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Base-Catalyzed Depolymerization of Solid Lignin-Rich Streams Enables Microbial Conversion

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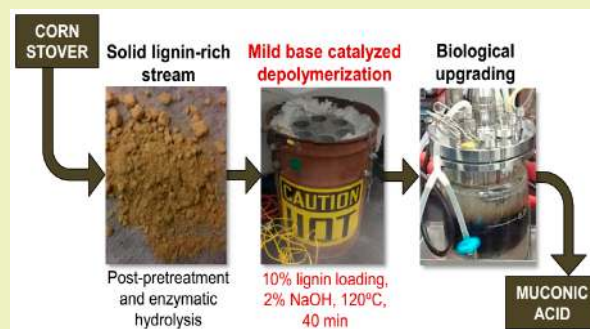
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Supporting Information

ABSTRACT: Lignin valorization offers significant potential to enhance the economic viability of lignocellulosic biorefineries. However, because of its heterogeneous and recalcitrant nature, conversion of lignin to value-added coproducts remains a considerable technical challenge. In this study, we employ base-catalyzed depolymerization (BCD) using a process-relevant solid lignin stream produced via deacetylation, mechanical refining, and enzymatic hydrolysis to enable biological lignin conversion. BCD was conducted with the solid lignin substrate over a range of temperatures at two NaOH concentrations, and the results demonstrate that the lignin can be partially extracted and saponified at temperatures as low as 60 °C. At 120 °C and 2% NaOH, the high extent of lignin solubility was accompanied by a considerable decrease in the lignin average molecular weight and the release of lignin-derived monomers including hydroxycinnamic acids. BCD liquors were tested for microbial growth using seven aromatic-catabolizing bacteria and two yeasts. Three organisms (*Pseudomonas putida* KT2440, *Rhodotorula mucilaginosa*, and *Corynebacterium glutamicum*) tolerate high BCD liquor concentrations (up to 90% v/v) and rapidly consume the main lignin-derived monomers, resulting in lignin conversion of up to 15%. Furthermore, as a proof of concept, muconic acid production from a representative lignin BCD liquor was demonstrated with an engineered *P. putida* KT2440 strain. These results highlight the potential for a mild lignin depolymerization process to enhance the microbial conversion of solid lignin-rich biorefinery streams.



KEYWORDS: Lignin liquor, Biological conversion, Base-catalyzed depolymerization, Lignin monomers, Muconic acid

INTRODUCTION

Currently there is a strong interest in developing new technologies that enable the efficient conversion of lignocellulose into value-added products. Beyond polysaccharide conversion, lignin conversion is of growing interest. This heterogeneous aromatic polymer is the third most abundant biopolymer on Earth and the largest natural source of aromatic compounds.¹ Its core structure is composed of phenylpropanoid units derived from three hydroxycinnamyl alcohol monomers—*p*-coumaryl, coniferyl, and syringyl alcohols—linked through a variety of C–O and C–C bonds by

enzyme-mediated reactions involving free-radical coupling.² In addition, other types of monomers can be incorporated into lignin, such as the hydroxycinnamic acids, *p*-coumarate and ferulate,³ and flavonoids such as tricetin,^{4,5} introducing additional layers of structural complexity. Moreover, the amount and composition of lignin vary significantly between plant species and depend on developmental and environmental factors.⁶

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These features result in a heterogeneous, hydrophobic polymer that exhibits physicochemical properties to provide plants with structural support, the ability to transport nutrients through vascular tissue, and protection against microbial degradation. Unfortunately, its heterogeneity and recalcitrance to chemical or enzymatic depolymerization make lignin difficult to efficiently process into defined low molecular weight (LMW) fractions, which is a major barrier to its valorization.^{7,8} Many lignin depolymerization approaches have been tested, but they are often not fully connected to downstream valorization efforts.^{7,9,10} However, this connection is essential for lignin valorization,¹¹ and is therefore the central goal of this study.

One strategy to convert heterogeneous depolymerized lignin streams into value-added products is to use microorganisms with the natural ability to assimilate a wide range of lignin-derived compounds as energy and carbon sources and to engineer those microorganisms to convert lignin into bioderived fuels and chemicals.^{12–16} This ability, dubbed “biological funneling”, has been reported in a variety of bacteria and relevant lignin streams, such as alkaline pretreatment liquors for the production of polyhydroxyalkanoates, fatty acids, and muconate.^{12,14,17–19} Some advantages of this approach are that it avoids the need for separation of individual lignin depolymerization components, and it also helps to minimize the repolymerization of LMW components by removing them from the system.²⁰ Furthermore, this approach enables the exploitation of the natural biological diversity of aromatic catabolic pathways to stack desired traits into a single organism, the redirection of intracellular lignin intermediates to individual bioproducts, and the increase in volumetric productivities through high cell densities in bioreactors.^{17,19} However, the main drawback of using microbes in lignin upgrading processes is that they typically cannot utilize solid and/or high molecular weight lignin in a rapid manner. One way to circumvent this drawback is to couple biological funneling approaches with upstream chemo-catalytic lignin depolymerization methods that can generate readily consumable LMW species.¹⁸

In this study, base-catalyzed depolymerization (BCD) was investigated as a potential biocompatible chemo-catalytic lignin depolymerization method. BCD has been widely used to depolymerize lignin extracted from different lignocellulosic feedstocks (hardwoods, softwoods, and grasses), usually generating an array of aromatic monomers and oligomers when a strong base is used as a catalyst.¹⁰ BCD offers the potential advantage of using cheap reagents, and it allows for adjustment of the yield of lignin monomers and oligomers by varying reaction parameters such as temperature, pressure, base concentration, solids loading, and residence time.²¹ With the objective of maximizing the solubilization and yield of LMW compounds from lignin, BCD experiments have been typically performed at temperatures around 300 °C, with pressures ranging from 30 to 250 bar and base concentrations ranging from 2% to 10% w/v.^{21,22} However, these harsh conditions tend to generate compounds such as phenol, catechol, syringol, and guaiacol, among others, that can be toxic to certain microorganisms.²³

Considering these criteria, the goal of this study was to develop a biocompatible BCD process from a solid lignin-rich residue. The lignin substrate chosen for BCD is derived from corn stover subjected to deacetylation, mechanical refinement (DMR), and enzymatic hydrolysis (EH), which has been shown to be similar to native corn stover lignin.²⁴ Unlike the aforementioned alkaline pretreatment,^{12,14,17} which generates

soluble lignin streams and where the retention of carbohydrates is critical,^{25,26} carbohydrate retention is not important for the BCD process, so it can be performed at higher temperatures to improve lignin depolymerization. Therefore, a range of temperatures (60–240 °C) at two base concentrations was examined to identify conditions that could generate a biocompatible depolymerized DMR–EH lignin stream. After determination of the BCD conditions that generate high concentrations of monomeric compounds known to be readily consumed by some microbes (e.g., *p*-coumaric acid, ferulic acid), a screen was conducted to identify aromatic-catabolizing microorganisms that have high biocompatibility with the BCD lignin. Microbes that grew well in this stream were examined further to assess the extent of lignin conversion and whether they could elicit changes in the lignin molecular weight distribution. Finally, as a proof of concept, the conversion of BCD lignin liquors into *cis,cis*-muconic acid was demonstrated using an engineered strain of *Pseudomonas putida* KT2440. This compound was chosen because it is a precursor of adipic and terephthalic acids^{14,27} and a potential functional replacement chemical.^{28–31} Our results indicate that mild BCD of DMR–EH lignin is a potential method to solubilize solid lignin and generate a biocompatible lignin fraction that can be upgraded into value-added bioproducts.

■ MATERIALS AND METHODS

BCD of DMR–EH Lignin. The “DMR–EH lignin” represents the lignin-rich residual fraction obtained after the pretreatment (deacetylation and disk refining) and enzymatic hydrolysis (DMR–EH) of corn stover as previously described.²⁰ For the BCD reaction, the dried substrate was added as 10% (w/v) solids to 1% or 2% NaOH solutions, loaded into 50 mL stainless steel reactors (Swagelok Denver, CO), and maintained at the target temperature (60–240 °C) at a pressure of 0.7–20 bar for 40 min in a sand bath. These reactors consisted of a stainless steel tube (diameter, 1 in.; wall thickness, 0.83 in.; length, 6 in.) with 1 in. Swagelok tube fittings (SS-1612-1 and SS-1610-61). After the reaction, the reactors were quenched to room temperature with water. The pH of the BCD liquors was adjusted to a value of 7 with 5 N HCl, and the aqueous fraction was separated from the remaining solids by centrifugation. The liquors from all the BCD reactions were sterilized by filtration, through 0.20 μm pore size, for gel permeation chromatography (GPC) analysis and microbial growth experiments. A defined liquor volume was also freeze-dried and weighed to calculate the percentage of total DMR–EH lignin in solution, after correcting for the amount of salts added in the alkaline reaction and neutralization.

A larger depolymerization reaction was conducted at the selected BCD reaction condition to produce sufficient liquor for further biological treatments. For this purpose, the reaction was conducted in a 350 mL stainless steel Miniclave drive 3 pressure reactor (all factory parts) equipped with an impeller and temperature controller (Buchiglas). Batches were prepared by adding 30 g of solids to 300 mL of 2% NaOH in the reactor and the reaction proceeding as a 35 min ramp from 25 to 120 °C, a 30 min reaction at 120 °C, and a 25 min ramp from 120 to 40 °C, while keeping the stirrer speed constant at 1500 rpm. The pressure reached 2 bar during the reaction. The pH of the resultant liquor was adjusted to 7 with 5 N H₂SO₄, and the soluble and insoluble fractions were separated by centrifugation in 50 mL preweighed tubes (3 serial centrifugations at 10 000 × g) and filtered through 0.45 μm surfactant-free cellulose acetate (SFCA) filtration chambers. The solids recovered from the pellets and filters were combined and washed 2 times with 20 mM H₂SO₄ to remove salts, and then freeze-dried and weighed to determine the amount of insoluble material resulting from this reaction. The same procedure was applied at small-scale to the solid DMR–EH dissolved in water at room temperature and the BCD liquors (before and after neutralization). For this assay, 100 μL of sample volume was

centrifuged in 1.7 mL tubes, and the solids were washed and dried as before. All measurements were done in triplicate, and the resultant weights were expressed as percentage relative to the initial biomass.

Microbial Strains, Media, and Seed Preparation. The organisms utilized in the current study were *Rhodococcus rhodochrous* (soil isolate), *Rhodococcus opacus* (Klatte et al. ATCC 51882), *Delftia acidovorans* (den Dooren de Jong, Wen et al. ATCC 11299b), *Corynebacterium glutamicum* (Kinoshita et al. ATCC 13032), *Rhodotorula mucilaginosa* (soil isolate), *Exophiala alcalophila* (soil isolate), *Rhodococcus jostii* RHA1, *Pseudomonas putida* KT2440, and *Escherichia coli* MG1655. Among these, *R. opacus*, *D. acidovorans*, and *C. glutamicum* were purchased from ATCC. *R. rhodochrous*, *R. mucilaginosa*, and *E. alcalophila* were isolated after growing a microbial community taken from top soil (Alameda, CA) on alkali lignin (Sigma-Aldrich) as a single carbon source at 30 °C for 5 days on plates (2% agar, 1× M9 salts, 0.5 g/L lignin). The organisms were identified by analysis of their 16S/18S rRNA genes, amplified using the universal primers U515F and U1390R, and subsequent genome sequencing.

A 10-fold stock solution of M9 salts was prepared containing the following (per liter): Na₂HPO₄ (67.8 g), KH₂PO₄ (30.0 g), NaCl (5.0 g), (NH₄)₂SO₄ (19.8 g), and this was added to the neutralized and filtered BCD lignin liquors. Then, MgSO₄ (240 mg), CaCl₂ (12 mg), and 1 mL of a trace element mixture containing CuSO₄, MnSO₄, FeSO₄, and ZnSO₄ (100 μM each) were supplemented per liter of medium. For initial biocompatibility screenings, BCD lignin liquors were used at concentrations of 20% and 90%. Table 1 shows the

Table 1. Compositional Analysis Data of Corn Stover, DMR–EH Lignin Residue, and BCD Liquor

	corn stover, %	DMR–EH, %	BCD liquor, ^a %
lignin	14.8	53.5	36 (31 g/L) ^b
ash	2.5	2.2	43
glucan	36.5	9.5	0.13
xylan	30.2	10.8	1.17
galactan	1.8	1.4	0.84
arabinan	3.52	2.6	0.85
acetate	2.71	0.9	0.37
proteins	NA	9.3	NA
mass balance	92.03	90.2	82.36

^aThe composition corresponds to the soluble liquor produced at 120 °C and 2% NaOH after neutralizing at pH 7, removing solids, filter-sterilizing, and adding 10% salts from M9 for the bacterial cultures.

^bThe corresponding concentration of lignin in the liquor is shown in parentheses.

composition of the BCD liquor after adjusting at pH 7 and at a concentration of 90% plus the M9 salts. The composition of corn stover and DMR–EH lignin residue is also presented for comparison purposes.

Seed cultures for the different organisms were prepared in LB (lysogeny broth) medium at 30 °C, agitated at 200 rpm for 12 h, and added (one hundredth of the final volume) to the test tubes to start the cultivations.

Microbial Screening in BCD Lignin Liquors. First, *P. putida* KT2440—utilized as the control in the current study because of the extensive information regarding its robustness in toxic environments^{12,17,32}—was screened in 90% concentrated BCD lignin liquors resulting from the different BCD reactions to evaluate bacterial growth. Cultures were performed in 15 mL glass tubes containing 5 mL reaction volume and incubated at 30 °C and 200 rpm for 48 h. For the evaluation of bacterial growth, 1 mL was centrifuged, and the cells were washed with water to remove color interferences. Optical density at 600 nm (OD₆₀₀) was measured. Two biological replicates were performed for each condition.

Second, for the microbial screening, cultivations in 20% and 90% BCD liquor concentrations (prepared at the selected BCD conditions, 120 °C and 2% NaOH) were set in 15 mL glass tubes with 5 mL

reaction volume at 30 °C and agitated at 200 rpm for 4 days. Biomass concentration was measured as optical density at 800 nm in a SpectraMax M2 spectrophotometer (Molecular Devices) using 96-well Costar assay plates (Corning). Three biological replicates were performed for each organism, and the obtained values were corrected for media coloration with noninoculated controls.

Flask Experiments with Selected Microbial Strains for Growth and Aromatic Consumption Kinetics and Lignin Characterization Studies. Microbial cultivations were performed in 250 mL flat-bottom shake flasks with 50 mL of medium volume. The microorganisms were grown for 5 days under the same conditions and inoculation train described above, with the exception of *Corynebacterium glutamicum* cultures, which were conducted at pH 8.5 (by increasing the pH with NaOH), and in the presence of biotin (0.4 mg/L) to counteract the known auxotrophy to this vitamin.³³ Samples from the cultivations were collected and centrifuged at defined intervals in 1.7 mL tubes, and the supernatants were stored at –20 °C for LC–MS analysis. Cells were centrifuged, then resuspended and diluted in water for spectrophotometric determination of growth via OD₆₀₀. At the end of the cultivations, 25 mL were collected per flask, centrifuged, and the supernatant was frozen for GPC and compositional analyses. Correlation factors between OD₆₀₀ and cell dry weights (CDWs) of 0.44, 0.37, and 0.49 (g CDW/L)/OD₆₀₀ for *C. glutamicum*, *R. mucilaginosa*, and *P. putida*, respectively, were obtained and used to calculate the biomass concentrations. The specific growth rate (μ) was determined as the slope of the linear fit to graphs obtained by plotting the natural logarithm of four biomass values versus time during the exponential phase, with R^2 values ≥ 0.98 . The volumetric consumption rate (Q_s) was calculated by linearly fitting at least three *p*-coumaric acid concentration values as a function of time during the growth phase, with R^2 values ≥ 0.96 . The biomass-to-substrate yield ($Y_{b/s}$) was calculated with the combined total amounts of consumed *p*-coumaric and ferulic acids at the time when maximum biomass concentration was reached. The displayed graphs and calculations represent the average values obtained from two biological replicates, where the error bars represent the error range, obtained as the absolute value of the numeric difference between replicates.

Production of Muconic Acid from BCD Lignin Liquor by Engineered *P. putida* KT2440 in a Bioreactor. The muconate-producing strain used in this work, *P. putida* KT2440-CJ103, was constructed as reported previously.¹⁴ Prior to cultivation in the bioreactor, the lignin liquor (obtained after neutralization and centrifugation) was filtered through 0.8–0.2 μm cartridges (Sartopore 2 XLG) and then through 0.2 μm Millipore filters to ensure sterility. The cultivation strategy consisted of 3 different stages to avoid initial growth lags due to high lignin content, but also catabolic repression due to high glucose concentrations.³⁴ Specifically, the first stage consisted of a batch phase containing 50% BCD lignin liquor, 20 mM glucose, and M9 (10× as described above) in a total volume of 250 mL. In the second phase, 100% BCD DMR–EH lignin (100 mL) and 5 mM glucose were added. In the last stage, for assurance that all of the aromatic compounds were utilized, a continuous feeding (4 mL/h) from a 100 mL feed containing 90 g/L glucose and 18 g/L (NH₄)₂SO₄ was conducted. The cultivations were performed in a 0.5 L working volume BioStat-Q Plus bioreactor (Sartorius AG) sparged with air (1 vvm, volume of air per reactor volume per minute), and the pH was maintained at 7 with 4 N NaOH. The oxygen saturation was maintained between 40% and 60% in the first phase (increasing agitation from 350 to 650 rpm) and then automatically controlled by agitation to maintain a dissolved oxygen level of 30%. Samples (1.5 mL) were taken throughout the fermentation to analyze bacterial growth (OD₆₀₀), glucose concentration (analyzed by YSI 7100 MBS), aromatic compound consumption, accumulation of intermediates, and muconic acid production (analyzed by LC–MS/MS). In addition, 25 mL were removed from the fermentation broth before the third feeding stage as well as at the end of the cultivation to analyze lignin molecular weight (MW) by GPC. A control sample (before the bacterial inoculation) was also analyzed for the lignin MW distribution. For the calculation of muconic acid yield, the dilution effect due to the feeding and base addition was considered.

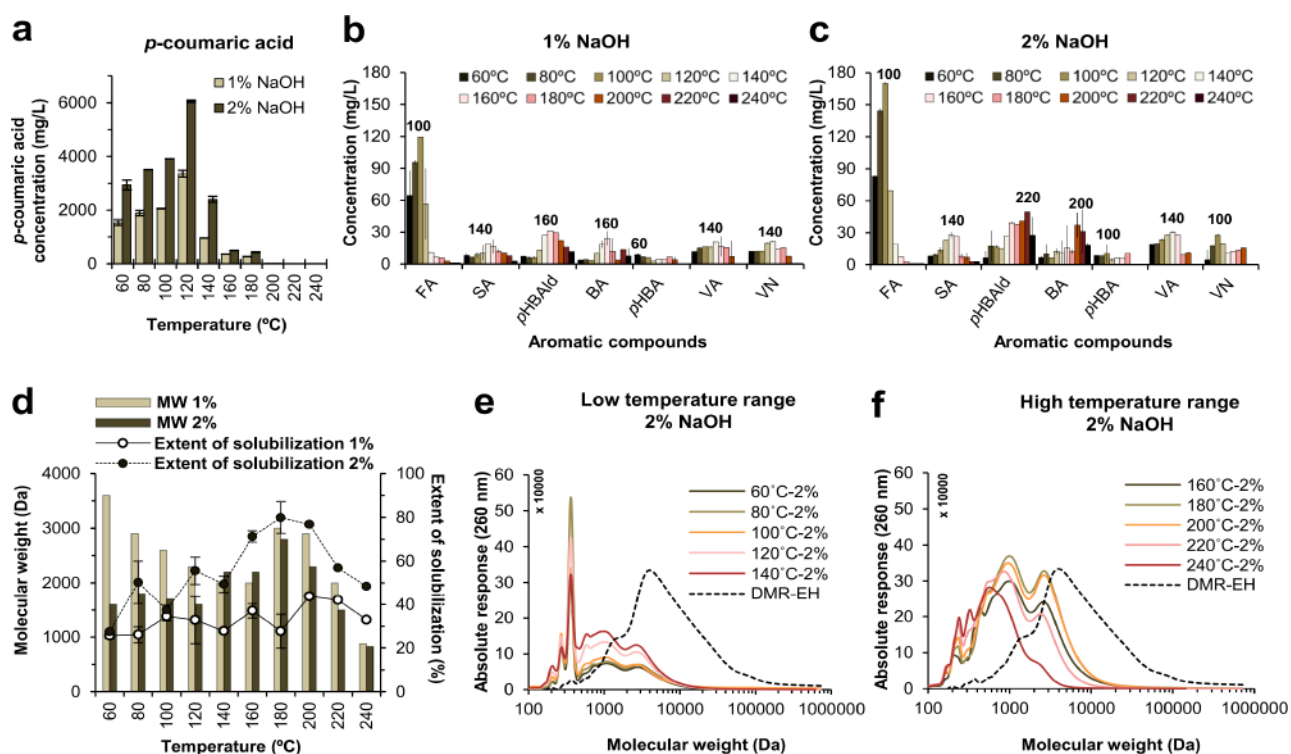


Figure 1. Characterization of the lignin liquors resulting from BCD reactions at different temperatures and base concentrations. (a) *p*-Coumaric acid concentration in the liquor. (b) Minor phenolic compounds detected in the liquor generated with 1% NaOH. (c) Minor phenolic compounds detected in the liquor generated with 2% NaOH. The numbers on top of graphs indicate the temperature that generated the highest concentration of each aromatic compound. (d) Solubilization extent of the solid lignin stream after BCD reactions and further neutralization and lignin M_w of the corresponding liquors. (e) GPC chromatograms from lignin liquors generated at 2% NaOH in the lower (60–140 °C) temperature range. (f) GPC chromatograms from lignin liquors generated at 2% NaOH in the higher (160–240 °C) temperature range. GPC chromatograms from lignin liquors generated at 1% NaOH are shown in Figure S1. FA = ferulic acid; SA = syringic acid; *p*HBAl = *p*-hydroxybenzaldehyde; BA = benzoic acid; *p*HB = *p*-hydroxybenzoic acid; VA = vanillic acid; VN = vanillin. The error bars indicate the error range from two technical replicates.

Liquid Chromatography Mass Spectrometry (LC–MS) Analysis To Determine Aromatic Compounds in BCD Lignin Liquors. Samples from BCD liquors and culture supernatants were filtered through Nanosep 3K centrifugal devices (Pall Laboratories) before analysis. LC–MS analysis of phenolic compounds was carried out with a method similar to the one described by Eudes et al.,^{35,36} where the mobile phase was composed of 10 mM ammonium acetate in water (solvent A) and 10 mM ammonium acetate in 90% acetonitrile and 10% water (solvent B). The only differences were the MS mass range (from 100 to 1600 m/z) and the LC gradient elution method, which is described as follows: increased from 30% B to 80% B in 12 min, increased from 80% B to 100% B in 0.1 min, held at 100% B for 0.5 min, decreased from 100% B to 30% B in 0.2 min, and then held at 30% B for a further 2.8 min. The flow rate was increased from 0.5 mL/min at 12.1 min to 1 mL/min at 12.6 min, and held at 1 mL/min for the remaining 3 min. The total LC run time was 15.6 min.

Alternatively, for the separation of aromatic monomer consumption in microbial cultivations, a Hypersil ODS C18 column was employed (5 μ m beads, 4.6 mm i.d. \times 250 mm length; ThermoFisher). Sample volumes of 5 μ L were run at a column temperature of 25 °C with an isocratic separation method using 20% acetonitrile in water. Flow from phenyl–hexyl or the C18 column was directly analyzed by MS by electrospray ionization (ESI) in the negative-ion mode.

The production of muconic acid in bioreactors and subsequent pathway analyte-tracking from engineered *P. putida* was performed using a Waters Acquity system including an ultraperformance liquid chromatography pump, a TQD triple quadrupole mass spectrometer with in-line ESI, and a tunable UV detector (TUV) (Waters Inc.). An injection volume of 5 μ L was loaded onto an Acquity BEH300 C4 column, 1.7 μ m, 2.1 mm i.d. \times 150 mm length (Milford). Separations were performed at 30 °C at a flow of 0.15 mL/min with a gradient as follows: (solvent A) 4 mM ammonium formate in water and (solvent

B) 4 mM ammonium formate in acetonitrile/water (9:1, v/v) starting with 100% A, held until 0.14 min; 93% A, 4.48 min; 91% A, 6.14 min; 90% A, 10.48 min; 75% A, 14.48 min; 70% A, held from 16.14 to 17.14 min; 50% A, 19.48 min; and lastly, 0% A, held from 20.48 to 26.48 min before equilibrium. Flow from the LC–TUV was directly routed in series to the ESI-MS TQD. The TUV was used to monitor chromatography at 210 and 264 nm for a direct comparison to MS data. The following source parameters were used for negative-mode ionization and analysis: capillary 2 kV, extractor 3 V, RF lens 0.1 V, source temperature 150 °C, desolvation temperature 250 °C, desolvation gas 500 L h⁻¹, and cone gas 10 L h⁻¹. Multiple reaction monitoring (MRM) experiments were performed for analyte tracking and quantitation between a mass range 50–250 Da. Cone voltage and collision energy optimization varied from 16–25 V and 10–20 eV, respectively, corresponding with each analyte. MassLynx 4.1 software was used for analysis of the production of muconic acid and pathway tracking. All quantitative standard curves were maintained with an R^2 value ≥ 0.995 , while samples were diluted accordingly to fit within the linear regions of the calibration curves. Authentic standards were obtained in the highest purity available to determine detector response. These compounds are listed in Figure S1.

Gel-Permeation Chromatography (GPC) Analysis to Analyze the Molecular Distribution of Lignin in BCD Liquors. The methodology for the GPC analysis employed in this work has been reported previously.²² Briefly, samples consisting of 20 mg of dried material from culture supernatants, BCD lignin liquors, or DMR–EH lignin, were acetylated with a mixture of acetic anhydride (0.5 mL) and pyridine (0.5 mL) at 40 °C for 24 h. Methanol was added (0.2 mL) to terminate the reaction, and all solvents were evaporated with nitrogen gas. The samples were dried in a vacuum oven at 40 °C overnight, dissolved in tetrahydrofuran (THF), and filtered with 0.45 μ m polytetrafluoroethylene (PTFE) filters. GPC analysis was performed

using an Agilent HPLC with 3 GPC columns (Polymer Laboratories, 7.5 mm i.d. \times 300 mm length) packed with polystyrene-divinylbenzene copolymer gel (10 μ m beads) with THF as eluent at a flow rate of 1 mL/min. Absorbance at 260 nm was quantified with a diode array detector, and the retention time was converted to molecular weight using a calibration curve made with polystyrene standards. It should be noted that this method only detects the lignin fraction of the solubilized DMR–EH substrate in the BCD liquor.

Full compositional analysis. Compositional analysis from the DMR–EH residue and BCD lignin liquors (before and after the microbial treatments) was performed according to the procedure in NREL LAP/TP-510-42618 64 to determine lignin, sugar, and other organic compound concentrations³⁷ with two exceptions (i.e., the samples were dried in a 40 °C oven, instead of 105 °C, and the analysis was scaled-down by a factor of 6). It is worth noting here (as it is specified in the procedure) that both insoluble and soluble lignin were considered for lignin quantification. Before this procedure was carried out, samples were freeze-dried and then weighed. The initial volume of the liquor subjected to freeze-drying and the final weight of the dried material were used to calculate the lignin concentration in g/L. The final concentration of lignin was also corrected for evaporation (in the shake flask experiments). The reported values represent the average of two technical replicates from each of two biological replicates.

RESULTS AND DISCUSSION

Characterization of Lignin Liquors Derived from BCD Performed at Different Temperatures and Base Concentrations. For an assessment of the impact of BCD reaction conditions on the solubilization and depolymerization of solid DMR–EH lignin, a range of temperatures (60–240 °C) and NaOH concentrations (1% or 2% w/v) were explored, using a fixed-solids loading (10% w/v) and residence time (40 min). The concentration of monomeric aromatic compounds released from the solid lignin stream at the different BCD reaction conditions was measured, and the most abundant species (according to their peak area values) were identified and quantified with external standards by LC–MS (Figure 1a–c).

The lignin-derived hydroxycinnamic acid, *p*-coumaric acid, was the most abundant monomer detected when using BCD reaction temperatures between 60 and 180 °C (Figure 1). The highest *p*-coumaric acid concentration of 6.1 g/L was achieved at 120 °C and 2% NaOH (Figure 1a). Ferulic acid was the second most abundant aromatic monomer identified, but its concentration was more than 10-fold lower than that of *p*-coumaric acid (<180 mg/L). This result is expected as corn stover lignin can contain up to 15–18% of esterified *p*-coumaryl groups, while ferulate is typically found at lower abundance, forming both ether and ester linkages with lignin and hemicellulose, respectively.^{3,38–40} The high concentrations of *p*-coumaric acid observed after BCD also support previous observations that mechanically refined lignins from corn stover appear to retain many of the linkages present in native biomass,²² including acylated *p*-coumaryl groups, relative to lignin pretreated more severely with, e.g., dilute acid or hydrothermal conditions. Other low-abundance lignin depolymerization products detected in the BCD lignin liquors were syringic acid, *p*-hydroxybenzaldehyde, benzoic acid, *p*-hydroxybenzoic acid, vanillic acid, and vanillin, all at concentrations below 50 mg/L (Figure 1b,c). Each compound exhibits a unique concentration profile as a function of BCD reaction temperature, with most compound concentrations peaking at temperatures at or below 160 °C. The NaOH concentration also impacts the quantity of released aromatic compounds from BCD. A concentration of 2% NaOH generally improves the release of aromatic compounds compared to 1% NaOH (Figure

1a–c). This observation agrees with the higher substrate solubility detected when using 2% NaOH (up to 80% at 180 °C), compared to that with 1% NaOH (up to 40% at 200 °C) (Figure 1d).

Regarding the lignin MW distribution, BCD treatments decrease the weight-average lignin molecular weight (M_w) significantly compared to the control (DMR–EH solid residue) (Figure 1d), with a concomitant production of LMW compounds with masses in the range 100–1000 Da (Figure 1e,f, Figure S2). The GPC profiles obtained from BCD treatments at 1% and 2% NaOH follow the same trends, as shown in Figure S2 and Figure 1e,f, respectively. Lignin M_w values were in general lower and more uniform among different temperatures when using 2% NaOH than those when using 1% NaOH, suggesting that depolymerization is more efficient and less dependent on temperature at higher NaOH concentrations (Figure 1d). Moreover, the GPC profiles for the reactions with 2% NaOH highlight considerable depolymerization even at the lowest temperature tested, 60 °C, which produces two distinct peaks between \sim 200 and 500 Da that presumably correspond to monomers and dimers (Figure 1e). BCD reaction temperatures higher than 140 °C increased lignin solubilization but, at the same time, drastically reduced the monomer concentrations, such as *p*-coumaric and ferulic acids (Figure 1a–c). At the extreme temperatures of 220 and 240 °C, lignin solubilization decreases, and *p*-coumaric and ferulic acids were not detected, even though peaks corresponding to LMW species in the GPC chromatograms are still observed (Figure 1f). The disappearance of *p*-coumaric and ferulic acids from the liquors at the high temperature range is likely resulting from pH-dependent decarboxylation reactions.⁴¹ As a reference, the profiles obtained with *p*-coumaric or ferulic acid standards are shown in Figure S3.

Since *p*-coumaric and ferulic acids are readily consumed by many microbes, their decrease in concentration in the BCD lignin liquor produced at higher temperatures may negatively affect the extent of microbial growth on these BCD liquors. For a test of this hypothesis, growth of *P. putida* KT2440, a robust aromatic-catabolizing bacterium⁴² that has been previously demonstrated to grow in alkaline pretreatment liquors from corn stover,^{12,17} was first examined in each of the BCD lignin liquors. Figure S4 shows that *P. putida* KT2440 was not able to grow in BCD liquors produced at temperatures higher than 200 °C. These results indicate that those liquors are toxic or do not contain sufficient consumable aromatic compounds for growth and suggest that BCD streams produced at reaction temperatures below 140 °C may be more useful for biological lignin valorization. This usefulness goes beyond these BCD streams, as other methods of lignocellulose fractionation that generate these hydroxycinnamic acids^{43,44} could also be coupled to this biological conversion process. Differences in abundance of these and related aromatic compounds in specific plant species^{3,45} can be used to guide biomass selection when using this approach.

Selection of BCD Conditions for Microbial Screening. Since BCD at 120 °C and 2% NaOH generates the highest concentration of *p*-coumaric acid and does not present significant toxicity to *P. putida* KT2440, this condition was selected for further biocompatibility screening (Figure 2). At a 10% (w/v) DMR–EH solids loading, the BCD reaction conducted at 350 mL solubilized 85% of the solids before neutralization. The pH of the BCD liquor was subsequently lowered from 12 to 7, a process known to cause lignin

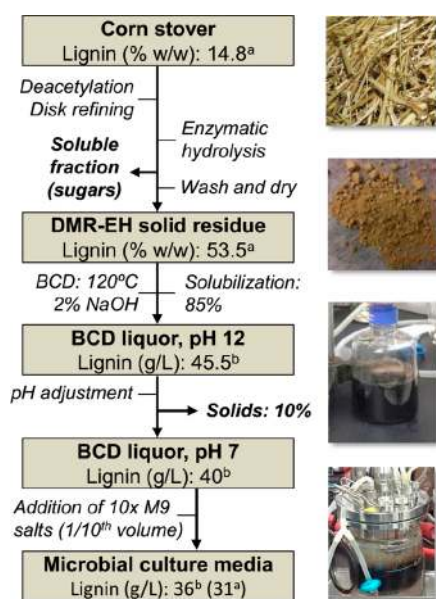


Figure 2. Scheme of the steps required to obtain the lignin-enriched solid substrate DMR–EH and the BCD lignin liquor for further biological upgrading. Lignin content is shown for each of the substrates, from corn stover to the microbial culture medium. Measured values are indicated by a; calculated values are indicated by b.

precipitation. After pH adjustment, and centrifugation and filtration of the BCD eluents, 73% of the liquid fraction was recovered, and 76% of the DMR–EH remained in solution relative to the starting amounts, which corresponds to 40 g/L of lignin. This indicates that about 10% of the solubilized material is being precipitated during pH adjustment. The degree of solubilization obtained when using a 350 mL scale stirred reactor (76%) was higher than the one measured at 50 mL scale (56%) (Figure 1d), which can likely be attributed to enhanced mixing. For the enabling of microbial growth, the BCD liquor was then supplemented with phosphate, sulfate, and nitrogen salts, and trace elements, resulting in a 10% dilution and a final concentration of ~36 g/L of lignin (Figure 2).

As a result of this process, slightly higher concentrations of the main aromatic monomers were obtained in the 350 mL scale BCD reaction, resulting in final concentrations of 7.1 g/L of *p*-coumaric acid and 0.48 g/L of ferulic acid, representing a combined monomer yield of 14 wt % relative to the initial lignin content (53.5 g/L). BCD processes have typically been performed at temperatures near 300 °C, which have resulted in the production of syringol, guaiacol, phenol, and alkylated phenolics with a wide range of monomer yields. However, it is difficult to directly compare their yields to this study because of the different substrates, catalysts, conditions, and separation processes employed in each case.^{10,46,47} Overall, these different monomer profiles indicate that BCD process conditions can be tuned to best match the desired downstream lignin valorization application, highlighting the versatility of the BCD process. For example, a mixture of monomeric and oligomeric phenol derivatives produced at 300 °C could be subjected to hydrodeoxygenation to valorize lignin into fuels,⁴⁸ but they are perhaps not as biocompatible as *p*-coumaric and ferulic acids, released by milder BCD conditions.

Microbial Screening on BCD Lignin Liquor. There are many organisms known to utilize lignin-derived compounds as carbon and energy sources via metabolic pathways, such as the β -ketoacid pathway, which catalyze the oxidative cleavage of aromatic rings and their conversion into common metabolic intermediates.⁴⁹ Since the ability of a specific organism to consume a lignin-derived substrate will depend on the substrate composition and concentration, media composition, and cultivation conditions, eight different organisms known to consume lignin-like aromatic monomers were evaluated for compatibility with the BCD liquor, similar to previous work conducted on alkaline pretreated liquors.¹⁷ *E. coli* was used as a negative control since it does not catabolize lignin-derived aromatic compounds. Small-scale cultivations were performed in minimal media containing low (20% v/v) and high (90% v/v) BCD liquor concentrations. As expected, growth differences were observed at the two different liquor concentrations (Figure 3). At a concentration of 20%, only two organisms, *R.*

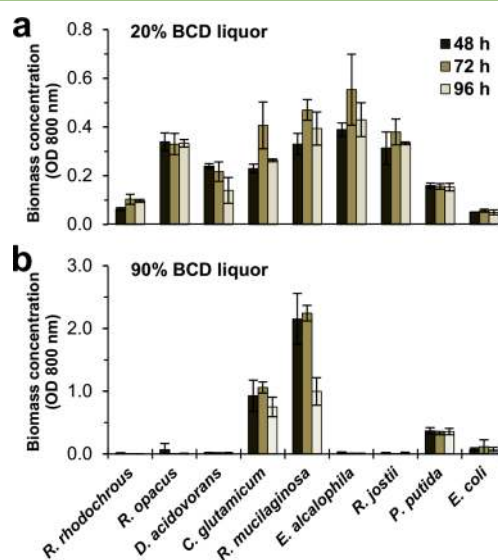


Figure 3. Microbial growth tests performed in BCD liquor at different concentrations. Samples for biomass measurements were collected after 2, 3, and 4 days of incubation using a concentration of (a) 20% and (b) 90% v/v. The error bars indicate the standard deviation from three biological replicates.

rhodochrous and *E. coli*, exhibit insignificant growth after 4 days of incubation (Figure 3a). In fact, the minor growth observed for these 2 strains was likely due to the low amounts of sugar-derived compounds and acetate present in BCD liquors (Table 1). *R. rhodochrous* has been reported to consume a number of aromatics,⁵⁰ but it may not prefer the carbon sources present in the BCD liquor, or it may have experienced some level of toxicity. Conversely, when a BCD liquor concentration of 90% was used, only three of the nine organisms were able to grow: *C. glutamicum*, *R. mucilaginosa*, and *P. putida*. Maximum cell growth was achieved after 2–3 days of cultivation, which was proportionally higher in the medium with 90% BCD liquor (Figure 3b) than that with 20%. It should be noted that the resultant lignin liquor also contains a high concentration of sodium salts, which will limit growth of organisms with low salt tolerance. As such, *C. glutamicum*, *R. mucilaginosa*, and *P. putida* were selected for further characterization in shake flasks containing 90% BCD liquor medium.

Biological Conversion of BCD Liquors by *C. glutamicum*, *R. mucilaginosa*, and *P. putida*. For a better understanding of the interaction of the three microbes that have high biocompatibility with the BCD liquor, a more in-depth analysis on bacterial growth kinetics, lignin modification, and aromatic utilization was performed. For optimization of cultivation conditions, the optimal pH for each microbe was determined in the BCD liquor. *C. glutamicum* grew best at pH 8.5, while *R. mucilaginosa* and *P. putida* preferred pH 7. The selection of optimal pH was based on growth rate and final cell density in cultures containing *p*-coumaric acid as a sole carbon source and in BCD lignin liquors adjusted to pH 7–10 (Figure S5). Under optimal pH conditions, *C. glutamicum*, *R. mucilaginosa*, and *P. putida* each utilized *p*-coumaric and ferulic acids simultaneously during the exponential growth phase in BCD liquor, and their maximum cell biomass concentration was achieved between 36 and 48 h of cultivation (Figure 4).

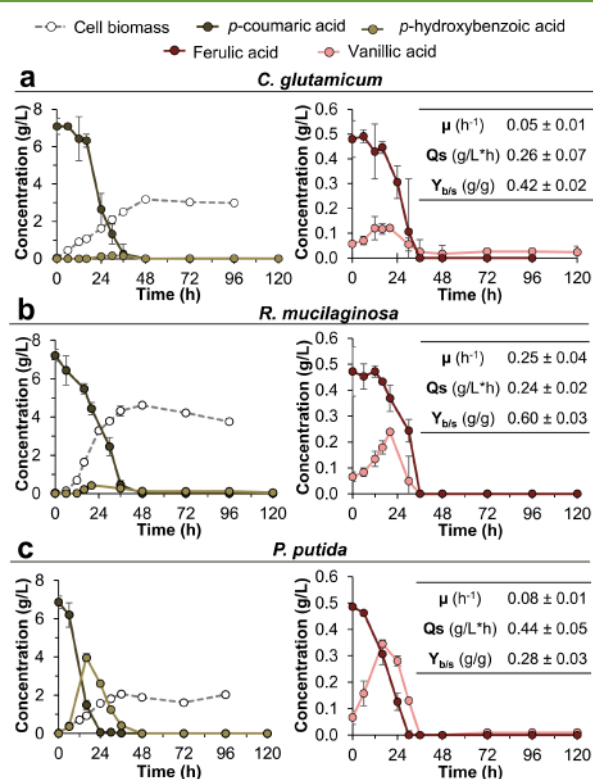


Figure 4. Kinetic profiles of three organisms in shake flasks, grown in 90% final BCD liquor concentration for 5 days. (a) *C. glutamicum*. (b) *R. mucilaginosa*. (c) *P. putida*. μ = specific growth rate, Q_s = *p*-coumaric acid volumetric consumption rate, and $Y_{b/s}$ = biomass-to-substrate yield. The error bars indicate the error range from two biological replicates.

The end of the exponential growth phase was correlated with the depletion of *p*-coumaric and ferulic acids in the supernatants, suggesting that these compounds are among the primary carbon and energy sources.

C. glutamicum generated 3.1 g/L of cell biomass with a specific growth rate (μ) of 0.05 h^{-1} , a *p*-coumaric acid volumetric consumption rate (Q_s) of $0.26 \text{ g}/(\text{L h})$, and a biomass-to-substrate yield ($Y_{b/s}$) of 0.42 (g/g) (Figure 4a). Furthermore, *C. glutamicum* is generally regarded as a robust organism that can be grown to very high cell densities and has many tools available for genetic manipulation.³³ This bacterium

is capable of assimilating diverse aromatic compounds through the formation of catechol, protocatechuate, gentisate, 1,2,4-trihydroxybenzene, and other central aromatic intermediates that can be further converted into tricarboxylic acid cycle intermediates.⁵¹

In contrast to *C. glutamicum*, the red yeast *R. mucilaginosa* has limited available information, and was able to produce 4.6 g/L of biomass with a 5-fold higher μ and a 43% higher $Y_{b/s}$, making evident differences in substrate assimilation pathways and yields of biomass precursors (Figure 4b). *R. mucilaginosa* naturally produces a high concentration of pigments and lipids, offering attractive opportunities for valorization of lignin-derived compounds.⁵² This organism appears to express proteins belonging to the protocatechuate branch of the β -ketoacid pathway but lacks enzymes of the catechol branch, and it is therefore unable to metabolize catechol.⁵³ One drawback for this organism is that there are few genetic tools currently available for red yeasts compared to *P. putida* and *C. glutamicum*, and therefore a more extensive effort is required to pursue genetic engineering strategies in this organism.

Finally, the Gram-negative bacterium *P. putida* exhibited the highest Q_s , and produced the lowest amount of biomass (2.0 g/L) with a slow rate (0.08 h^{-1}), which means that the substrate conversion rate per cell is higher (Figure 4c). Of the three organisms, *P. putida* allocated the smallest fraction of the consumed carbon into biomass, offering the possibility to redirect a larger portion of metabolic intermediates into bioproducts and, thus, to present higher product yields. This organism has been extensively studied for its ability to thrive in many environments, and thus detailed physiological information is available.^{54,55} For example, it is known that the *P. putida* KT2440 genome harbors enzymes belonging to the catechol and protocatechuate branches of the β -ketoacid pathway, and it has been engineered to convert lignin-derived compounds into molecules of interest.^{12,14,19,56} The data presented in this work demonstrate that high initial concentrations of *p*-coumaric acid can be tolerated by *P. putida* and consumed with a rate that is on par with other carbon sources. Notably, during the consumption of *p*-coumaric and ferulic acids, a simultaneous increase in the extracellular concentrations of *p*-hydroxybenzoic and vanillic acids was observed (Figure 4c). These aromatics are intermediates in *p*-coumaric and ferulic acid catabolic pathways, respectively.^{42,51} Thus, the accumulation of these compounds suggests that there may be bottlenecks within the β -ketoacid pathway under the conditions studied in this work. Both *p*-hydroxybenzoic and vanillic acids were detected in all organisms, but this effect was more evident in *P. putida*, likely because of the faster consumption of *p*-coumaric acid.

In addition to the analysis of monomer utilization, the lignin MW profiles after the microbial treatment were also examined. Two peaks in the LMW region were present in the uninoculated control profile but disappeared after the microbial cultivation (Figure 5a,b), which is in agreement with the depletion of *p*-coumarate and ferulate from the culture supernatants. Overall, *C. glutamicum* reduced the lignin content from 29.6 to 25.3 g/L, *R. mucilaginosa* from 28.6 to 25.5 g/L, and *P. putida* from 28.6 to 26.0 g/L (Figure 5c), representing a total lignin conversion of 15%, 11%, and 9%, respectively. Previous publications have reported lignin weight losses during microbial cultivations from 10% to 44%; however, those lignins had different origins, and their concentrations were considerably lower, ranging from 0.5 to 10 g/L.^{17,57,58} Here we

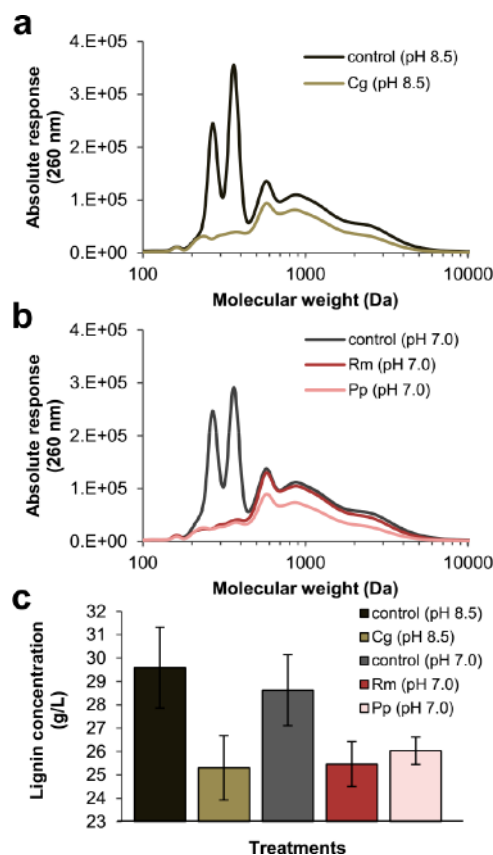


Figure 5. Lignin molecular weight profiles and lignin content from the BCD liquors before and after the microbial cultivation. (a) GPC chromatograms from experiments performed at pH 8.5 with *C. glutamicum* (Cg, green) and a noninoculated control (black). (b) GPC chromatograms from experiments performed at pH 7 with *R. mucilaginosa* (Rm, red), *P. putida* (Pp, pink), and a noninoculated control (gray). (c) Lignin content calculated from the BCD liquors from the controls and after 5 days of microbial cultivation. The error bars indicate the error range from two biological replicates.

demonstrate that lignin concentrations up to 30 g/L can be tolerated by certain microorganisms and that a lignin conversion up to 15% can be attained. As detailed above, the proportion of aromatic compounds relative to total lignin is approximately 14%, and considering the molecular weight profiles, the lignin conversion presented in the current study is likely due primarily to the utilization of monomeric compounds, not high molecular weight lignin.

Production of Muconic Acid from BCD Lignin Liquor by Engineered *P. putida*. In this study, *P. putida* presents several advantages over the other two organisms tested when growing on BCD liquors (e.g., higher aromatic compound utilization rates and lower production of cell biomass). Thus, we selected an engineered *P. putida* strain that is able to produce muconic acid from diverse lignin-derived compounds, KT2440-CJ103,¹⁴ to demonstrate that a fraction of the aromatic compounds present in the BCD liquor could be upgraded to a value-added molecule. Since the aromatic compounds in the BCD liquor will primarily be converted into muconic acid in this strain, another carbon source, glucose, was added to maintain the bacterial metabolism. For avoidance of catabolic repression due to high glucose concentrations,³⁴ a fed-batch strategy in a pH-controlled and aerated bioreactor was employed. *P. putida* KT2440-CJ103 grew adequately in the

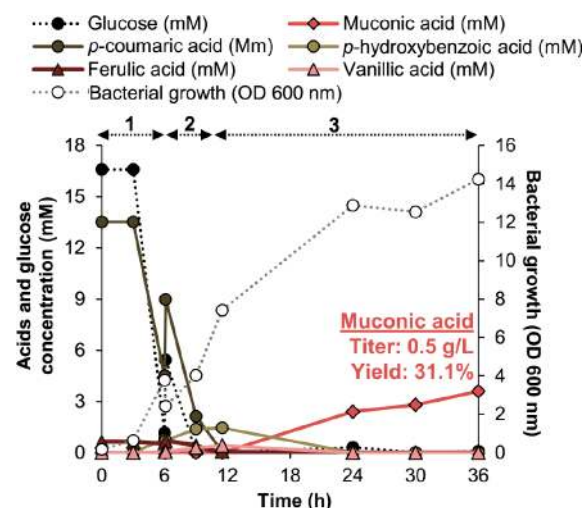


Figure 6. Production of muconic acid by engineered *P. putida* KT2440-CJ103 from BCD lignin liquors. The cultivation and feeding strategy consisted of three different stages (see the [Materials and Methods](#) section), which are highlighted as discontinuous arrows at the top of the graph.

BCD liquor and consumed 3.6 g/L (~22 mM) of *p*-coumaric acid in less than 15 h (Figure 6). Glucose was consumed in parallel with *p*-coumaric acid, and it did not accumulate during the continuous feeding phase. *p*-Hydroxybenzoic acid accumulated slightly at the beginning of the cultivation but was later reassimilated. Final titers of muconic acid reached over 0.5 g/L (~3.6 mM). GPC analysis of the final cultivation medium showed a disappearance of LMW lignin peaks (Figure S6), consistent with the complete utilization of *p*-coumaric and ferulic acids. The metabolic yield, calculated as mole of muconic acid per moles of *p*-coumaric and ferulic acid, and corrected by the dilution factor, was over 30%. In a previous report, the same *P. putida* strain achieved a muconate yield of 67% from a different lignin stream, alkaline-pretreated liquor.¹⁴ Several factors may account for this difference in yield. For example, the BCD liquor in this study contains a lignin concentration of 31 g/L compared to 10 g/L for the alkaline pretreated liquor, and this 3× higher lignin concentration could cause an inhibitory effect on yield.¹⁷ In addition, *P. putida* KT2440-CJ103 overexpresses a promiscuous phenol monooxygenase that might cause unspecific hydroxylations of various intermediates during the catabolism of aromatics, thus decreasing their availability for further conversion to muconic acid.⁵⁹

Overall, the results presented in this paper show that a low-temperature BCD of lignin-rich substrates can generate partially depolymerized lignin streams that are amenable to biological upgrading and the production of value-added compounds.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.7b01818.

Additional figures showing growth comparisons and changes in molecular weight profiles obtained as a response to BCD reaction parameters, microbial treatments, and cultivation conditions (PDF)

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Notes

The authors declare no competing financial interest.

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