH was adjusted to 7.0. The first four ingredients were mixed with DI water and autoclaved. The last magnesium sulfate, calcium chloride, and iron sulfate were filter sterilized and added after autoclaving to avoid the formation of a precipitate. For testing syringol, which oxidized easily indicated by an orange color, sodium sulfite was added to a concentration of 1 g/L before autoclaving. Sodium sulfite was chosen because it has been used in the food industry as an antioxidant [20]. Results from the syringol experiments can be viewed in the supplementary material. *S. multivorrum* was purchased from ATCC as strain ATCC® BAA-1093™. Stocks of *P. putida*, *P. putida* KT240 LJ015, *Amycolatopsis*, *R. jostii* RHA1, *R. erythropolis* U23A, and *P. protegens* were from Dr. Gregg Beckham from the National Renewable Energy Lab.

**Cell culture and preparation**

The bacteria were pre-cultured in LB medium in 250 mL flasks with a working volume of 10 mLs, and the following conditions: *P. putida*, *P. putida* LJ015, *P. protegens*, *R. erythropolis* U23A, *S. multivorrum*, 30°C, 200 RPM, overnight; *R. jostii* RHA1 30°C, 200 RPM, two overnights, *Amycolatopsis* 37°C, 200 RPM two overnights. Cells were harvested by centrifugation at room temperature 3,500 X g for 2 minutes. Cells were washed 3 times
with modified M9 medium without magnesium sulfate, calcium chloride, or iron sulfate. Each centrifugation step was at room temperature 3,500 X g for 2 minutes, and each resuspension was the same volume as the original volume harvested from the culture. 100 µL of the washed cells were spread with a hockey stick spreader or glass beads on the agar plates.

**Liquid cultures**

For liquid cultures, cells were resuspended in 20 mLs of modified M9 medium with varying concentrations of vanillin in 250 mL flasks. They were shaken in the same conditions as the pre-cultures. All liquid cultures started with an OD$_{600}$ of 0.05. Cultures were monitored periodically by measuring the OD$_{600}$.

**Agar Plate Preparation**

Modified M9 with agar was prepared as described in the materials section. For syringol which was easily oxidized, 1 g/L of sodium sulfite was also added. 10 mLs of hot M9 agar were added to empty petri dishes. The filter paper disks were cut into circles with a 12.7 mm diameter from Whatman 55 mm filter papers. To impregnate the disks with the phenolic of interest, the pre-weighed autoclaved disks were placed in the wells of a 12-well plate, and the phenolic solutions were added to each well. The final concentration if the phenolic fully diffused throughout the disk would be 0.002 g/mL. This concentration of 0.002 g/mL has been used as a starting point in studies of aromatic utilization [12]. The disks were dried in a laminar flow hood until there was no more liquid visible on the disk surface. The disks were weighed again to calculate the amount of phenolic on each disk. The disks were placed on top of the agar plates using tweezers immediately after the agar plates were spread with cells. The plates were incubated for 72 hours at the same temperatures described in the culturing section.
Imaging and Image Analysis

Images of the plates were taken with a Canon EOS Rebel Ti3 camera in a dark room with black velvet under the plates. A fluorescent light was placed next to the plate to illuminate it without casting shadows. The lid of the plate was removed during image acquisition to eliminate glare.

The images were analyzed with a modified version of the image analysis program diskImageR [19]. This program was originally written to quantify drug susceptibility and tolerance from drug disk diffusion assays. In these assays, there is typically uniform growth beyond the zone of inhibition. To capture phenolic assay growth, additional options were provided to existing functions, and additional functions were written to quantify growth beyond the zone of inhibition. The primary workflow in diskImageR uses ImageJ to convert images of disk diffusion assays to pixel intensity values. The program then uses a maximum likelihood frame to fit a logistic model to this data, which is utilized to determine where the zone of inhibition is by identifying the point on the curve where growth is reduced by a specified amount (typically 20%, other values are also supported; [19]).

The primary difference from a computational perspective for phenolic assay growth is the absence of a growth asymptote beyond the zone of inhibition. To account for this, the maximum likelihood function was modified to provide an option to use the maximum point of growth intensity to inform the asymptote, in essence, to force the asymptote of the resultant logistic function to match the point of maximum growth intensity rather than the area beyond this point where growth is diminished due to growth requirements and a concentration reduction of the required compound (Figure 1). The existing diskImageR parameters that assess the zone of inhibition can then be determined from this analysis (i.e, the radius of inhibition for 20%, 50% and 80% reduction in growth). The slope of inhibition
or SLOPE as it is referred to in the diskImageR package, is calculated by taking the slope of the ten data points above and below the point at which growth is decreased by 50% on the disk adjacent side (Figure 2) [19].

To quantify resource limitation beyond the zone of inhibition, a new function “growthInhib()” was developed and added to diskImageR. This function determines the point of maximum intensity (both distance from the disk as well as pixel intensity), as well as the distances beyond this point that corresponds to growth reductions of 90%, 75%, 50%, 25% and 10% (where appropriate) (Figure 2). Because we sometimes observed this local maximum most likely due to a higher concentrations at the edges of the plate (see diffusion model with cells), the analysis of parameters was restricted to 30 mm away from the center of the disk. diskImageR is freely available to be downloaded from github (http://www.github.com/acgerstein/diskImageR). A detailed vignette and script walkthrough are available at http://acgerstein.weebly.com/diskImageR).

**Measurement of phenolic monomer diffusion**

Sample collection and preparation was adapted from Arcelloni et al. [21]. Samples of the solid media were obtained using a Pasteur pipette with a 2mm opening to cut a cylinder into the agar, and then tweezers were used to pull the cylinder out of the plate. 20 ug of ethylvanillin was added to all samples as an internal standard. 60 uL of acetonitrile was added to the sample and heated at 80°C in a dry bath for 40 minutes. The treatment melted the agar, and then all samples were filtered by a 0.2 um PTFE syringe filter to remove any residual solids. Samples were silylated by adding 70 uL of BSTFA and incubating for 30 minutes at 60°C.

GC-MS was carried out in the W.M. Keck Metabolomics Laboratory at Iowa State university on a 6890N gas chromatograph in tandem with a 5973 Agilent MSD. The
separation was obtained on an Agilent-HP5MSI (30 m long, 0.250 mm ID, 0.25 μm film thickness) column. The oven program was as follows: Initial temperature of 70°C for 1 min followed by, 8°C/min ramp to 120 and 25°C/min ramp to 320°C with a final hold for 3 min. The inlet and the interface temperatures were maintained at 280°C. The detection mass range was set from 40–800 m/z. The GCMS was controlled by the Agilent ChemStation software. Guaiacol and vanillin were identified using their total ion mass spectrum and comparison to NIST 14 library and also confirmed by the running of the standards on the same method.

Results and Discussion

The DISC assay (diffusive inhibition with substrate consumption) aims to assess both microbial utilization of and resistance to inhibitory substrates, such as phenolic monomers. Our focal monomers and organisms were selected based on reported characterization of thermally depolymerized lignin [22] and microbial utilization of aromatic monomers (Table 1, Table 2). Because these monomers were solubilized in ethanol, disks soaked only in ethanol were used as a negative control. The traces for all experiments are in the supplemental data file. Quantitative assessment was performed by diskImageR.

For cases in which the microbe was able to utilize the associated monomer as carbon source, a zone of no growth surrounds the disk followed by a region of microbial growth further away from the disk (Figure 3), similar to the standard antibiotic resistance assay. However, the assay described here differs from the standard antibiotic resistance assay in that there is a decrease in intensity of microbial growth approaching the edge of the plate, as would be expected in the case of limiting substrate concentration. Each organism/monomer combination has a variety of associated metrics. For example, the size of the inhibition zone can be readily estimated based on visual inspection. The distance from the disk at which growth is at a maximum value requires careful assessment of the position associated with
maximum growth, such as via quantitative image analysis. Rigorous image analysis also enables estimation of the inhibition slope and the limitation slope (Figure 4). These slopes describe the relationship between cell growth and distance from the disk. The inhibition slope describes the transition from the zone of inhibition to the point of maximum growth. Similarly, the limitation slope describes the transition from the point of maximum growth to the outer edge of microbial growth. These metrics each provide additional insight into utilization and tolerance of the monomer by the organism.

**Detection of monomer utilization via inhibition slope**

The primary outcome of the DISC assay is determination of whether a certain microbial species can utilize a specific phenolic monomer as sole carbon source. The use of the inhibition slope as a numerical metric enables rigorous comparison to the negative control for this determination. This metric (SLOPE in *diskImageR*) quantifies the transition between the regions of no growth and full growth.

When the DISC assay is performed with an organism that cannot utilize the associated monomer as sole carbon source, it is expected that there will be no microbial growth or perhaps a small amount of growth due to utilization of the trace amounts of ethanol. In this case, the slope of inhibition should be very small. Contrastingly, when the organism is able to utilize the incoming front of phenolic monomer as carbon source, there is a gradient of growth reflecting movement of the monomer front due to diffusion and microbial consumption of the monomer.

In our initial development of this assay, inhibition slopes significantly greater than the corresponding ethanol control were interpreted as evidence of utilization of the monomer for growth (Figure 4). Using this criteria, five of the six microbial species characterized here show utilization of vanillin. These results are the first reports of vanillin utilization by *R.*
*erythropolis* and *S. multivorum*. Our results for *P. putida* and *Amycolatopsis* are consistent with prior reports [23,24]. Vanillin utilization by *P. protegens* is consistent with the detection of a putative vanillin dehydrogenase within its genome by a BLAST search against the *P. putida* vanillin dehydrogenase [8], as well as the fact that other *Pseudomonads* such as *P. fluorescens* HR199 are known to degrade vanillin [25]. The utilization of vanillin as sole carbon source by *R. erythropolis, S. multivorum* and *P. protegens* was verified by growth in liquid culture, as discussed below.

Among our pool of focal organisms, only *R. jostii* did not have a vanillin inhibition slope that significantly differed from the ethanol control. This was surprising given that *R. jostii* was previously reported to grow on 0.2 mg/mL vanillin as sole carbon source [26]. Further characterization verified that *R. jostii* does grow in liquid cultures containing 0.1 and 0.2 mg/mL vanillin, but not at higher concentrations, as described below (Figure 5). Based on the amount of monomer applied to the disc, it is expected that the bulk concentration of vanillin in the solid media at equilibrium would be greater than 0.5 mg/mL. Thus, the vanillin concentration in the DISC assay may have been prohibitively high for detection of *R. jostii* growth during the proscribed incubation period.

Among the six species tested here, *Amycolatopsis* and *R. jostii* were unusual in that the inhibition slopes indicated utilization of guaiacol. This result is consistent with previous reports that both organisms express DyP enzymes, which have activity on guaiacol [27,28]. Also, growth of *R. jostii* on in liquid culture with guaiacol as sole carbon source has been previously reported [29], and was also observed here.

*P. putida* on 1,2-benzenedimethanol had an inhibition slope significantly higher than the negative control, though only at 24 and 72 hrs and not at 48 hrs. Also, the magnitude of
these inhibition slopes was much lower than those observed during utilization of vanillin by the same organism. Liquid cultures were performed with 0.1 and 0.2 mg/mL 1,2-benzenedimethanol to corroborate the disk diffusion results, but cultures did not show any growth over 120 hours of observation. This suggests that inhibition slopes that are relatively small and inconsistent across time points should be cautiously interpreted as evidence of growth and verified by other experimental methods, such as liquid culture.

**Ranking of monomer tolerance**

The inhibition slope metric can be used to determine if a given compound is utilized by a specific microbe. However, it is desirable for a microbial biocatalyst to not just utilize phenolic monomers, but to also tolerate the monomers at relatively high concentrations. The metrics calculated by diskImageR allow comparison of monomer tolerance across organisms and monomers.

As described above, all of the organisms characterized here are able to utilize vanillin as sole carbon source, though the vanillin sensitivity of *R. jostii* prevented detection of vanillin utilization in the DISC assay. We probed the ability of these organisms to tolerate this monomer in liquid culture across a range of vanillin concentrations (Figure 5). The inhibitory concentration in liquid culture was determined based on a lack of growth. For example, for *P. putida* the vanillin inhibitory concentration was 2.2±0.2 mg/mL. Contrastingly, over the standard observation period of 72 hrs, growth of *R. jostii* was, on average, not detected in the presence of 0.6 mg/mL vanillin, though variability in the length of the lag time between replicates resulted in a large confidence interval for this assessment.

Instead of using liquid cultures to compare microbial tolerance of a single monomer across a range of concentrations, this comparison can be easily performed using the RAD\textsubscript{20} value determined by the DISC assay. This RAD\textsubscript{20} value was previously correlated with the
log₂(MIC50) in the standard antibiotic disk diffusion assay [19]. Microbial utilization of a monomer in the DISC assay results in a position from the disc at which growth is at its maximum intensity. The gradient from the zone of no growth towards this position of maximum growth includes a position at which growth is 80% of the maximum value. This position is referred to as RAD₂₀, in that growth is reduced by 20% relative to the maximum. A smaller RAD₂₀ indicates less inhibition because the cells are growing closer to the disk. The 20% cutoff has been determined to be what the average human eye would classify as a prominent reduction in growth in the standard antibiotic disk diffusion assay [19]. The standard zone of inhibition [30] can be compared to the RAD metric by doubling the RAD value and adding the diameter of the diffusion disk. Here, we observed that the vanillin RAD₂₀ value from the DISC assay is significantly correlated with the inhibitory vanillin concentration in liquid culture (Figure 5). For example, the RAD₂₀ value was significantly lower for *P. putida* at all time points relative to other microbes, consistent with the high tolerance of vanillin in liquid cultures.

These characterizations also included *P. putida* strain EM42 LJ015 due to its reported 200 fold improved tolerance over *P. putida* in fast pyrolyzed, fractionated biomass and with individual phenolic monomers [31]. We observed that both *P. putida* KT2440 and *P. putida* EM42 LJ015 had the smallest RAD₂₀ values amongst all strains tested on vanillin, indicating a relatively high vanillin tolerance. However, our results do not indicate that EM42 LJ015 has increased vanillin tolerance in the DISC assay. This difference from previous reports may be due to the use of glucose in prior studies, while no glucose is present in the DISC assay. The EM42 LJ015 strain was previously engineered for improved protein folding [31,32]. The
energy required for protein folding may make this tolerance strategy less effective in the absence of glucose.

We also explored the ability of the RAD$_{20}$ metric to reflect the guaiacol sensitivity of *Amycolatopsis* and *R. jostii* (Figure 5). For both organisms, the RAD$_{20}$ value was smaller on guaiacol than on vanillin and the inhibitory concentration of guaiacol was higher than the inhibitory concentration of vanillin. Thus, it appears that the RAD$_{20}$ metric can be used as a robust and facile alternative to liquid cultures in both comparisons across organisms for a single monomer and across monomers for a single organism.

**Ranking monomer scavenging ability**

An ideal microbial biocatalyst for valorization of depolymerized lignin can utilize a range of phenolic monomers, tolerate these monomers at high concentrations, but also be able to scavenge the monomers when they are present at low concentrations. Scavenging ability can be assessed in liquid cultures with a range of monomer concentrations, similar to assessment of monomer tolerance. However, just as the DISC assay can quantitatively assess monomer tolerance, it can also quantitatively assess monomer scavenging.

Here we define the limiting concentration of substrate as the concentration in liquid culture at which the maximum OD is decreased by 50% relative to the overall maximum OD. For instance, the overall maximum OD for *P. putida* during growth on vanillin was 1.5±0.1. Half of that value is 0.76±0.06, which was observed at a vanillin concentration of 0.84 mg/mL (Figure 5). This value is much higher than the limiting concentration of 0.2 mg/mL vanillin observed for *Amycolatopsis*, indicating improved vanillin scavenging ability relative to *P. putida*.

An increase in maximum OD over a concentration range followed by a sharp drop off where the cells no longer grew in higher concentrations was observed for all strains (Figure
5). The increase in maximum OD$_{600}$ over a concentration range can be thought of as the liquid-culture version of the zone of limitation in the disk diffusion assay. The sharp drop-off at higher concentrations can be thought of as the liquid-culture version of the zone of inhibition in the disk diffusion assay.

We observed that the limiting concentration in liquid culture is significantly correlated with the DIST$_{10}$ metric of the DISC assay (Figure 5). As shown in Fig 5 (Amycolatopsis/vanillin), monomer utilization results in a position of maximum growth. As one continues outward from this position towards the edge of the plate, growth intensity decreases, as reflected by the limitation slope. The position at which growth intensity is decreased by 10% relative to the maximum value is quantified by the DIST$_{10}$ metric. A larger DIST$_{10}$ value indicates less limitation because the cells are growing closer to the edge of the plate.

The vanillin DIST$_{10}$ values, excluding R. jostii, were significantly correlated with the limiting vanillin concentration in liquid culture (Figure 5). For example, P. putida had the smallest vanillin DIST$_{10}$ and also was the worst at scavenging vanillin, as evidenced by the high limiting concentration. Contrastingly, Amycolatopsis had a much larger DIST$_{10}$ value and was much better at growing on low concentrations of vanillin.

This ability to infer scavenging ability based on DIST$_{10}$ values also seems to apply in the comparison of monomers, not just the comparison of organisms. Specifically, Amycolatopsis has a smaller DIST$_{10}$ value for guaiacol than the value observed for vanillin. This is consistent with the observation that guaiacol is limiting for Amycolatopsis growth at concentrations of 0.84 mg/mL in liquid culture, while the limiting vanillin concentration is 0.2 mg/mL. Scavenging of vanillin and guaiacol by R. jostii was not considered here due to
poor growth in the vanillin DISC assay and an inability to determine a DIST\(_{10}\) value for \(R. jostii\) on guaiacol, as no tapering of growth was observed.

\(P. putida\) EM42 LJ015 had a higher DIST\(_{10}\) at 72 hours and also had a lower vanillin limiting concentration in liquid culture which suggests it is better at scavenging vanillin than \(P. putida\) KT2440. This difference may be due to previous engineering of the EM42 LJ015 parent strain for genome reduction with the goal of increasing energetic efficiency. This increased energetic efficiency may enable increased biomass production during carbon source scarcity.

The DIST\(_{10}\), metric is unique metric to the DISC assay and has not been previously used in assessment of antibiotic resistance. We observed that the DIST\(_{10}\) metric correlated with the limiting monomer concentration in liquid cultures with a Kendall’s \(\tau\) value of -0.6 with a \(p\) value of \(6 \times 10^{-4}\). We also investigated the limitation slope as an indicator of scavenging ability, but the correlation was stronger for the DIST\(_{10}\) metric. Thus, the DIST\(_{10}\) metric is another useful output from the DISC assay as an alternative to the use of liquid cultures for assessing the microbial utilization of phenolic monomers at a range of concentrations.

**Confirmation of monomer diffusion and consumption**

The results presented above demonstrate how the DISC assay can be used to recognize microbial consumption of a phenolic monomer and quantitatively compare tolerance and scavenging ability across monomers and species. This assay relies on diffusive transport of the monomer outward from the disk, combined with the consumptive sink of microbial activity. The relative timescales of monomer diffusion, microbial growth, and monomer consumption merit consideration.
In the absence of microbial activity, the monomer concentration in the solid media should reach a uniform value after a sufficient length of time. We confirmed that this uniform concentration was reached within the standard incubation period of 72 hrs using guaiacol as a model monomer. This analysis was performed at both 30°C, the most commonly used temperature for the data presented, and at 37°C to accommodate the growth requirements of *Amycolatopsis*. At 30°C, the monomer fully diffused throughout the plate between 24 and 48 hrs. At 37°C, the diffusion was complete within 10 – 24 hrs. The faster diffusion of guaiacol at 37°C relative to 30°C is consistent with the expected impact of temperature on mass transfer. These results demonstrate that a concentration gradient across the plate does exist during the course of the DISC assay but also that the monomer diffuses to the edge of the plate by the end of the observation period. The diffusivity values of molecules in agar have previously been reported to vary with molecular weight [33]. The monomers used here have a molecular weight within 25% of the guaiacol molecular weight (Table 1) and thus it is expected that this conclusion is valid across monomers.

Experimental assessment of the guaiacol profile in the presence of *Amycolatopsis* also follows the expected trends (Figure 6). After 10 hrs, the concentration profiles with and without cells are experimentally similar, as any consumption of the monomer by the cells is not yet of sufficient magnitude to detect. A similar trend was observed at 24 hrs (not shown). However, at 48 hrs a clear difference is observed between plates with and without cells. Specifically, plates with cells have essentially no detectable levels of guaiacol (Figure 6). This confirms that the observed microbial growth was due to utilization of the guaiacol and demonstrates that the scale and rate of microbial activity is sufficient to decrease monomer abundance to below the detectable limit within the assay period.
Diffusion model illustrates the difference between tolerance and scavenging abilities

The experimental analysis described above shows microbial activity is sufficient to decrease monomer concentration to below the detection limit over the course of the assay. For example, at the position 20 mm from the disk, the guaiacol concentration was approximately 1 mg/mL at the 10 hr timepoint, but then was below the detection limit of 3.5E-5 mg/mL at 48 hrs. This raises the concern that microbial activity may outpace monomer diffusion, and thus cells further from the disk may not have the opportunity for monomer utilization. This scenario would possibly interfere with interpretation of the limitation slope values. Mathematical modeling of the diffusion and microbial activity were used to explore this concern.

The concentration profile due to diffusion outward from the disk and the sink of microbial consumption were modeled with a 1-dimensional transport equation (Equation 1). The consumption of guaiacol by *Amycolatopsis* at 37°C was used as a test case to establish and validate the model. The diffusion components of the model were tuned using the experimental concentration profiles in the absence of cells. Microbial consumption of the monomer was modeled using the Monod equation (Equation 6). Cell density as a function of position and time was based on experimental data from *diskImageR* using a previously-reported double logistic equation [19] modified here to include an exponential decay component to account for limitation. The maximum specific growth rate $\mu_{\text{max}}$ of *Amycolatopsis* on guaiacol on solid media was estimated based on the image intensity of the plates over time. The model of *Amycolatopsis* on guaiacol and the experimental data on which the model is based are shown in Figure 7 a,b.

The parameters of the growth part of the model (Cc) were varied to understand how the growth profile relates to the diffusion (Figure 7). Varying Asyma and Asymb which
relate to the asymptote at the maximum intensity had little effect on the diffusion model.

Scala and Scalb which relate to the curving of the logistic model also had little effect on the diffusion model. Little variation with asym and scal parameters can be attributed to the larger effect of the Umax parameter. However varying OD50 shifts the whole growth model on the x axis. This did have a visible effect on the diffusion model.

**Partial differential equation**

Eq 1) \( \frac{\partial (CS)}{\partial t} = D \frac{\partial^2 (CS)}{\partial x^2} - s(x,t) \)

**Initial Condition**

Eq 2) \( Cs(x,0) = Cs_{disk} \) at \( x<R_{disk} \)

Eq 3) \( Cs(x,0)=0 \) at \( R_{disk}<x<R_{plate} \)

**Boundary Conditions**

Eq 4) \( \frac{dCs}{dx} = 0 \) at \( x = 0 \)

Eq 5) \( \frac{dCs}{dx} = 0 \) at \( x = R_{plate} \)

**Consumption of inhibitor by cells**

Eq 6) \( s(x,t) = \frac{\mu_{max} \cdot Cs \cdot Cc}{K_s + Cs} \)

Eq 7) \( Cc = \frac{\text{asma} \cdot \exp(\text{scala} \cdot (\log x - \text{OD50a}))}{\exp(\text{scala} \cdot (\log x - \text{OD50a}))} \) at \( R_{disk} \leq x \leq b \)

Eq 8) \( Cc = \) logistic equation if no limitation or \( c \cdot e^d \) if limitation \( b > x \)

Eq 9) \( \frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_s}{\mu_{max} \cdot C_s} \)

**Terms**

\( Cs = \) conc. of inhibitor  
\( Cc = \) conc. of cells  
\( D = \) diffusivity set to 10 mm\(^2\)/h diffusivity  
\( R = \) radius of disk  
\( K_s = \) Monod constant  
\( b = \) distance to maximum intensity  
\( I_{max} = \) maximum intensity  
\( I_0 = \) initial intensity

The diffusion model predicts lower phenolic concentrations near the edge of the plate with a good scavenger.

We predicted that an ideal microbial candidate would have high tolerance and good scavenging ability. To explore this hypothesis, the growth model of *Amycolatopsis* on guaiacol (Figure 7d) was replaced with the growth model of *Amycolatopsis* on vanillin...
Amycolatopsis on vanillin showed lower tolerance and higher scavenging compared with growth on guaiacol. The percent difference in growth (Figure 7f) illustrates that there are more cells near the edge of the plate on vanillin compared with guaiacol. The predicted percent difference in phenolic concentration (Figure 7b 30 hours, 7c 48 hours) is larger near the edge of the plate as expected. Although Amycolatopsis on guaiacol was more tolerant than on vanillin, the predicted percent difference in phenolic concentration near the edge of the disk is no significant in this case.

Discussion

The DISC assay, modified from a widely accepted antibiotic resistance assay, highlights the strengths of interdisciplinary research. Just as in antibiotic resistance screening, the DISC assay allows for testing a range of concentrations in one discreet disk which would normally take a number of flasks or wells in liquid culture. Therefore, it is useful when a large number of microbes and monomers need to be tested; even when little is known about the acceptable concentration range. Here we demonstrated the utility of the assay by screening six microbial candidates on nine phenolic monomers. We compared the tolerance and scavenging abilities of microbes on vanillin and guaiacol. Amycolatopsis on guaiacol and P. putida on vanillin were among the most highly tolerant while Amycolatopsis on vanillin was one of the best scavengers. The DISC assay can complement genetic engineering work as we demonstrated when comparing P. putida KT2440 for P. putida EM42 LJ015. We found that although the strains did not differ in tolerance on vanillin as sole carbon source, KT2440 was a better scavenger. A model of the diffusion in the DISC assay complemented the image analysis by predicting different profiles for different growth models. For instance, Amycolatopsis on vanillin was a better scavenger than Amycolatopsis
on guaiacol and the percent difference in concentration near the edge of the disk reflected more consumption on vanillin. Interesting future applications of the DISC assay include screening of genetically engineered or evolved strains on lignin monomers or even other bioconversions of interest.

References


Figure 1. (a) The standard calculation for the logistic equation determined through maximum likelihood (red line). (b) The new modification of the maximum likelihood calculation better captures growth when there is inhibition and resource limitation by forcing the asymptote of the determined logistic equation through the global maximum. The grey points are pixel intensities determined by computational analysis of photographs in ImageJ. Distance from the center of disk is in units of mm.
Figure 2. The new function “growthInhib()” determines the point of maximum intensity (black arrow) and various cutoff points for resource limitation beyond that point (grey arrows). It also calculates the slope up to the maximum intensity point (“inhibition slope”, red line) and from the maximum intensity point (“limitation slope”, blue line).
Table 1. Phenolic monomers tested here. Chemical structures were produced using ChemDraw Professional. Aqueous solubility was estimated based on the LogS value predicted by ChemDraw Professional.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>IUPAC Name</th>
<th>CAS number</th>
<th>MW</th>
<th>Predicted solubility (g/L)</th>
<th>Chemical Formula</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-benzenedimethanol</td>
<td>[2-(hydroxymethyl)phenyl]methanol</td>
<td>612-14-6</td>
<td>138</td>
<td>72</td>
<td>C₇H₁₀O₂</td>
<td><img src="image1.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>2,3-dimethoxytoluene</td>
<td>1,2-dimethoxy-3-methylbenzene</td>
<td>4463-33-6</td>
<td>152</td>
<td>27</td>
<td>C₇H₁₀O₂</td>
<td><img src="image2.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>2-methylanisole</td>
<td>1-methoxy-2-methylbenzene</td>
<td>578-58-5</td>
<td>122</td>
<td>19</td>
<td>C₇H₁₀O</td>
<td><img src="image3.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>4-ethylguaiacol</td>
<td>4-ethyl-2-methoxyphenol</td>
<td>2785-89-9</td>
<td>152</td>
<td>20</td>
<td>C₇H₁₀O₂</td>
<td><img src="image4.png" alt="Chemical Structure" /></td>
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<tr>
<td>4-vinylguaiacol</td>
<td>2-methoxy-4-vinylphenol</td>
<td>7786-61-0</td>
<td>150</td>
<td>18</td>
<td>C₇H₁₀O₂</td>
<td><img src="image5.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Creosol</td>
<td>2-methoxy-4-methylphenol</td>
<td>93-51-6</td>
<td>138</td>
<td>26</td>
<td>C₇H₁₀O₂</td>
<td><img src="image6.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Guaiacol</td>
<td>2-methoxyphenol</td>
<td>8021-39-4</td>
<td>124</td>
<td>34</td>
<td>C₆H₈O₂</td>
<td><img src="image7.png" alt="Chemical Structure" /></td>
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<tr>
<td>Phenol</td>
<td>phenol</td>
<td>108-95-2</td>
<td>94</td>
<td>27</td>
<td>C₆H₆O</td>
<td><img src="image8.png" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Vanillin</td>
<td>4-hydroxy-3-methoxybenzaldehyde</td>
<td>121-33-5</td>
<td>152</td>
<td>40</td>
<td>C₆H₈O₃</td>
<td><img src="image9.png" alt="Chemical Structure" /></td>
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Table 2. Organisms tested here.

<table>
<thead>
<tr>
<th>Organism Name</th>
<th>Aromatic Catabolites</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amycolatopsis</em> sp. 75iv2 ATCC^® 39116™ previously <em>Streptomyces setonii</em> ATCC* 39116™</td>
<td>vanillin^</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>guaiacol</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td><em>p</em>-coumaric acid</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>ferulic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vanillic acid</td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em> U23A</td>
<td>poly-chlorinated biphenols (PCBs)</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td><em>p</em>-coumaric acid</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>ferulic acid</td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus jostii</em> RHA1</td>
<td>vanillin*</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>phenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-ethoxyphenol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>styrene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>guaiacol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>toluene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>protocatechuate</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>o</em>-xylene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vanillic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>p</em>-coumaric acid</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>ferulic acid</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas protegens</em> PF-5 previously <em>Pseudomonas fluorescens</em> PF-5</td>
<td>vanillin; putative enzyme activity</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>ferulic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vanillic acid</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> EM42 LJ015 EM42 PP_1584::Ptac::clpB-groES-groEL</td>
<td>thermochemical wastewater streams</td>
<td>[31]</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> KT2440</td>
<td>vanillin*</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td><em>p</em>-coumaric acid</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>ferulic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vanillic acid</td>
<td></td>
</tr>
<tr>
<td><em>Sphingobacterium multivorum</em> ATCC^® BAA-1093™</td>
<td>Growth observed on petroleum-contaminated soil by <em>S. multivorum</em> environmental isolate</td>
<td>[36]</td>
</tr>
</tbody>
</table>

*sole carbon source
^observed as metabolic intermediate
Figure 3. The DISC method enables identification and assessment of phenolic monomer utilization. For organism/monomer combinations with no microbial utilization of the monomer, the trace is indistinguishable from, or lower than, the control assay in which the disk was soaked in ethanol with no monomer. Microbial utilization of the monomer is evidenced by a trace with higher intensity than the negative control, though typically with a notable zone of no growth attributable to inhibition of the microbe by the monomer, similar to the standard antibiotic resistance assay. Distinct from the standard antibiotic resistance assay, growth in the DISC assay displays a global maximum, beyond which there is a tapering of the microbial growth. This decrease in growth is attributable to a decrease in substrate availability. The images and data traces are with *Amycolatopsis* after 72 hours. The inhibition slope was calculated with the function `maxLik` in `diskImageR` which averages the slopes of 10 points above and below RAD_{50}. The limitation slope was calculated with the function `growthInhib` in `diskImageR` which calculates the slope from the maximum intensity to XXX. X and Y error bars indicate one standard deviation above and below the average. From at least 3 replicates.
Figure 4. Quantification of the slope of inhibition enables distinction of growth and no growth. * indicates inhibition slopes that are significantly greater than the ethanol control (p<0.05) based on a one-way ANOVA test followed by a Tukey-Kramer test.

Figure 5. The disk diffusion assay provides information on inhibition by and utilization of monomers, such as vanillin. The disk diffusion assay provides information on inhibition by and utilization of monomers, such as vanillin. 7a Maximum OD600 of microbes in vanillin liquid culture. 7b Rad20. 7c inhibiting vanillin concentration versus Rad20. 7d limitation slope 7e Dist90 7f limiting vanillin concentration versus Dist90. P. protegens, R. erythropolis, P. putida KT2440, S. multivorum, P. putida KT2440 LJ015, and R. jostii were cultured at 30°C. Amycolatopsis was cultured at 37°C. Error bars indicate one standard deviation. The tables below Figure 7 b, d, and e, indicate statistically different groups based on an ANOVA test followed by a Tukey-Kramer HSD test. Figures 7c and f are correlations calculated using the Kendall’s τ correlation.
Figure 6. The time to equilibrium in the disk diffusion experiment is between 24 and 48 hours at 30 °C (b) and 10-24 hours at 37°C (c). We see the expected high concentration of guaiacol at the edge of the disk at 0 hours followed by a sharp drop off (a). There was not a significant difference between cell and no cell experiments (d) until 48 hours where we did not detect any guaiacol at 20 and 30 mm in the cells experiment (e). The diffusion model followed the same trend as the experiments. The table gives slopes to approximate change over distance.
Figure 7. The experimental concentrations fall within the bounds of the base case diffusion of Amycolatopsis on guaiacol (a). The growth model used for the base case also closely mirrors the intensity (d). Amycolatopsis was highly tolerant to guaiacol but it was not the top scavenger. Amycolatopsis on vanillin growth model (e, f) was less tolerant but better at scavenging. Here we show the predicted percent difference in concentration between Amycolatopsis on guaiacol and vanillin at 30 hrs (b) and 48 hrs (c). The table below gives the parameter values of the model.
CHAPTER 5. YEAST BIOLOGICAL MEMBRANE MODEL INFORMED BY SYNTHETIC and WHOLE CELL VESICLE CHARACTERIZATION

Kirsten Davis, Laura Jarboe, Jeffery Klauda
To be submitted

Abstract

Saccharomyces cerevisiae is heavily utilized to produce food, beverages, health products, fuels, polymers, and adhesives. Chemicals and fuels produced with S. cerevisiae from a biomass feedstock have the potential to contribute to improved environmental quality, a decrease in carbon footprint, improved rural economies, and decreased dependence on petroleum. Even though S. cerevisiae is a robust biocatalyst, competitive product titers and yields are at odds with the organism’s survival. Desirable fermentation products such as fatty acids and organic solvents are toxic to S. cerevisiae. Fermentation products can damage the plasma membrane which is essential for the cell’s survival. The biomolecular effects of key fermentation products such as solvents and fatty acids on the plasma membrane are not well understood. To increase knowledge regarding the biomolecular interactions that solvents and fatty acids have with bio-model artificial vesicles, we used unilamellar phospholipid vesicles composed of commercially available 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) as well as extruded whole cells as experimental systems. Vesicles were treated with various solvents and fatty acids, and the size, polydispersity, leakage and fluidity were characterized. 100% POPC and 5:95% POPE:POPC vesicles did not swell or aggregate during alcohol treatment and therefore were ideal systems for membrane characterization. Leakage and an increase in fluidity was observed during alcohol treatment although the addition of 5% POPE decreased the leakage in tertbutanol and
n-butanol and decreased the change in fluidity in n-butanol. Experimental evidence was used to inform an *in silico* model of a yeast plasma membrane.

**Introduction**

**Butanol production**

The production of biofuels and biochemicals from a cheap renewable resource is an attractive alternative to the production of petroleum-derived products [1]. Longer chain alcohols such as butanol are more similar to gasoline as compared to ethanol. Butanol is more energy dense and less hygroscopic than ethanol [2]. The 2017 Science by Liu, Qureshi, and Hughes reviews butanol production and tolerance in a number of promising microbial hosts. *S. cerevisiae* is an attractive microbial host because is genetically tractable. *S. cerevisiae* can also tolerate environmental oxygen unlike the anaerobic, butanol-producing *Clostridia* species.

A number of interventions enabled and increased *S. cerevisiae* butanol production. The heterologous acetoacetyl-CoA derived *Clostridia* pathway was integrated into *S. cerevisiae*. The new pathway and externally provided pantothenate enabled *S. cerevisiae* to produce 130 mg/L ± 20 mg/L n-butanol from glucose at a yield of 0.012 g/g glucose [3–5]. Alternatively, pathways have also been constructed using reverse β-oxidation (20 mg/L no yield reported) and a synergistic endogenous threonine pathway and citramalate synthase mediated pathway (835 mg/L, 42 mg/g glucose in anaerobic glass tubes and 1.05 g/L in a bioreactor) [6,7].

**Inhibition**

Many successful engineering strategies have made it possible for *S. cerevisiae* to produce butanol and carboxylic acids. However, it is still inhibited by its products. Membrane damage is one of the fundamental causes of toxicity. Overtone’s rule states that
the solubility of a lipid is related to its membrane permeability. Thus, amphipathic molecules such as carboxylic acids and longer chain alcohols are expected to be membrane permeable and thus have the potential to damage the membrane.

There are several reports of microbial membrane damage by alcohols. Most cells can only tolerate 1.5-2% (v/v) 1-butanol. Many microbial membranes are perturbed under butanol stress [8–11]. For instance, lipids extracted from Clostridia cells and artificial vesicles made of dimyristoylphosphatidylcholine showed increased fluidity in ethanol and butanol [12].

A number of membrane engineering strategies successfully decreased membrane damage. Some examples include engineering the length of the fatty acid tails, the degree of saturation, the conformation of the fatty acid tails, and the composition of the phospholipid heads [13–17]. Recently Besada-Lombada et al. replaced a key serine residue (1157) with an alanine on an S. cerevisiae acetyl-CoA carboxylase with the intention of increasing the fatty acid production. The membrane properties were studied in an effort to understand the increased fatty acid production. The strain with the altered carboxylase produced 2.3-fold higher oleic acid than the baseline strain. The minimum inhibitory concentration of octanoic acid for the new strain was 2.5 mM compared to the baseline strain which was 1.25 mM [18].

**Yeast Plasma Membrane**

The yeast cell’s first line of defense against membrane damage is the plasma membrane. Although there is abundant evidence which supports that alcohols and carboxylic acids damage the cellular membrane, the biomolecular and cellular mechanisms of this damage are still somewhat unclear. The yeast cell has a cell wall which is mainly composed of glycopolypeptides and polysaccharides, but its role is mainly to scaffold and hold the cell together with its high turgor pressure [19,20]. For the scope of this paper, the plasma
membrane composition was the focus, and the membrane proteins were not integrated into the computational model or the experiments. The yeast plasma membrane contains five kinds of phospholipids: phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositol phosphatides (PI), and inositol phosphoceremides (IPC). The plasma membrane is asymmetric. The inner leaflet is composed of PE, PS, and PI phospholipids. The outer leaflet is composed of PC and IPC phospholipids. In yeast, the main sterol is ergosterol, and it makes up about 50% of the phospholipids in the plasma membrane [21]. The main IPC in yeast are mannose-(inositol-P)\(_2\)-ceramide MIP\(_2\)C which makes up more than 70% of the IPCs in yeast [22]. About 70% of the lipids in the membrane contain one double bond, and the rest are saturated [23]. The recent *Science* publication highlighted the importance of membrane composition. The altered sterol composition was thought to be the cause of increased thermotolerance in an evolved yeast strain [24]. To-that-end, a computational model of a yeast membrane was informed with experimental evidence.

**Computational models of membranes**

The use of molecular modeling to study membranes is a well-established technique [25–29]. There have been several previous studies using molecular models on the effect of alcohols on the membrane [30–34]. The effect on PC-lipid membranes in ethanol [30,32] and C-8, C-10, and C-14 alcohols were studied [29]. Alcohols with more than eight carbons resulted in more chain ordering of the lipids and less surface area per lipid. Alcohols with less than eight carbons resulted in more chain disordering and an increase in surface area per lipid. There has only been one study using butanol, and model bilayers were made of dipalmitoylphosphatidylcholine (DPPC) bilayers [32]. Some studies studied more complex lipid compositions to better simulate mammalian membranes, for instance PC/PS [32] or PC/sphingomyelin/cholesterol [31,35]. In the PC/sphingomyelin/cholesterol study,
cholesterol increased the membrane tolerance to ethanol [31]. The membrane insertion molecules oligo-polyphenylenevinylene-conjugated oligoelectrolytes conferred butanol resistance to an E. coli in silico membrane [34].

Klauda et al. developed E. coli and yeast membrane models [36–48]. Klauda’s recent work highlights the important lessons that can be learned from increasing the complexity of yeast membrane models to mirror the composition of actual yeast cells. Ceramides which are a component of sphingolipids had a profound-effect on the following properties of a yeast membrane model: surface area per lipid, chain order and tilt, area compressibility moduli, bilayer thickness, hydrogen bonding, and lipid clustering [48]. In the current study, we expanded on previous models by integrating the experimentally observed effects of alcohols and carboxylic acids on membrane permeability, surface hydrophobicity, and fluidity with the in silico yeast plasma membrane model.

**Methods (Experimental)**

**Artificial vesicle preparation**

To prepare the artificial vesicles for the experiments, the following phospholipids were purchased at Avanti lipids: (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE). These lipids were dried under nitrogen. Lipids were suspended in vesicle buffer (100 mM sodium chloride, 10 mM 2-[4-(2-hydroxyethyl]-1-piperazinyl]ethane sulfonic acid (HEPES) in nanopore water, pH 7.4). For the leakage experiments, lipids were suspended in ANTS/DPX buffer (20 mM sodium chloride, 12.5 mM 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS) fluorescent dye, 45 mM p-xylene-bis-pyridiniumbromide quencher (DPX) at pH 7.4). The suspended lipids were heated at 42°C for about 10 minutes and vortexed. The lipids in solution were subjected to 5 cycles of 1.5 minutes freeze in liquid nitrogen and 2.5 minutes...
thaw in a 65°C water bath. After each thaw, the lipids were vortexed. The lipids were extruded 20 times through a 0.2 um pore polycarbonate membrane from Avanti with an Avanti mini-extruder.

**Dynamic light scattering**

Vesicles were monitored by dynamic light scattering to determine the quality of the sample with a Malvern Zetasizer series nanoZS90. The refractive index (RI) was set to 1.47. The temperature was set to 30°C. The dispersant was selected as phosphate buffered saline with a dispersant dielectric constant of 78.5, which was the closest material type in the catalogue.

**Leakage**

The leakage protocol was adapted from Muller et al [49]. Non-encapsulated ANTS dye was removed by size exclusion chromatography with two rounds of spin column purification (PD-10 GE Healthcare). The columns were conditioned with the vesicle buffer. Centrifugation took place at 1000 x g for 2 minutes.

The vesicles were diluted to 0.01 mM with the vesicle buffer and inhibitor. The vesicles were added to a 96-well plate, and the fluorescence was measured on a Biotek Synergy HT multiplate reader at 30°C for 10 minutes. The excitation was at 380/20 nm, and the total fluorescence emission intensity was measured at 600/40 nm. The point at which intensity no longer increased with ethanol concentration was selected as the maximum intensity ($I_{sample\ max}$). Percent leakage was calculated using equation 1.

$$\frac{I_{sample} - I_{no\ ANTS} - I_{no\ inhibitor}}{I_{sample\ max} - I_{no\ ANTS\ max} - I_{no\ inhibitor}} \times 100\% \ \ Equation\ 1$$

$I_{sample} = intensity\ of\ sample$

$I_{no\ ANTS} = intensity\ of\ sample\ without\ ANTS$

$I_{no\ inhibitor} = intensity\ of\ sample\ without\ inhibitor\ added$

$I_{no\ ANTS\ max} = intensity\ of\ maximum\ sample\ with\ no\ ANTS$
**Fluidity**

Fluidity was measured with the fluorescent probe diphenylhexatriene (DPH) which imbeds in the membrane and reacts to polarized light [50]. The DPH solution was solubilized in ethanol at 0.4 mM. The protocol was adapted from Liu et al [51]. The vesicles were diluted by HEPES/NaCl and the alcohol of choice to a total volume of 1 mL and incubated at 30°C for one hour. 0.5 mL of each tube was removed to use a control without DPH. DPH solution (0.5 uL) was added to the remaining 0.5 mL and incubated again at 30°C for one hour. Samples were aliquoted into black bottomed Nunclon delta surface 96-well plate. A Synergy Multi-Mode microplate reader from BioTek was used to measure the polarization. The 360/40 nm was used for excitation. The excitation was set in the vertical position. The 460/40 nm filter was used for emission was set in the vertical position (I_{VV}) or the horizontal position (I_{VH}). During reading, the plate reader was kept at 30°C. Polarization was calculated with Equation 2.

\[
P = \frac{I_{VV} - I_{VH}G}{I_{VV} + I_{VH}G} \quad \text{Equation 2}
\]

G is the grating factor which was assumed to be 1.

**Results and Discussion**

**Stability of 100% POPC and 5:95 POPE:POPC vesicles determined with DLS**

Dynamic light scattering (DLS) was used to find the appropriate concentration range for each treatment (Figure 1). The vesicles aggregated or were highly disrupted outside of the appropriate concentration range as evidenced by a polydispersity index over 0.4, an increased count rate, and/or poor cumulant and distribution fits. The z-average diameter and polydispersity of 100% POPC and 5:95 POPE:POPC vesicles increased with concentration but stayed within the acceptable ranges. The diameters were expected to be about 200 nm, and the polydispersity index was expected to stay below 0.4 for a moderately polydisperse
sample. However, even with low ethanol concentrations (<20 volume percent) the diameter of the POPS vesicles increased with alcohol concentration within the acceptable ranges while the polydispersity stayed constant (data not shown) indicating the pure POPS vesicles aggregated or swelled. Z-average diameter and polydispersity changed dramatically outside of the acceptable ranges for all samples indicating that as the vesicles were disrupted, they became too polydisperse to analyze with dynamic light scattering.

**Vesicle leakage is directly related to alcohol concentration**

Vesicles exhibited signs of leakage directly related to concentration in all alcohols tested (Figure 2). The change in percent leakage with concentration was greatest for POPC vesicles in n-butanol which was expected considering the larger chain length. Interestingly, isopropanol had the greatest effect on the leakage of 5:95 POPE:POPC vesicles which may be due to the unique ability of isopropanol to “jump” through the membrane (based on unpublished data from Klauda et al.) There was significantly less leakage of 5:95 POPE:POPC vesicles in tert-butanol and n-butanol compared with 100% POPC vesicles.

**A decrease in DPH polarization indicates an increase in vesicle fluidity**

Fluidity was measured for 100% POPC and 5:95% POPE:POPC vesicles in the alcohols. The DPH polarization decreased with alcohol concentration which corresponds to an increase in fluidity. The increase in fluidity was expected, because it has been observed previously in vesicles and lipids extracted from Clostridia treated with ethanol and butanol [12]. Here, n-butanol increased the fluidity of both vesicle types more than ethanol, isopropanol, or tert-butanol. The polarization slope of 100% POPC vesicles in tert-butanol was significantly higher than in 5:95 POPE:POPC.
Discussion

This project was unique in that it was the merging of biological and \textit{in silico} modeling. It was extremely important to keep the experimental systems simple so that correlation can be made in the future with the \textit{in silico} model. Pure phospholipid vesicles proved very useful in that we could precisely control the composition of our lipid bilayers. However, as we learned, only specific formulations of these vesicles remain intact for characterization. Here we found it useful to use pure POPC vesicles and spike in POPC to probe the changes. Just 5\% POPE showed decreased fluidity in n-butanol and decreased leakage and fluidity in tert-butanol. Interestingly, isopropanol had the most effect on the leakage of the 5:95 POPE:POPC vesicles whereas n-butanol had the most effect on the leakage of the pure POPC vesicles. Our results confirm that the phospholipid head composition plays a role in the behavior of the lipid membrane.

References


Figure 1 The polydispersity index (PDI) and z-average diameter of 100% POPC and 5:95 POPE:POPC vesicles in alcohol concentration ranges with good cumulant and distribution fits. The size of the vesicles with these alcohol concentrations are within the expected ranges of about 200nm. The PDI is also below 0.4 indicating a moderately polydisperse sample. Each point is the average of at least 3 dynamic light scattering runs, with the error bars indicating one standard deviation.
Figure 2 Vesicle leakage was directly related to the concentration of the alcohols. The bar chart gives the slopes of percent leakage with molarity. Here we report the averages of at least 2 sample replicates with error bars indicating one standard deviation. * = P value of <0.05 based on a Student’s t-test. The lines in the scatter plots show the range of values used to calculate the slopes.

Figure 3 Vesicles decreased in DPH polarization with alcohol concentration which corresponds to an increase in fluidity. The bar chart gives the slopes of DPH polarization with molarity. Here we report the averages of at least 2 sample replicates and 3 technical replicates with error bars indicating one standard deviation. * = P value of <0.05 based on a Student’s t-test. The lines in the scatter plots show the range of values used to calculate the slopes.
CHAPTER 6. GENERAL CONCLUSION

Here I have described three tools which allowed us to learn more about microbial performance in biorenewable applications. Our emulsion formulation allowed for growth of *P. putida* in a lignin-rich fraction of biomass pyrolysate. From our DISC assay, we know that *P. putida* can utilize vanillin which is present in the pyrolysate. However, there is still more testing required to fully understand how *P. putida* survived and grew on the lignin-rich fraction. Testing an expanded set of aromatics using the DISC assay would be one way to unravel this mystery. We have also only tested growth on a sole carbon source, and it would be interesting to test growth on multiple phenolics at once. The DISC assay allowed us to screen several microbial candidates for utilization, and *Amycolatopsis* stood out as utilizing two carbon sources out of the 10 tested. It would be interesting to continue with *Amycolatopsis* as a potential host for lignin utilization. The DISC assay could also be expanded to test engineered organisms and carbon sources that are toxic. The final project was an exploration into mechanisms of biological membrane damage. Even just slight perturbations in the make-up of the vesicles changed the response. This really highlights the benefits of modulating the makeup of the membrane to make cells more robust toxic molecules. These tools should prove useful in the greater context of microbial conversions of renewable feedstocks to fuels and chemicals. We need microbes which can convert not only simple sugars but other more recalcitrant feedstocks as well as ones that tolerate the toxicity due to feedstock and product. Within the context of greenhouse gas mitigation and other types of renewables, microbial conversions fit more easily into the production of actual material goods as opposed to energy. Still with most of our everyday products containing a petroleum-based component, this technology could contribute of greenhouse gas mitigation.