Engineered Microbial Production of 2-Pyrone-4,6-Dicarboxylic Acid from Lignin Residues for Use as an Industrial Platform Chemical

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As one of the most abundant materials in nature, lignin has been used widely in co-generation operations and for fine chemicals and bio-fuels production. These uses, although important, are of relatively low value. Lignin contains many aromatic compounds with useful structures, and it is potentially more profitable to produce high-value fine chemicals from the low-molecular weight lignin fraction while using the high-molecular weight fraction for fuel or other applications. A transgenic *P. putida* bacterial strain PDHV85 was developed with the capability to convert vanillin, vanillic acid, and syringaldehyde to 2-pyrone-4,6-dicarboxylic acid (PDC), a novel platform chemical that can produce a variety of bio-based polymers. Initial testing with vanillin showed promise for lignin conversion. Testing for this, we used kraft lignin, Japanese cedar (*Cryptomeria japonica*), or birch (*Betula platyphylla*) to represent some of the most abundant industrial lignin sources from softwood and hardwood. Repeated manipulation of culture conditions and strain adaptation allowed conversion of these extracts to PDC by PDHV85, which has not previously been reported in a bacterial strain. No inhibition was observed at 0.14 mg/mL kraft lignin extract, 1.14 mg/mL Japanese cedar extract, nor 1.15 mg/mL birch extract when using the optimized growth conditions.

Keywords: Pseudomonas putida PDHV85; 2-Pyrone-4,6-dicarboxylic acid (PDC); Japanese cedar (Cryptomeria japonica); Birch (Betula platyphylla); Kraft lignin; Bio-based platform chemicals

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INTRODUCTION

Lignin is the second most abundant natural raw material available in terrestrial ecosystems; representing nearly 30% of the organic carbon sequestered in plant materials; it has an annual industrial production of 500 to 3,600 million tons (Gosselink *et al.* 2004; Lora 2008; Austin and Ballare 2010). However, lignin is considered to be a low-quality, low-value residual material because of the difficulty and high cost associated with its deconstruction and purification to useful moieties (Zabaleta 2012; Yoshikawa *et al.* 2013). Only 1% to 2% of the lignin in the pulp and paper industry is converted to commercial products (Gosselink *et al.* 2004; Lora 2008; Stewart 2008). Kraft lignin is the primary source for industrial lignin, representing approximately 90% of the total amount of lignin produced, with an annual global production of 6 to 9 million tons (Azadi *et al.* 2013; Berlin

and Balakshin 2014). Commercial applications for kraft lignin are focused on derivatives such as sulfonated kraft lignin and ethoxylated sulfonated kraft lignin, with product derivatives including dye dispersants, emulsifiers, and antioxidants (Ten and Vermerris 2015). Pulp mills typically generate a portion of their energy by burning kraft lignin in co-generation operations. Lignin contains many aromatic compounds, and it is potentially more profitable to produce high-value fine chemicals from the low-molecular weight lignin fraction while using the high-molecular weight fraction for fuel or other applications. Most chemical pulping processes can convert a portion of the high-molecular weight lignins into various low-molecular weight compounds, but only a few of these low-molecular weight compounds have been used to produce high-value chemicals such as vanillin (Araújo *et al.* 2010) because the purification of aromatic compounds from the other fractions has generally been too expensive. Furthermore, little work has been done to generate purified monomers from lignin for use as platform chemicals for cost-effective production of polymers and other products.

Both softwoods and hardwoods are used extensively in the pulping industry. Lignin extract composition depends on the plant source and the variations that occur in different species with respect to the generation of lignin precursors in plant/tree biosynthetic pathways (Pettersen 1984). For example, softwood crude lignin extracts typically contain vanillic acid and vanillin, while hardwood lignin extracts contain vanillin, vanillic acid, syringic acid, and syringaldehyde. Because of the complexity of the aromatic mixtures generated in pulping, it is difficult to convert and then purify pulping liquor to generate useful platform chemicals *via* chemical methods. However, microorganisms with appropriate enzymatic machinery for metabolizing many different types of aromatic compounds provide unique pathways to address the issue – if manipulated appropriately. A select few microorganisms have been identified that have ability to degrade complex substrates that contain lignin; these organisms include both fungi (Goodell 2003; Thevenot *et al.* 2010; Ayyachamy *et al.* 2013) and bacteria (Wang *et al.* 2013; Brown and Chang 2014). *Sphingomonas paucimobilis* SYK-6, which was isolated from pulping wastewater, has the ability to completely degrade a variety of low-molecular weight aromatic compounds, although to date only a few select purified aromatic compounds have been used as substrates in research with this organism. Metabolic pathways for the degradation of aromatics, as well as enzymes, produced by *S. paucimobilis* have been well studied (Masai *et al.* 1991, 1999, 2000, 2007a; Peng *et al.* 1998, 1999). When metabolizing low-molecular weight aromatic compounds, *S. paucimobilis* first converts aromatics into vanillin, vanillic acid, and syringic acid. The organism can be engineered to metabolize these compounds to other useful chemicals, including 2-pyrone-4,6-dicarboxylic acid (PDC), *via* the protocatechuate 4,5-cleavage pathway, through the introduction of *ligA*, *ligB*, and *ligC* genes (Sonoki *et al.* 2000; Otsuka *et al.* 2006). However, the SYK-6 strain grows slowly, and for this reason there have been efforts to develop an enhanced system, including work with engineered *Pseudomonas putida*. The introduction of *ligA*, *ligB*, and *ligC* genes from SYK-6 into a mutant strain of *P. putida* PpY1100 produced a new strain that facilitates the metabolic conversion of intermediate compounds into PDC (Otsuka *et al.* 2006; Sonoki *et al.* 2014). However, to date, the organism has only been grown on relatively expensive purified substrates.

PDC is a novel platform chemical that is not readily synthesized by chemical methods (Otsuka *et al.* 2006) but has many potential applications. With its pseudo-aromatic ring and two carboxyl groups, it has great potential as a platform chemical in the production of bio-based polymers and has already been used to produce polyesters (Michinobu *et al.*

2009) and polyamides (Shigehara *et al.* 2002). Recently, it has also been used as a selective chelator (Yamamoto *et al.* 2010) to sequester radioactive cesium for potential application in the cleanup of nuclear contamination (Otsuka *et al.* 2014). PDC-based polyesters are biodegradable and have a strong binding capacity for certain metals. Moreover, their metal-bonding adhesive properties are unique (Michinobu *et al.* 2009, 2011). If low-value substrates that permit efficient synthesis of PDC can be identified, the use of this monomer could potentially increase, with many future applications.

In terms of the resources required for fermentation and platform chemical production, sugars are typically required in the growth media for metabolic function of the organism as well as for platform chemical production. If the use of sugar can be reduced or eliminated, this will reduce competition with feedstocks that also are needed for human and animal nutrition and thus are of high value for other purposes. The use of lignin as a feedstock in bioconversion processes to reduce sugar requirements can therefore be considered an enhancement of fermentation systems for platform chemical production. In this paper, extracts from kraft lignin (the largest source of industrial lignin globally), Japanese cedar (softwood), and birch (hardwood) were used to study the microbiological conversion of lignin extracts to PDC by the transgenic bacterium PDHV85.

EXPERIMENTAL

Cultivation of Bacterium and Preparation of Base Fermentation System for PDC Production

A transgenic bacterium PDH (described below) and two plasmids, pVapoligVABC (for kanamycin sulfate (K_m) resistance) and pJFV2Z85 (for tetracycline hydrochloride (T_c) resistance), were obtained from the Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan. *Pseudomonas putida* PpY1100 is a derivative of *P. putida* mt-2 (ATCC 33015) (Fukuda and Yano 1985), but it is unable to metabolize any aromatics and does not have the TOL plasmid (tol^-) (Franklin *et al.* 1981). PDH is a pcaD and pcaH mutant of *P. putida* PpY1100 that cannot metabolize *cis-cis* muconate. Genes *vanA* (vanillate demethylase A, accession: AE015451.1), *vanB* (vanillate O-demethylase oxidoreductase, accession: AE015451.1), *ligV* (vanillin aldehyde dehydrogenase, accsession: AB287332.1), *ligA* (protocatechuate dioxygenase A, accession: AB073227.1), *ligB* (protocatechuate dioxygenase B, accession: AB073227.1), and *ligC* (4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase, accession: AB073227.1) were cloned into a pKT230 plasmid (ATCC37294) to form pVapoligVABC. Genes *ferA* (feruloyl-CoA synthetase, accession: AB110975.1), *ferB* (feruloyl-CoA hydratase/lyase, accession: B110975.1), and *desZ* (3-O-methylgallate dioxygenase, accession: AB110976.1) were cloned into a pJB866 plasmid (accession no. U82001.1) to form pJFV2Z85. A transgenic bacterium, PDHV85, was engineered from PDH with pVapoligVABC and pJFV2Z85 inserted. The pVapoligVABC and pJFV2Z85 were previously found to contain all the genes (Masai *et al.* 2002, 2007b, 2012; Nelson *et al.* 2003) required to convert vanillin, vanillic acid, and ferulic acid into PDC (unpublished data, Otsuka, Katayama, Masai, Okamura, and Nishimura). The *P. putida* PpY1100 strain was initially selected because it has the ability to take up many low molecular weight aromatic compounds. However, it is not able to metabolize these compounds. Further, the strain was selected because it can produce high-density cultures that would be desirable in later scale up.

To initiate growth, cultures were maintained on *Pseudomonas* Agar F (PAF) medium supplemented with 50 μg/mL K_m and 20 μg/mL T_c. The liquid pre-culture media (per 50 mL total) contained 53 mM $(NH_4)_2SO_4$, 33 mM, 72 mM Na_2HPO_4, and 50 μg/mL K_m (all purchased from Fisher Scientific Co. L.L.C., Pittsburgh PA, U.S.A.), 0.47% w/v yeast extract (Nacalai Tesque Inc., Tokyo), 1.8 % w/v glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 20 μg/mL T_c (Research Products International Corp., Mount Prospect, IL, U.S.A.), with 0.5 mL of stock salt solution (Yano and Nishi 1980) added to the media mix to provide minerals for cell growth. The final cultures were grown in bioreactors (total volume of 400 mL of media) with media initially consisting of 44 mM $(NH_4)_2SO_4$, 28 mM KH_2PO_4, 123 mM $Na_2HPO_4 \cdot 7H_2O$, 2.84% w/v yeast extract, 50 μg/mL K_m, and 20 μg/mL T_c, with 4 mL of stock salt solution and 6 mL of 45.3% concentrated glucose solution. Where specified, modified conditions were used; 15 mL of 45.3% concentrated glucose solution and 8.5 mL of stock salt solution were ultimately added to the final culture medium. The PDHV85 strain was refreshed on PAF medium plates with K_m and T_c for 1 day, followed by pre-cultivation in liquid pre-culture medium with K_m and T_c for 1 day. After pre-cultivation, 1 mL of the pre-culture was added to 400 mL of the final culture medium, with K_m and T_c in bioreactors as the base bioreactor state to which amended media would then be added.

A BIOFLO 110 Fermenter/Bioreactor system (New Brunswick) with BioCommand software (New Brunswick) was used to cultivate the *P. putida* strain under controlled conditions, which included a vessel with working volume of 0.4 to 1.0 L, temperature of 28 °C, a stirrer speed of 700 rpm, and pH 6.5. The stirrer was fitted with dual Rushton type impellers, each 52 mm in diameter and fitted with six straight flat blades. Airflow was set at 2 L/min. The strain was grown overnight (14 h) in the bioreactor in the final culture medium. Lignin extracts were then dissolved and subsequently added to the culture (details below). Cultivation was continued for 23 h using the same culture parameters as those used in the pre-cultivation stage.

Extraction of Aromatic Compounds from Softwood Kraft Lignin, Japanese Cedar, and Birch

For test feedstock materials, the following materials were prepared:

Kraft lignin extract: Ten grams of commercial softwood kraft lignin powder (Kraft Sphere® Pine Chemical, Mead Westvaco, USA) was suspended in 50 mL of ethyl acetate and extracted overnight by stirring. The suspension was then filtered through a 0.2-μm membrane and the filtrate was dried under a stream of N_2 gas to yield 0.058 g of crude kraft lignin extract as a dark brown solid.

Cedar and birch extracts: To extract low-molecular weight aromatic compounds using alkaline nitrobenzene oxidation, Japanese cedar (*Cryptomeria japonica*) or birch (*Betula platyphylla*) powder (0.3 g each) were reacted at 160 °C for 2.5 h with 0.4 mL of nitrobenzene and 7 mL of 2 N NaOH under constant rocker mixing. After cooling, the mixture was extracted (3X) with 7 mL of diethyl ether to remove the nitrobenzene and its reduction products. The diethyl ether layer was decanted and collected, and the aqueous layer was acidified with concentrated HCl to pH 2.0 and re-extracted (3X) with 7 mL of diethyl ether to recover additional phenolic residues. Anhydrous Na_2SO_4 was added to the combined diethyl ether extracts to remove water, and samples were then rotary-evaporated to produce the final solid aromatic extracts.

HPLC and Analysis

Typically, 0.10 mg of the extracts was dissolved in 2 mL of solvent consisting of 10% acetonitrile and 90% 10 mM H_3PO_4, with the resulting solution filtered through a nylon membrane syringe filter (porosity, 0.2 μm) prior to injection onto the HPLC column. Spent culture medium was also filtered before injection onto the HPLC column. Extracts and spent culture medium were resolved by HPLC on a C-18 reverse-phase column (Inertsil ODS-3, 5 μm column, 4.6 x 250 mm) at 40 °C with an isocratic mobile phase (10% acetonitrile and 90% 10 mM phosphoric acid) flowing at 1 mL/min. Aromatic compounds and PDC eluting from samples were detected using a UV/vis detector (SPD-10AV UV/VIS detector, Shimadzu Corp., Japan) set at 280 nm and 310 nm and identified by comparing their residence times with those of standard purified compounds. Fractions were also collected and compared with standards *via* thin layer chromatography (TLC) on a silica gel TLC plate (catalog no. M1057150001, EMD Millipore Corp., Billerica MA, U.S.A.) using an organic solvent cocktail (10 mL of chloroform, 8 mL of acetal acetate, and 1 mL of formic acid mixed together) as the mobile phase. The components separated on the TCL plate were detected by UV light.

Lignin-Amended Media for Bioreactors

To determine how the PDHV85 strain may metabolize lignin extracts, the following materials were prepared. The "final medium" listed below was generated after multiple early trials and optimization of the bioreactor system, as described in the Results and Discussion section:

Kraft lignin extract preparation: Crude kraft lignin extracts (58 mg) were dissolved in 2 mL of water and 20 μL of 1 M NaOH.

Cedar or birch extract preparation: Japanese cedar or birch extracts (0.5 g each) were added to 10 mL of 0.10 M NaOH, with sonication for 20 min.

Final medium: The desired amendment (kraft lignin or cedar or birch extract) was tested separately in different culture batches. Kraft lignin crude extract was mixed with 2 mL of 45.3% glucose solution and 1 mL of stock salt solution, while Japanese cedar and birch extract was mixed with 8.5 mL of the stock salt solution and 15 mL of 45.3% glucose solution. Each mixture was then added to the final base *P. putida* culture medium for the various experiments conducted. Sampling was then performed during the fermentation, with analysis by HPLC, to assay for the consumption of the aromatic compounds and PDC biosynthesis. Two trials were performed for each lignin extract experiment.

RESULTS AND DISCUSSION

Pure vanillin was first used to check the bioconversion capability of the *P. putida* PDHV85 strain that was developed, as many aromatic structures can be degraded *via* the same pathways as vanillin. The engineered PDHV85 strain was effective in converting vanillin to PDC; however, other organisms have previously been reported to convert purified compounds like vanillin to useful platform chemicals (Otsuka *et al.* 2006). The present research expanded beyond this to explore optimal growth conditions (initially also with vanillin) where inhibition would be prevented in preparation for later work with lignin extracts. In preliminary studies to assess growth conditions potentially inhibitory to PDC production, 5 g of vanillin was dissolved in 50 mL distilled water with 4 mL stock salt

solution, 6 mL 43.5% glucose solution and 2.5 mL ammonium hydroxide. This resulted in inhibition of PDC production, so we experimented with batch-injection of glucose into a bioreactor containing 400 mL of the pre-cultured *P. putida* strain in media, with an injection rate of 15 mL/h (determined after multiple trials). The pH and temperature were maintained at 7.5 and 28 °C, but both vanillate and vanillin were observed in the culture after 23 h. To eliminate this inhibition the levels of glucose and stock salt were adjusted, and ultimately a 15 mL glucose (43.5% glucose solution) and 8.5 mL salt solution were used to eliminate inhibition. Repeated trials ultimately showed that this ratio and feed rate would support a robust cellular metabolism of 10.8 mg/mL vanillin, resulting in rapid and complete conversion of vanillin to PDC. Nearly 100% conversion was achieved, with no intermediates or inhibitor compounds detected. PDC produced during bioconversion of vanillin was detected after extraction using purified standard compounds for reference. This initial work was important to provide direction in developing appropriate conditions for conversion of lignin-derived compounds."

Extraction of Aromatic Compounds and Conversions by *P. putida*

HPLC analysis showed that the crude extracts from kraft lignin contained vanillic acid and vanillin as the two major components, with a total percentage of approximately 0.04% (Fig. 1a). This low level of extractable aromatics (Table 1) was expected because the raw material is the product of black liquor after pulping and was obtained after several washings. Because the present research focus is on the use of low-molecular weight waste lignin fractions, this mixture was appropriate for the bioreactor tests.

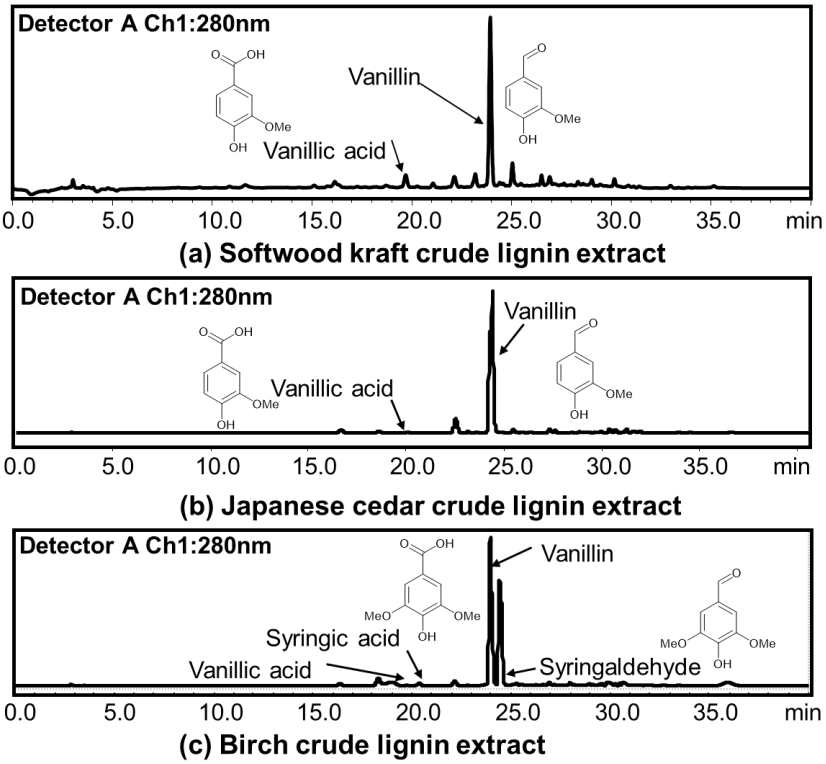

Fig. 1. Major aromatic components in the crude lignin extracts analyzed in this research as determined by HPLC analysis

The extractable aromatic content in this kraft lignin was much lower than the extractable aromatics from Japanese cedar or birch, which totaled in both cases approximately 30%. Vanillin was the major aromatic component in the Japanese cedar crude lignin extracts, along with a small amount of vanillic acid (Fig. 1b). Birch crude lignin extracts were observed to have vanillin and syringaldehyde as major components, as well as very small peaks for vanillic acid and syringic acid (Fig. 1c).

In this work, the level of low-molecular weight lignin extracted from the kraft lignin was much less than that from woody materials. For example, the vanillin extracted from kraft lignin powder was determined to be only 0.04% by HPLC in this study; while from other studies it has been demonstrated the amount of vanillin that can be recovered from black liquor ranges from 0.12 to 0.15 wt.% (Löwendahl *et al*. 1978). By simply changing the oxidation parameters, including temperature and oxygen content during the kraft cooking process, the vanillin yield varied from 2.8 to 10.8% (Borges da Silva *et al*. 2009). Therefore, if appropriate industrial procedures are developed in the future for the isolation of the aromatic compounds from black liquor directly prior to washing steps, low-molecular weight lignin fractions from this source could be used rather than from direct extraction of the woody biomass.

Table 1. Percentages of Major Aromatic Compounds in the Crude Lignin Extracts Used in this Research

	Aromatic compounds	Major aromatic compound percentage of total extract (% HPLC peak area)
Kraft lignin extract	Vanillin	0.04
	Vanillic acid	< 0.01
Nitrobenzene oxidation extracts from Japanese cedar	Vanillin	29.80
	Vanillic acid	0.19
Nitrobenzene oxidation extracts from Birch	Vanillin	14.00
	Vanillic acid	0.61
	Syringaldehyde	15.81
	Syringic acid	0.07

PDC Production from Lignin-amended Media

Conversion of lignin extracts to PDC

HPLC and TLC were used to monitor PDC production; however, as the TLC results were used only for regular routine assessment of the potential for PDC production and were not specifically quantitated, only the HPLC results are shown. Initial growth conditions were found not to be appropriate for efficient conversion of the lignin extracts to PDC. For convenience, only two trials are listed in Table 2; each shows the final efficient conversion rates when using the media composition listed as "Final medium" in the Experimental section - "Lignin-amended Media for Bioreactors". When crude kraft lignin extracts were used as a substrate, vanillic acid and vanillin were completely converted to PDC by *P. putida* over a 24-h period (Fig. 2). With birch extract, almost no vanillin remained after 23 h of cultivation, and from 500 mg of extract (at a concentration of 1.15 mg/mL in the total culture volume), on average, 193 mg of PDC was produced (Table 2), yielding a

concentration of 483 µg/mL of PDC (average value from two trials) in the spent media (Fig. 2). For Japanese cedar extract, no vanillin remained after 23 h. From 495 mg of Japanese cedar extract (1.14 mg/mL), the average PDC yield was 262 mg (Table 2), yielding a PDC concentration of 655 µg/mL (average value from two trials) in the spent media (Fig. 2). The average production of PDC from the kraft lignin extract (0.14 mg/mL) was 53 µg/mL (average value from two trials in 50 mL spent media) (Fig. 2).

Table 2. Theoretical and Actual PDC Yields Derived from *P. putida* Strain PDHV85 in Metabolism of Lignin Extracts from Three Sources

Name		Raw extracts (mg)	Aromatic compounds (mg)		Theoretical PDC production (mg)	PDC produced (mg)	PDC produced / theoretical PDC production
Kraft lignin	Trial 1	58	Vanillin	10.3	12.5	2.5	17.1%
			Vanillic acid	1.9	2.1		
	Trial 2	58	Vanillin	10.3	12.5	3.0	20.5%
			Vanillic acid	1.9	2.1		
Japanese cedar	Trial 1	500.2	Vanillin	149.0	177.0	248	140.1%
	Trial 2	490.0	Vanillin	146.3	176.9	276	156.0%
Birch	Trial 1	509.4	Vanillin	71.4	166.6	178.3	107.0%
			Syringaldehyde	79.5			
	Trial 2	490.8	Vanillin	68.7	160.0	208.2	130.1%
			Syringaldehyde	76.6			

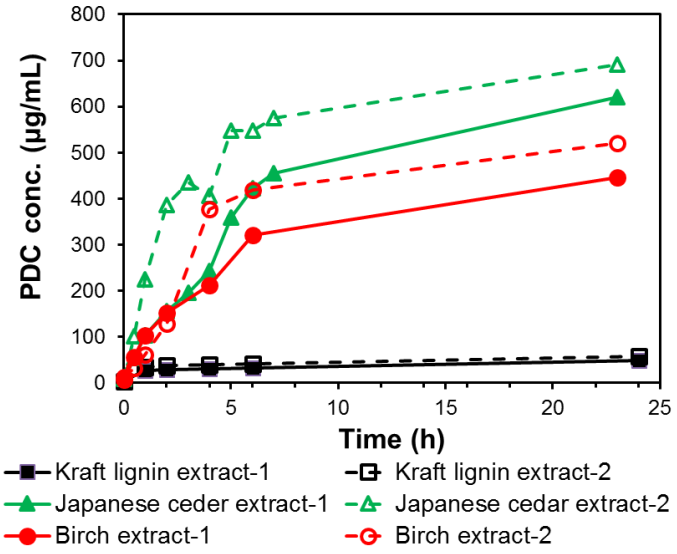

Fig. 2. Average PDC concentration from the fermentation of Japanese cedar crude lignin extracts, birch crude lignin extracts, and Kraft lignin crude extracts

Condensation of aromatic structures, such as would have occurred in kraft lignin, produced insoluble aromatic compounds that could not be metabolized. However, complete and rapid conversion of all the low-molecular weight lignin compounds to PDC

demonstrated that the *P. putida* engineered strain was able to produce PDC from crude lignin extracts of kraft lignin, Japanese cedar, and birch with no, or very limited, inhibition.

This work demonstrated that modification of media and culture conditions allowed the PDHV85 strain to metabolize lignin extracts efficiently and ultimately improve the yield of PDC from crude lignin extracts by enhancing the biosynthetic pathways of this strain. Protocatechuate 4,5-dioxygenase (LigAB) functions in the presence of oxygen to convert protocatechuate to 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) (Noda *et al.* 1990), and CHMS can then be converted to PDC by dehydrogenase (LigC) in the pathway. However, this reaction is dependent on the presence of NAD^+ and/or $NADP^+$ (Masai *et al.* 2000). Vanillic acid demethylase VanA and VanB also require NAD^+ and/or $NADP^+$ (Hibi *et al.* 2005). We hypothesize that the addition of glucose in our cultures would have enhanced metabolic flux through the glycolysis pathway and increased the availability of NAD^+ for enzymes such as LigC, VanA, and VanB (Otsuka *et al.* 2006). This is one of the reasons why increasing glucose content would have enhanced PDC production. Alternately, a simple enhancement in cell density, resulting from an increase in glucose and mineral content, would also increase the conversion of aromatic compounds into PDC. In our work, increasing the glucose and mineral concentration did not result in any observed inhibition, and conversion efficiency to PDC was enhanced.

Refinements to the media composition were required to permit the PDHV85 strain to convert extracts from the different lignin materials completely to PDC. The actual PDC yields from crude extracts of Japanese cedar in particular, but also birch, were higher than the theoretical values (Table 2). A possible reason for this may be that the two crude lignin extracts contained some soluble aromatic oligomers that were not detected by HPLC analysis, but which may have been depolymerized into monomeric form during fermentation and then metabolized by *P. putida* (Linger *et al.* 2014). In addition, the lignin extracts tend to form "sticky" colloidal precipitates in aqueous solution, and these precipitates will likely dissolve slowly during stirred cultivation, potentially releasing more soluble oligomers than originally assayed.

The high conversion rates to PDC from the crude extracts of Japanese cedar and birch suggest the potential for the future application of this strain in the bioconversion of industrial kraft lignin. Although the conversion of softwood kraft lignin in Table 2 was very low, this was a commercial kraft lignin that was obtained after repeated washing, which removed almost all of the soluble lignin components. Application in a mill situation might allow crude kraft lignin, including the low-molecular weight fraction, to be used more efficiently in the fermentation process. In this scenario, the higher-molecular weight, condensed fractions of lignin could still be used as a source of energy in co-generation operations, thus allowing pulp mills to maintain operations without major shifts in production processes while still permitting the generation of a high-value co-product, PDC.

Lignin extracts are complicated mixtures of various aromatic compounds, and the compounds are very difficult to separate and purify. Conversion of a complex mixture of aromatic compounds to pure, platform chemical products has long been a challenge, and the time and expense required to achieve this has typically prevented successful conversion by chemical means. In this research, we achieved our goal of finding an efficient means to convert complex lignin waste streams using a microorganism that is efficient in its processing. Bacterial conversion of crude lignin extracts to useful platform chemicals like PDC has not previously been achieved. Scale up is the next challenge.

CONCLUSIONS

1. Using enhanced media and bioreactor conditions, a novel engineered *P. putida* strain was effective at converting:
   a. Kraft lignin extracts containing vanillin and vanillic acid to PDC as the sole product without inhibition at a media concentration of 0.14 mg/mL.
   b. Japanese cedar (1.14 mg/mL) and/or birch (1.15 mg/mL) crude lignin extracts, containing vanillin, vanillic acid, syringic acid, and syringaldehyde, to PDC with a high yield. No inhibitory effects were observed when growth conditions were optimized.

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