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Complete List of Authors:	Liu, Zhi-Hua; Texas A&M University, Shinde, Somnath; University of Tennessee Knoxville, Chemical and Biomolecular Engineering Xie, Shangxian; Texas A&M University, Plant Patho & Microbiol. Hao, Naijia; State Key Laboratory of Fine Chemicals, Dalian University of Technology Lin, Furong; Texas A&M University Li, Man; Texas A&M University Yoo , Chang Geun; Oak Ridge National Laboratory Ragauskas, Arthur; University of Tennessee, Yuan, Joshua; Texas A&M University, Institute for Plant Genomics and Biotech

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Cooperative Valorization of Lignin and Residual Sugar to Polyhydroxyalkanoate (PHA) for Enhanced Yield and Carbon Utilization in Biorefineries

Zhi-Hua Liu [1, 2], Somnath Shinde [3], Shangxian Xie [1, 2], Naijia Hao [3], Furong Lin [1, 2], Man Li [1, 2], Chang Geun Yoo [4], Arthur J. Ragauskas [3, 4, 5, 6], Joshua S. Yuan [1, 2, *]

[1] Synthetic and Systems Biology Innovation Hub (SSBiH), Texas A&M University, College Station, TX, 77843, USA

[2] Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843, USA

[3] Department of Chemical & Biomolecular Engineering, University of Tennessee, Knoxville, TN 37996, USA

[4] Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

[5] Joint Institute for Biological Sciences, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

[6] Center for Renewable Carbon, Department of Forestry, Wildlife, and Fisheries, University of Tennessee Institute of Agriculture, Knoxville, TN 37996, USA

*Corresponding contributor. E-mail: syuan@tamu.edu

Abstract

Lignin valorization is essential for a sustainable and economically viable biorefinery. Despite the recent efforts, it is still unclear how the reactivity of native lignin can be improved by pretreatment in a biorefinery and how different components in biorefinery residue especially residual sugar contribute to bioconversion in biorefineries. In the present study, co-utilization of lignin and limited glucose was first evaluated and proved to facilitate lignin conversion and polyhydroxyalkanoate (PHA) production. A new integrated biorefinery was then designed to cooperatively valorize lignin and residual sugar for improving the PHA yield and utilization efficiency of biorefinery residue. By the design of integrated biorefinery, the glucose and xylose yield were 91% and 73%, respectively, which increased by 18% and 12% compared with that only using sodium hydroxide pretreatment. Approximately 85% of the lignin was dissolved into a liquid stream with the integrated biorefinery, corresponding to an increase of 69% compared with that using only sodium hydroxide pretreatment. More than 70% of the residual sugar was released from the biorefinery residue, producing the soluble lignin stream that contains both lignin and residual sugar for synergistic bioconversion. Bioconversion of soluble lignin stream with *Pseudomonas putida* KT2440 produced 1.5 g/l PHA, representing the record titer of PHA from biorefinery residue. The lignin characterization results from nuclear magnetic resonance and gel-permeation chromatography showed that the integrated process significantly reduced the lignin molecular weight, broke down more β-O-4 and β-5 linkages, and enriched the H-lignin content. Alongside the increased residual sugar concentration, the bioconversion performance of soluble lignin to PHA was significantly improved. Overall, the integrated biorefinery increased the fermentable sugar yield and improved the PHA production from biorefinery waste by cooperative valorization of lignin and residual sugar, which shows potential advantages for biorefinery sustainability.

Keywords: Lignin valorization; Residual sugar; Integrated biorefinery; Mixed enzyme treatment; Polyhydroxyalkanoate; *Pseudomonas putida* KT2440

1 Introduction

Biorefineries produce renewable fuels and chemicals from lignocellulosic biomass (LCB) to reduce fossil energy use and greenhouse gas emissions. [1-3] Conventional biorefineries focus on converting carbohydrates into fuels and chemicals, [4-6] whereas lignin-enriched streams are considered wastes to be utilized in a low-value way. However, lignin is the world's most abundant terrestrial organic polymer after cellulose and thus represents a major potential feedstock for renewable products. [7-9] The success of biorefineries depends on the full utilization of the three components of the plant cell wall (cellulose, hemicellulose, and lignin), and lignin valorization thus offers unique opportunities to improve the profitability of biorefineries. [10-13] Biological lignin conversion was recently established as a potential route for lignin valorization to produce fuels and chemicals. [12, 14-17] Similar to cellulose processing, the macromolecular lignin should also be depolymerized into low-molecular-weight lignin or aromatic monomers for bioconversion by pretreatment and/or lignin-degrading enzymes (Figure 1). Although recent breakthroughs have provided a foundation for efficient lignin bioconversion, several issues still need to be addressed to increase the lignin-based product yield toward commercial relevance.

One of the most intriguing questions lies in how residual sugars in the biorefinery waste could contribute to lignin bioconversion. It should be noted that biorefinery residue is a complex substrate comprising lignin, residual sugar, and other components (Figure 1). The utilization of biorefinery residue must take into considerations of all components. Approximately 10-30% of total sugars are retained in biorefinery residue with most of traditional pretreatments. [12, 18, 19] These unconverted residual sugars are often highly crystallized, intricately intertwined, and embedded with lignin, which could block their further hydrolysis. The unprocessed residual sugars not only prevent the processing of lignin, but also reduce the overall efficiency of LCB conversion. Even if they are further released, the sugar concentration in the waste stream will be too low to be utilized alone. Considering all of these factors, the residual sugar negatively impacts the overall economics and reduces the biorefinery sustainability. [20-22] Despite the importance, no research has

evaluated how the residual sugar in biorefinery residue can be processed via bioconversion. Neither do we understand if and how the cooperative effect between lignin and residual sugar can be achieved in bioconversion.

In addition, lignin is a heterogeneous polymer consisting of phenylpropane units obtained by cross-linking three aromatic monolignols: *p*-coumaryl, coniferyl and sinapyl alcohol (Figure 1). [4, 23] The monolignols are conjugated together via radical coupling reactions to form a variety of chemical bonds, including *β*-O-4, *β*-*β* and *β*-5 linkage. [24, 25] Their chemical properties contribute to the heterogeneity and recalcitrance of lignin, hinder LCB deconstruction, and impede the depolymerization, purification, and processing of lignin. [10, 26, 27] However, pretreatment and/or fractionation present the potential capacity to deconstruct LCB and overcome lignin recalcitrance by modifying the structure of LCB and lignin. These modifications not only affect the yield of fermentable sugar but also impact the changes in lignin reactivity and eventually define the product yield from biorefinery residue. [28, 29] Generally, lignin reactivity is closely related to its molecular-weight, linkages, unit types, and functional groups. [30-32] Previous studies have reported that different technical lignins possess different chemistries and thus reactivities for bioconversion.[1, 15, 29, 33, 34] Furthermore, the pretreatment has the capacity to tune the chemistry and thus increase the processability of lignin for lignin-based products. [29, 35, 36] Despite these progresses, the correlations among biorefinery procedures, lignin structure alterations, and lignin reactivity for bioconversion have not been fully investigated. Most importantly, the conventional biorefinery procedures have not taken into consideration of the balance among hydrolysis efficiency, lignin reactivity, residual sugar utilization, and overall yields of both fermentable sugar and lignin stream.

To address these challenges, the multi-step conversion process was required in a biorefienry to fractionate and convert three components of LCB and hence to make a sustainable biorefinery by co-producing multi-products. Herein an integrated biorefinery was designed to increase the complete utilization of corn stover biomass with the balance of hydrolysis efficiency and lignin yield and reactivity, and improve

the co-valorization of lignin and residual sugar in biorefinery residue. In detail, the optimization of cultivation using lignin as a carbon source was carried out to assess the potential production of lignin-based product, polyhydroxyalkanoate (PHA). Biorefinery strategies were then designed to release the residual sugar and fractionate lignin in biorefinery waste for cooperative valorization of lignin and residual sugar. In these biorefineries, leading pretreatments and mixed enzyme treatment were employed to deconstruct corn stover, yield fermentable sugar platform, improve lignin reactivity, and fractionate residual sugar from waste stream. The cooperative bioconversion of the lignin and the residual sugar in biorefinery waste was assessed by producing PHA using *Pseudomonas putida* KT2440. A mechanistic study of the relationship between lignin chemistry, residual sugar content, and reactivity was then conducted to interpret the outstanding performance of the new integrated biorefinery.

2 Materials and Methods

2.1 Integrated biorefinery strategy

Corn stover biomass was harvested from Comanche, Texas, USA. Corn stover was air-dried to the moisture content of 5% to 10%. For the pretreatment, corn stover was milled and passed through a 10-mesh screen. Integrated biorefinery configurations by employing pretreatment, enzymatic hydrolysis, mixed enzyme treatment, and cultivation were designed to improve the fractionation and utilization efficiency of corn stover biomass (Table 1 and Figure 2). At stage 1 of pretreatment (Cases 1-7), 60 g corn stover (dry weight, dw) was loaded into a 1.0-L screw bottle (VWR International, CAT. No. 10754-820) with 10% (w/w) solid loading and heated by Amsco LG 250 Laboratory Steam Sterilizer (Steris, USA). The residence time of pretreatment in Table 1 presented the maintenance time for reaction. The heating time for pretreatment was about 5 min, while the cooling time was less than 25 min for all pretreatments. The pretreated slurry was then filtrated by Brinell funnel with filter paper (Whatman, cat. no. 1001-110) to separate the pretreated solid from liquid stream. After conditioning, the liquid stream containing lignin produced from stage 1 of Case 1 was used for cultivation. For Cases 2 and 3, pretreated corn stover from stage 1 was treated at stage 2, followed by enzymatic hydrolysis at stage 3. For Cases

4-7, pretreated corn stover from stage 1 was enzymatically hydrolyzed at stage 2, followed by alkaline treatment at stage 3. To further depolymerize lignin polymer and release residual sugar, mixed enzyme treatment of solid residues was conducted at stage 4 of Cases 6-7. After the stage 1 of pretreatment, the slurry was filtered by vacuum filtration to separate the solid from the liquid stream. The solid fraction was post-washed 10 times with ddH_2O. The solid fraction was used for further treatment, while the liquid stream and washing stream were collected for further analysis.

Enzymatic hydrolysis of pretreated solid was conducted by using Cellic CTec2 and HTec2 (kindly provided by Novozymes, USA) at 50 °C and 200 rpm for 168 h in a 0.05 M citrate buffer solution (pH 4.8). Filter paper activity (FPU) and protein content of Cellic CTec2 is 96 FPU/ml and 178±19.9 mg/ml, respectively. The cellobiase activity of *β*-glucosidase is 1270 CBU/ml. The protein content of Cellic HTec 2 is 103 ± 9.6 mg/ml. 10 FPU/g solid of Cellic CTec2 and the volumetric ratio 10:1 of CTec2 and HTec2 was used. At the end of hydrolysis, the hydrolysate was collected for sugar analysis. Sugar conversion was calculated based on the sugar content in pretreated solid.

$$\text{Glucan conversion (\%)} = (\text{Glucose}_{\text{Hydrolysate}} \times 162/180)/\text{Glucan}_{\text{Pretreated solid}} \times 100\% \quad (1)$$

$$\text{Xylan conversion (\%)} = (\text{Xylose}_{\text{Hydrolysate}} \times 132/150)/\text{Xylan}_{\text{Pretreated solid}} \times 100\% \quad (2)$$

For mixed enzyme treatment, the biorefinery residues containing lignin and residual sugar produced from Cases 6-7 were depolymerized by laccases and limited cellulases. Laccases (specific activity 0.53 U/mg) from *Trametes versicolor* were purchased from Sigm-Aldrich (St. Louis, MO, USA). 1-Hydroxybenzotriazole hydrate (HBT) was used as the mediator. Laccase loading of 15 mg/g substrate and the ratio 3:5 of laccase and HBT were used. Cellic CTec2 loading of 5 FPU/g substrate and the volumetric ratio 10:1 of Cellic CTec2 and HTec2 were employed to release residual sugar. Mixed enzyme treatment was conducted at 50 °C and 200 rpm for 72 h in a 40 mM phosphate buffer (pH 7.0) in a 250-mL Erlenmeyer flask with a breathable sealing film. The pH 7.0 is the optimal one for laccase in the present study.

2.2 Bacterial strains and seed medium preparation

Pseudomonas putida KT2440 obtained from Dennis C. Gross' lab at Texas A&M

University was employed to produce PHA using lignin stream as carbon sources. The strain was stored on the Luria-Bertani plate containing 1.5% agar. For seed culture, a single colony on Luria-Bertani plate was inoculated into 10 ml Luria-Bertani broth and grown at 28 °C and 200 rpm. When stationary phase was reached, 1 ml culture solution was transferred into 100 ml M9 mineral medium supplemented with 20 g/l glucose and 1.0 g/l NH_4Cl, and cultivated at 28 °C, 200 rpm for 24 h [37, 38]. Cell growth was monitored using the optical densities at 600 nm.

Chemicals used for medium preparation were purchased from Sigma-Aldrich (St Louis, MO). 100 ml seed medium contains 20 g/l glucose, 1.0 g/l NH_4Cl, 10 ml 10X Basal salts, and 1 ml 100X Mg/Ca/B1/Goodies mixture. Basal salts were prepared by dissolving 30 g KH_2PO_4, 60 g $NaHPO_4$, and 5 g NaCl into 1 l using ddH_2O. For the preparation of 100X Mg/Ca/B1/Goodies mixture [39], 500 ml stock salt solution, 3.009 g $MgSO_4$, and 25 ml 1% $FeSO_4$ was mixed to make 1.0 l concentrated Goodies. 250 ml concentrated Goodies were then mixed well with 200 ml 1 M $MgSO_4$, 10 ml 1 M $CaCl_2$, and 10 ml 10 mM thiamine to make 1.0 l 100X Mg/Ca/B1/Goodies mixture. Stock salt solution was composed of 22.94 g/l $MgCl_2 \cdot 6H_2O$, 2.0 g/l $CaCO_3$, 4.5 g/l $FeSO_4 \cdot 7H_2O$, 1.44 g/l $ZnSO_4 \cdot 7H_2O$, 0.85 g/l $MnSO_4 \cdot H_2O$, 0.25 g/l $CuSO_4 \cdot 5H_2O$, 0.24 g/l $CoCl_2 \cdot 6H_2O$, 0.06 g/l H_3BO_3, and 51.3 ml HCl.

2.3 Polyhydroxyalkanoate (PHA) cultivation using lignin medium

The soluble lignin stream from each biorefinery was collected and used to prepare the medium for producing polyhydroxyalkanoate (PHA) by *P. putida* KT2440. Cultivation strategies and conditions were provided in Electronic supplemental information A (ESI A). For medium preparation, the lignin rich liquid stream produced was carefully adjusted to pH 7.0 using 1.0 M sulfuric acid, and then mixed well with 10 ml 10X Basal salts and 1 ml 100X Mg/Ca/B1/Goodies mixture to make 100 ml medium. The soluble lignin stream after filtration using a 0.22-μm vacuum-driven filter system (Genesee Scientific) was diluted to different soluble substrate concentrations (SSC) using sterilized ddH_2O (ESI A). *P. putida* KT2440 cell pellets were collected by centrifuging the seed culture at 4000 rpm for 10 min and used for inoculation. Cultivation was conducted in a 250-mL Erlenmeyer flask at pH

7.0, 28°C, and 200 rpm for 18 h. The time course experiments were conducted at pH 7.0, 28°C, and 200 rpm for 6 h, 12 h and 18 h, respectively.

2.4 Cell dry weight and PHA extraction

After cultivation, the cell biomass was harvested by centrifugation at 10,000 rpm for 10 min under 4 °C, washed two times by ddH_2O, and then lyophilized at -50 °C for 24 h (Labconco Corporation, USA). Cell dry weight in cultivation was defined as the dry weight of cell biomass per liter, g/l. PHA content in dried cell was determined by the gravimetric method.[38] In detail, the lyophilized cell biomass was mixed with chloroform at a ratio of 7:1 (mg/ml) in a screw-cap glass vial and incubated at 60 °C with a shaking speed of 180 rpm for 24 h. After cooling, 2 ml ddH_2O was added and the mixture was centrifuged at 2500 rpm for 10 min to separate the liquid from cell debris. The chloroform organic phase containing soluble polyesters was filtered using 0.45 μm polytetrafluorethylene membranes (VWR international), and then concentrated to approximately 1 ml using N_2 flux. 10-fold volume of pre-chilled methanol was added to precipitate the PHA. The PHA precipitant was separated by centrifugation at 2500 rpm for 10 min under 4 °C. To purify the PHA, the precipitant was re-dissolved in chloroform, and the above process was repeated twice. The PHA pellets were collected and dried in a vacuum desiccator.

2.5 Characterizations of the fractionated lignins

2D ^{1}H-^{13}C HSQC nuclear magnetic resonance (NMR) spectra of the fractionated lignin were determined using a Varian 500 MHz NMR spectrometer. 30 mg lignin sample was dissolved in 0.6 ml dimethylsulfoxide (DMSO)-d_6. The gradient-enhanced HSQC with adiabatic pulses (gHSQCAD) mode was employed using the following parameters: 1.0 pulse delay, 32 scans, 1024 data points for ^{1}H, and 256 increments for ^{13}C. The central solvent peak (δC/δH=39.5/2.49 ppm) was used for reference.

The hydroxyl groups in fractionated lignin were analyzed by ^{31}P NMR. 20-25 mg lignin sample was dissolved in 0.7 ml stock solution of pyridine/$CDCl_3$ (v/v = 1.6/1) containing 1.25 mg/ml $Cr(acac)_3$ and 2.5 mg/ml internal standard *endo*-N-hydroxy-5-norbene-2,3-dicarboxylic acid imide. 70 μl 2-chloro-4,4,5,5-

tetramethyl-1,3,2-dioxaphospholane was added to the vial and mixed well. ^{31}P NMR spectra were performed on a Varian 500 MHz spectrometer using an inverse-gated decoupling pulse sequence, 90° pulse angle, 1.2 s acquisition time, 25 s pulse delay, and 64 scans.

Gel-permeation chromatography (GPC) was employed to determine the molecular weight of the fractionated lignin in each biorefinery according to published methods. [29, 36]

2.6 Composition analysis method

Composition analysis of corn stover was performed according to the Laboratory Analysis Protocol (LAP) of the National Renewable Energy Laboratory (NREL), Golden, CO, USA.[40, 41] Sugar analysis was carried on an Ultimate 3000 HPLC System (Thermo Scientific, USA) equipped with an Aminex HPX-87P carbohydrate analysis column (Bio-Rad Laboratories, CA) and a refractive index detector. HPLC grade water was used as the mobile phase at a flow rate of 0.6 ml/min. Sugar yield in the whole fractionation process was calculated as follows:

$$\text{Glucose yield (\%)} = [\text{Glucose}_{\text{Liquid of fractionation}} + \text{GO}_{\text{Liquid of fractionation}} \times (180/162) + \text{Glucose}_{\text{Hydrolysate}}] / [\text{Glucan}_{\text{Feedstock}} \times (180/162)] \quad (3)$$

$$\text{Xylose yield (\%)} = [\text{Xylose}_{\text{Liquid of fractionation}} + \text{XO}_{\text{Liquid of fractionation}} \times (150/132) + \text{Xylose}_{\text{Hydrolysate}}] / [\text{Xylan}_{\text{Feedstock}} \times (150/132)] \quad (4)$$

$$\text{Residual sugar (\%)} = \text{Sugar}_{\text{Solid residue}} / \text{Sugar}_{\text{Feedstock}} \quad (5)$$

Where GO represents the glucose oligomers and XO represents the xylose oligomers. Residual sugar presents the glucan retained in solid residue after each fractionation.

Lignin concentration in the liquid stream was determined according to the LAP of the NREL, Golden, CO, USA.[40, 41]. Soluble substrate concentration (SSC) of the soluble lignin stream was determined by a gravimetric method. In detail, 10 ml liquid stream was added into a pre-weighted glass vial and dried in a 105 °C oven for 24 h. Error bars in the Figures represented the standard deviation of the duplicates.

3 Results and Discussion

3.1 Optimization of PHA cultivation using lignin as a carbon source

PHAs comprise a large class of polyesters and are synthesized by numerous bacteria as an intracellular carbon and energy reserve compounds.[42, 43] Due to their biocompatibility and biodegradability, PHAs are being extensively used in biomaterial, biomedical, and other fields. Lignin valorization to produce PHA could improve the overall competitiveness of biorefineries. [26, 44, 45] Alkaline pretreated lignin has been considered as a potential carbon source for ligninolytic bacteria due to its low molecular weight and high reactivity. [1, 11, 15] Our previous study reported the improvements in lignin bioconversion by combinatorial pretreatment.[35] However, this previous study focused only on the development of pretreatment technology to increase the lignin utilization and did not consider how to improve the release of different components of biorefinery residue, in particular, the residual sugar. Neither the previous study revealed how better fractionated lignin and residual sugar in biorefineries can synergistically improve lignin conversion and valorization. Additionally, the cultivation technology and process of PHA production using lignin stream remains underdeveloped in a biorefinery. Herein the effects of regulatory nutrients on PHA production by *P. putida* KT2440 were evaluated by using alkaline (NaOH)-pretreated lignin (Case 1) (ESI A, B, C, and D).

The accumulation of PHA in bacteria is influenced by growth conditions. Most bacteria require a threshold concentration of nitrogen source for PHA synthesis from an excess carbon source. [46, 47] ESI B shows that the cell growth and PHA formation were dependent on the carbon to nitrogen (C:N) ratio. *P. putida* KT2440 produced a higher cell biomass with a lower C:N ratio, whereas the PHA content depended on the limited nitrogen source. The highest concentration, content, and yield of PHA were obtained at a C:N ratio of 10 g/l:0.5 g/l, which was thus employed as the optimal C:N ratio. ESI C showed a direct correlation between inoculum OD and cell growth ability on lignin medium. As expected, as the inoculum OD increased *P. putida* KT2440 consumed more lignin to produce cell biomass and increase PHA concentration. Theoretically, a high inoculum OD may increase the adaptability of the strain to the lignin stream by reducing inhibitory effects of the degradation products generated from pretreatment. [48, 49] Besides that, a high inoculum OD itself should contribute to

increased cell biomass. However, considering a high inoculum OD will require more nutrients for the seed culture, an optimal OD of 1.0 was used for cultivation optimization. The bioavailability of trace elements such as Fe^{2+} dramatically affects the cell growth and the PHA accumulation in bacteria. [50] The highest PHA concentration, content, and yield were produced with the addition of 2.5 mg/l $FeSO_4$ (ESI D), which was used as the optimal concentration.

A high substrate concentration should be advantageous because it increases the titer of the target product while lowering energy input along with capital costs. ESI E shows that the PHA production depended on soluble substrate concentrations (SSC) in medium. The cell dry weight increased with the increase of SSC, while PHA content, PHA yield, and lignin consumption decreased. The concentration of degradation products such as weak acids, furans, and phenolic compounds in the lignin stream from pretreatment increased and some of these products might have inhibitory effects on the strain ability and thus PHA accumulation.[48, 51] Additionally, the rheology behavior of the medium could change at high SSC, [19, 52] which may influence strain growth. To overcome these issues, two fed-batch cultivation modes were evaluated (ESI F and ESI A). Compared to batch mode, the cell dry weight in fed-batch modes 1 and 2 increased by 33% and 51%, respectively, while the PHA concentration increased 61% and 123%. Fed-batch cultivation maintained a lower SSC by feeding new medium, which may reduce the potential inhibitory effects and rheological changes of the medium and thereby improved PHA accumulation. Overall, systematic optimization was performed using alkaline-pretreated lignin in terms of the C:N ratio, inoculum concentration, iron level, and fed-batch processing. The final optimized process achieved a PHA titer of 0.65 g/l, representing an increase of 0.6 times.

3.2 Potential synergy evaluation of lignin and limited glucose

Biorefinery concept emphasizes the full utilization of the three main components in LCB to deliver cost-effective biorefineries. Such utilization is hindered by low deconsturction performance, poor lignin reactivity, and undeveloped residual sugar utilization. In particular, the carbon utilization efficieny in a biorefinery significantly

depends on the processing of biorefnery residue. Therefore, new biorefinery need to be designed to simultaneously increase sugar yield, enhance lignin reactivity, and improve residual sugar utilization in biorefinery residues.

It is unclear if residual sugars will be synergistically utilized with lignin to promote bioconversion or not. Before designing a biorefinery, co-cultivation of lignin and limited glucose for PHA production had first been conducted to validate the potential synergy of lignin and residual sugar (Figure 3 and ESI A). Compared to the control without glucose, the cell dry weight increased from 3.4 to 4.9 g/l with the addition of 2 to 15 g/l glucose. As expected, the PHA concentration and yield also increased with increasing glucose concentration. Interestingly, glucose was nearly consumed at 18-hour cultivation with the addition of 2 and 5 g/l glucose. However, the pattern of glucose consumption differed when 10 or 15 g/l glucose was added. Correspondingly, lignin consumption reached the maximum value with the addition of 5 g/l glucose. Glucose concentrations higher than 10 g/l cannot be consumed completely, probably due to the short cultivation time. Previous studies have reported that consuming glucose to accumulate PHA may produce more acids, which in turn inhibit strain growth. [53, 54] However, the pH value of the lignin medium with 10 and 15 g/l glucose added was maintained at approximately 7.0-7.3 during the cultivation; and this pH range should not inhibit strain growth. PHA yield based on total carbon source in cultivation was also higher at lower glucose compared with that in other conditions (ESI G**).** Previous study reported the co-cultivation of aromatic compounds with limited glucose to produce lipids using *Rhodococcus jostii* RHA1. They found that in aromatic medium containing lower levels of glucose (below 5 g/l), adaptive-evolved *R. jostii* RHA1 consumed more aromatics and improved lipid synthesis. When the glucose concentration was beyond 20 g/l, adaption evolution necessarily enhanced lipid production.[55] Overall, these results highlighted that a limited amount of glucose in lignin medium promoted the lignin usage and PHA production using *P. putida* KT2440.

Based on the above results, biorefinery configurations were designed by employing an integrated fractionation and conversion process and considering the

co-processing of lignin and residual sugar (Table 1). In the integrated biorefinery, pretreatment employed dilute sulfuric acid (Cases 4-7) at stage 1 to deconstruct and purify corn stover by dissolving hemicellulose, depolymerizing lignin, and removing non-structural components. Following stage 1, enzymatic hydrolysis at stage 2 was performed to release sugars, whereas alkaline treatment using sodium hydroxide at stage 3 depolymerized and fractionated lignin. To improve the lignin reactivity and release residual sugar, mixed enzyme treatment was performed at stage 4 of Cases 6 and 7 for further depolymerizing both lignin and residual sugar, as the biorefinery residue contains lignin polymer and remaining lignin-carbohydrate complex (LCC) structure. Pretreatment using sodium hydroxide (Case 1) was conducted as a control.

3.3 Integrated biorefinery improves the fermentable sugar yield

The enzymatic hydrolysis efficiency was evaluated to ensure the improvement of fermentable sugar yield in a biorefinery. Figure 4 shows that compared with Case 1, Cases 2 and 3 increased glucan and xylan conversion by 12-17% and 11-16% during enzymatic hydrolysis, respectively. The composition analysis results (ESI H) showed that the use of dilute sulfuric acid at stage 1 in Cases 2 and 3 removed most of the hemicellulose, especially xylan from corn stover, whereas the use of sodium hydroxide at stage 2 fractionated most of the lignin. The removal rates of xylan and lignin in pretreated corn stover from Cases 2 and 3 were greater than 70% (ESI I), resulting in the exposure of more accessible surface area of cellulose and thus enhanced enzymatic hydrolysis. These results are consistent with those of previous studies in which the most effective pretreatment strategy was to selectively remove the main components and expose the cellulose fibers. [56-62] Cases 5 and 7, which featured a long residence time at stage 1, also increased glucan and xylan conversion as compared to Case 1, whereas Cases 4 and 6, which had short residence times at stage 1, produced approximately the same glucan and xylan conversions. The improved hydrolysis performance in Cases 5 and 7 was due to xylan removal and lignin deconstruction using dilute sulfuric acid at stage 1, as confirmed by the composition analysis results (ESI H) and previous studies. [56, 63] These results indicate that the integrated biorefinery significantly improved the hydrolysis performance

compared with Case 1.

More than 20% of the total sugars were retained in solid residues after enzymatic hydrolysis at stage 2 of Case 1. The residual sugars are intricately intertwined and embedded with lignin, hindering their further hydrolysis. The release of these residual sugars could extend the hydrolysis time and cost for a high dosage of enzymes. In addition, the residual sugars show the potential to promote the formation of condensed lignin during the further processing of lignin and thus prevent lignin utilization. However, it is essential to release the residual sugars as biorefinery sustainability depends on the full utilization of the three main components of LCB.

To further release residual sugars and improve the lignin reactivity, mixed enzyme treatment of the lignin-rich biorefinery residue was performed at stage 4 of Cases 6 and 7 using laccases and cellulases. Figure 5 shows the glucose and xylose yield in each process. Compared with Case 1, Cases 2-5 produced 8-12% higher glucose yield and 6% higher xylose yield. Cases 3, 5, and 7, which featured long residence times at stage 1, produced 3.6-9.2% higher glucose yields and 3.0-6.1% higher xylose yields than Cases 2, 4, and 6. Pretreatment with dilute sulfuric acid and a long residence time at stage 1 removed more hemicellulose, as confirmed by composition analysis. These changes should facilitate enzymatic hydrolysis, lignin fractionation, and residual sugar release. Thus, the results indicated that an integrated biorefinery, especially with a long residence time at stage 1, can improve the fermentable sugar yield from corn stover.

Cases 6 and 7 produced higher glucose and xylose yields. ESI J shows the mass balance in the whole biorefinery for Case 7 compared with Case 1. The glucose and xylose yields produced from Case 7 were 91% and 73%, respectively, representing increases of 18% and 12% compared with Case 1. The increased sugar yield resulted from the improved lignin fractionation and greater deconstruction of residual sugars in the alkaline treatment of the solid residue at stage 3 of Case 7 (ESI H, I and J). The mixed enzyme treatment at stage 4 further released the residual sugars via the synergy of the laccases and cellulases. As a result, the integrated biorefinery Case 7 ultimately improved fermentable sugar yield from corn stover.

3.4 Integrated biorefinery enhances lignin dissolution

Currently, biorefineries that employ pretreatment and hydrolysis to deconstruct LCB will yield lignin-rich streams via two modes: (i) extract the sugars and retain most of the lignin in the solid residue; (ii) deconstruct LCB to fractionate lignin into the liquid stream. Soluble lignin in water generally has low molecular weight and high reactivity and is suitable to be used as carbon source for bioconversion.

Figure 6A shows that lignin distributions in the solid and liquid stream were dependent on biorefinery design. In Cases 2 and 3, pretreatment using dilute sulfuric acid at stage 1 deconstructed the LCC matrix and the lignin from the inner part of the corn stover. Alkaline treatment using sodium hydroxide at stage 2 deconstructed the acidic pretreated solid and fractionated the lignin into the liquid stream, finally producing 32% and 50% higher lignin yield in the liquid stream than that in Case 1, respectively. In Cases 4 and 5, following pretreatment at stage 1, enzymatic hydrolysis at stage 2 released sugars and enriched lignin content in the solid residue, whereas alkaline treatment using sodium hydroxide at stage 3 fractionated the lignin into the liquid stream to produce 42% and 56% higher lignin yields, respectively. Interestingly, Cases 6 and 7 produced 79.1% and 85.2% lignin yield in the liquid stream, respectively, whereas only 17% and 13% of the lignin remained in the solid residue. The lignin yields from Cases 6 and 7 were 10.8% and 8.0% higher than those from Cases 4 and 5, and 57.3% and 69.3% higher than those from Case 1, respectively. These results suggested that mixed enzyme treatment can further depolymerize and dissolve the retained lignin. Taken together, these results highlight that the integrated biorefinery Case 7 significantly improved lignin fractionation performance.

3.5 Integrated biorefinery increases the residual sugar release

Figure 6B shows the residual sugars in the solid stream from the final stage of each biorefinery. More than 20% of the total sugars were retained in the solid residue produced from Case 1. However, Cases 2-5 reduced the residual sugar by 10-45% as compared to Case 1. Interestingly, Cases 6 and 7 retained only 8.7% and 6.2% of residual sugar, respectively, corresponding to decreases of 57.8% and 70.0%

compared with Case 1 (Figure 6B and ESI J). This result may be attributable to further deconstruction of the solid residue by the alkaline treatment in stage 3 and the mixed enzyme treatment of the solid residue at stage 4.

Figure 6C shows the glucose concentration in the soluble lignin stream. Case 1 had the lowest glucose concentration (3.6 g/l). It is worth noting that the glucose released from Case 1 should be the easily dissolved sugar in corn stover. Interestingly, the glucose concentration increased by 11-94% in Cases 2-5 compared with Case 1, whereas Cases 6 and 7 produced 3.4 and 3.8 times higher glucose concentration, respectively. The results further confirmed that mixed enzyme treatment in Cases 6 and 7 resulted in the release of more residual sugar and thus increased the glucose concentration in the soluble lignin stream. Notably, the glucose concentration was very low in the liquid steam, and thus the separation of glucose at such low concentrations would be costly. However, as mentioned previously for the co-cultivation of lignin and limited glucose (Figure 3), the residual sugar in the lignin stream could promote the bioconversion of lignin. As a result, the integrated biorefinery, especially Case 7, dissolved more residual sugar into the liquid stream, which should improve the overall economics of biorefinery.

3.6 Increased PHA production from the lignin stream in integrated biorefinery

The soluble lignin stream produced from each biorefinery was used as a carbon source by *P. putida* KT2440 to produce PHA. Figure 7 shows the cultivation time course of PHA production for each biorefinery. Compared to values obtained using lignin 1, the cell dry weight after 18-hour cultivation obviously increased by 25-104% while the PHA concentration increased from 0.34 to 1.09 g/l using lignins 2-7. The PHA content and yield obtained using lignins 2-7 increased by 13-58% and 11-99%, respectively. In addition, biorefinery Cases 3, 5, and 7, which featured a long residence time at stage 1, produced a higher cell dry weight, PHA concentration, and yield than Cases 2, 4, and 6. These results indicated an improvement of lignin reactivity via the modification of lignin structure by integrated biorefineries. In addition, Cases 6 and 7 released more residual sugar in the liquid stream compared with Case 1, and thus facilitated cell growth and PHA production, confirming the

co-cultivation results for lignin and limited glucose (Figure 3). Previous study reported the PHA production in *P. putida* KT2440 grown on different carbon sources and they found that only cells grown on the fatty acid dodecanoate accumulated high amounts of PHAs (17.9%) without nitrogen limitation, which was about 10-fold higher than that from cells grown on glucose. [64] Huijberts et al. reported that the PHA content reached 16.9% and 27.6% of the cell dry mass using glucose and deaconate as carbon source, respectively, by *P. putida* KT2442.[65] Davis et al. developed high cell density cultivation of bacteria with a fed batch bioprocess for PHA production. Using glucose as the carbon source for 21 h followed by the supply of nonanoic acid as a PHA precursor, *P. putida* KT2440 accumulated 32% of cell dry weight.[66] As confirmed by previous studies, PHA synthesis by bacteria is significantly dependent on the expression levels of relevant genes, the types of carbon source used, the cultivation mode employed, and growth condition developed.[16, 38, 64, 66]

To further improve PHA production, fed-batch mode was conducted with the addition of 30 g/l SCC at 0 h and 30 g/l SSC at 18 h (Figure 8). Compared to that obtained using lignin 1, the cell dry weight increased by 21-63% when lignins 2-5 were used and by 56% and 88% when lignins 6 and 7 were used, respectively. The PHA concentration produced from lignin 1 was only 0.55 g/l, suggesting low lignin conversion at high SSC. Compared to lignin 1, the PHA concentrations produced from lignins 2 and 3 were 1.6 and 2.0 times higher, respectively, whereas the concentrations produced from lignins 4 and 5 were 1.5 and 2.0 times higher. The highest PHA concentration, 1.54 g/l, was produced from lignin 7 and was 2.8 times higher than that obtained from lignin 1. Lignins 2-7 produced 20-51% higher PHA content and 13-52% higher PHA yield than lignin 1. Moreover, more lignin was consumed by *P. putida* KT2440 for lignin 2 to 7 compared to lignin 1. Linger et al. reported a PHA yield of approximately 0.25 g/l and PHA content of 32% cell dry weight using alkaline-pretreated lignin as the sole carbon source for *P. putida* KT2440 under shake-flask conditions. [1] They reported that *P. putida* KT2440 can convert mixed-model carbon sources (glucose, *p*-coumaric acid, ferulic acid, and acetate) to produce a PHA yield of 0.15 g/l.[1] When aromatic compounds were used as

carbon sources by *Pandoraea sp.* ISTKB, Kumarr et al. reported biomass and PHA accumulation of 0.52 g/l and 0.25 g/l from 4-hydroxybenzoic acid, 0.42 g/l and 0.17 g/l from *p*-coumaric acid, and approximately 0.09 g/l and 0.02 g/l from Kraft lignin, respectively. [67] These results suggest that the PHA cultivation performance depends greatly on the lignin type, the lignin reactivity and the strains employed. Overall, the integrated biorefinery, especially Case 7, indeed released more residual sugar, improved lignin fractionation and reactivity, eventually produced the best cell growth and PHA yield in fed-batch cultivation mode at high SSC and thus promoted the bioconversion of lignin-rich residue.

3.7 Improved lignin reactivity for bioconversion in a biorefinery

Similar to cellulose processing in bioconversion, lignin polymer needs to be depolymerized to generate low-molecular-weight lignin or aromatic monomers. The molecular weight of lignin is one of the most crucial factors determining the lignin reactivity. [67, 68] As shown in Table 2, the biorefinery options obviously impacted the molecular weight of lignin. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) decreased in the following order: corn stover native lignin (CSNL) > lignin 1 > lignin 3 > lignin 5 > lignin 7, suggesting that all biorefineries, especially Case 7, significantly depolymerized the lignin polymer and produced lower-molecular-weight lignin. As lignin with low molecular weight is more likely to be converted by lignin-degrading bacteria, the integrated biorefinery improved lignin reactivity for bioconversion. [69] The lignin produced from each biorefinery exhibited a higher polydispersity index (PDI), implying a much broader molecular weight distribution, as compared to CSNL. Zhao et al. reported that the PDI of the lignin varied from 8.9 to 12.7 after different biological treatments, representing a significant increase compared to CSNL (PDI 4.6). [68] However, Wei et al. found that the molecular weight of Kraft lignin decreased after the O_2-pretreatment, while the PDI dropped from 4.6 to 1.9. [34] The possible reason for these results may be attributable to differences in the lignin types and treatment methods employed. Notably, lignin 7 exhibited a lower PDI than lignin 5 and the other lignins, likely due to depolymerization of higher-molecular-weight lignin by the mixed enzyme

treatment at stage 4 of Case 7. After cultivation, M_n and M_w increased compared to the values before cultivation. This increase may have occurred because *P. putida* KT2440 had deconstructed the higher molecular weight lignin and/or consumed the lower molecular weight lignin, consistent with previous studies. [70] The obvious decrease in PDI after cultivation suggests that the lignin became uniform, as confirmed by the trends of M_n and M_w. As a result, the integrated biorefinery, especially Case 7, enabled the depolymerization of the lignin, reduced its molecular weight, and thus facilitated its conversion to PHA.

Figure 9 shows the subunits, hydroxycinnamates, and linkages of CSNL and fractionated lignin before and after cultivation. Compared to CSNL, all biorefineries enriched H-lignin content. Previous studies have confirmed that H-lignin is more readily consumed by lignin-degrading bacteria. [44, 71] As expected, H-lignin in lignin 7 decreased by 18% after cultivation, consistent with the ability of H-lignin to promote lignin bioconversion. The hydroxycinnamate content increased in the following order: lignins 1, 3, 5, and 7, suggesting that the integrated biorefinery Case 7 produced more ferulate (FA) and *p*-coumaric acid (*p*CA) in the fractionated lignin. More than 78% of *p*CA and 15% of FA in lignins 3, 5, and 7 were consumed during cultivation. Previous studies have also confirmed that FA and *p*CA are readily consumed by *P. putida* KT2440 to produce cell biomass and PHA. [1] As a result, the consumption of hydroxycinnamates by *P. putida* KT2440 likely contributed to the increased PHA concentration. In addition, this increased consumption may have been encouraged by the synergistic utilization of lignin and residual sugar, in which the residual sugar released by the mixed enzyme treatment in Case 7 actually promoted the utilization of hydroxycinnamates by *P. putida* KT2440.

All biorefineries significantly decreased the amount of *β*-O-4 linkages in fractionated lignin compared with that in CSNL. Compared with lignin 5, the mixed enzyme treatment at stage 4 further decreased the *β*-O-4 and *β*-*β* linkages in lignin 7 by 10.5% and 50%, respectively. These decreases correlated with the lower molecular weight of lignin 7 and indicated improved lignin fractionation, which could have contributed to the enhanced lignin reactivity. After cultivation, the amount of *β*-O-4

linkage slightly increased. This increase could result from the degradation of other linkages by bacteria in the cultivation. Overall, the integrated biorefinery, especially Case 7, enriched the H-lignin, FA, and *p*CA content and broke down more *β*-O-4 linkages, thus improving lignin reactivity and facilitating lignin bioconversion.

Further information regarding the changes in the functional groups of lignin was provided by ^{31}P NMR (Table 3). Compared with CSNL, all biorefineries significantly increased phenolic OH and COOH groups, indicating enhanced degradation and solubility of lignin and thus improved accessibility of lignin to ligninolytic bacteria. Lignins 3, 5, and 7 had higher phenolic OH group content than lignin 1, suggesting more deploymerization of lignin. After cultivation, the content of aliphatic OH, guaiacyl OH and COOH groups increased, whereas the C5-substituted OH group content decreased. Previous studies have reported that aliphatic OH, guaiacyl OH, and C-5 condensed OH groups in O_2-Kraft lignin decreased after cultivation for lipid production by *Rhodococcus opacus* DSM 1069 [34]. These results might be attributable to differences in stains and lignin types employed. Furthermore, as confirmed by previous studies, the reactivity of lignin depends on the fractionation method, and different strains also have different mechanisms of lignin degradation and metabolism. [16, 44, 72] The increased COOH group during cultivation may result from further degradation of fractionated lignin by *P. putida* KT2440. As a result, the integrated biorefinery, especially Case 7, deconstructed the lignin more significantly, as indicated by the increase in hydroxyl groups, and thus enhanced the lignin reactivity for bioconversion.

3.8 Improved biorefinery sustainability by cooperative valorization of lignin and residual sugar

Overall, integrated biorefineries were developed by cooperative valorization of lignin and residual sugar to improve biorefinery sustainability. The new integrated biorefinery configuration (Case 7) successively employed the dilute acid pretreatment, hydrolysis of the pretreated solid, and sodium hydroxide and mixed enzyme treatment of the lignin-rich residues. Pretreatment using dilute sulfuric acid at stage 1 deconstructed the corn stover by dissolving hemicellulose, thereby improving the

hydrolysis performance (stage 2). Alkaline treatment using sodium hydroxide at stage 3 further deconstructed the lignin-rich solid residue from hydrolysis to dissolve the lignin and expose the residual sugar to enzymes. The subsequent mixed enzyme treatment at stage 4 depolymerized lignin using laccases and facilitated the release of residual sugar. As a result, the integrated biorefinery increased glucose and xylose yields by 18% and 12%, respectively, as compared to Case 1, indicating improved fermentable sugar yield (Figure 5 and ESI J).

The integrated biorefinery also increased the lignin dissolution, enhanced the lignin reactivity for bioconversion, improved the utilization efficiency of residual sugar, and reduced the generation of biorefinery residue. By using this process (Case 7), the lignin yield in the liquid stream was 85.2%, which was 69.3% higher than that in Case 1. Only 6.2% of the residual sugar was retained in the solid residue, a decrease of 70.0% compared with Case 1 (Figure 6 and ESI J). Using this lignin stream as the carbon sources (Figure 8), the cell dry weight and PHA concentration were 9.1 g/l and 1.5 g/l, respectively, representing increases of 1.8 and 2.8 times compared with Case 1. These results demonstrated that cooperative valorization of lignin and residual sugar improved the PHA production (Figures 3, 8 and 9). Compared with Case 1, the add-on operations of post-treatments of the lignin-rich residue may increase the capital cost and reduce the profitability of the biorefinery. To improve biorefinery sustainability, the process optimization needs to be further evaluated to reduce the chemical and enzyme usage, to simplify the fractionation process, and to increase the lignin conversion and product yield. Overall, the new integrated biorefinery with cooperative valorization of lignin and residual sugar significantly improved fermentable sugar yield, lignin reactivity, and residual sugar utilization and finally facilitated the lignin bioconversion and the PHA production.

4 Conclusions

An integrated biorefinery was evaluated by cooperative valorization of lignin and residual sugar to make the potential of corn stover biorefinery a reality. By using this process, glucose and xylose yields increased by 18% and 12% while lignin yield in the liquid stream was 69.3% higher compared with that using Case 1. The residual

sugar decreased by more than 70.0%. Using the lignin stream as carbon sources, the highest cell dry weight and PHA concentration was 9.1 g/l and 1.5 g/l, respectively, which were 1.8 and 2.8 times as that using lignin 1. Chemical analysis revealed that this increase resulted from better lignin fractionation, more lignin dissolution, and increased residual sugar in waste stream. As a result, the integrated biorefinery increased the PHA yield by cooperative valorization of lignin and residual sugar and thus showed the potential to improve the carbon utilization efficiency.

Competing interests

The authors declare that they have no competing interests.

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Table 1 Biorefinery strategies by using sodium hydroxide pretreatment (Case 1), combinatorial pretreatment (Cases 2 and 3), and an integrated fractionation approach (Cases 4-7) for improving the utilization efficiency of corn stover biomass

Case	Stage 1		Stage 2		Stage 3		Stage 4
	Chemicals	Conditions	Chemicals	Conditions	Chemicals	Conditions	
1	1% NaOH	120°C, 60 min	EH	10 FPU/g solid, 168 h	-	-	-
2	1% H_2SO_4	120°C, 30 min	1% NaOH	120°C, 60 min	EH	10 FPU/g solid, 168 h	-
3	1% H_2SO_4	120°C, 60 min	1% NaOH	120°C, 30 min	EH	10 FPU/g solid, 168 h	-
4	1% H_2SO_4	120°C, 30 min	EH	10 FPU/g solid, 168 h	1% NaOH	120°C, 60 min	-
5	1% H_2SO_4	120°C, 60 min	EH	10 FPU/g solid, 168 h	1% NaOH	120°C, 60 min	-
6	1% H_2SO_4	120°C, 30 min	EH	10 FPU/g solid, 168 h	1% NaOH	120°C, 60 min	Mixed enzyme treatment
7	1% H_2SO_4	120°C, 60 min	EH	10 FPU/g solid, 168 h	1% NaOH	120°C, 60 min	Mixed enzyme treatment

* EH presents enzymatic hydrolysis; % is calculated based on the weight percent, w/w. Chemical loading used in the present study was based on the total weight of corn stover.

Table 2 Molecular weight distributions of the fractionated lignin produced from each biorefinery before and after cultivation.

Samples	Before cultivation			After cultivation		
	M_n	M_w	PDI	M_n	M_w	PDI
CSNL	1371	6241	4.5			
Lignin 1	435	2669	6.1	370	1116	3
Lignin 3	400	2463	6.1	603	2172	3.6
Lignin 5	311	2260	7.3	487	2385	4.9
Lignin 7	267	1396	5.2	477	2833	5.9

* CSNL represents corn stover native lignin; M_n represents number-average molecular weight; M_w represents weight-average molecular weight; PDI represents polydispersity index; Lignin 1 represents the fractionated lignin produced by Case 1 in Table 1. After cultivation represents the lignin samples collected at the end of cultivation

Table 3 Contents of hydroxyl groups in fractionated lignin produced from each biorefinery before and after cultivation

Samples	Aliphatic OH (mmol/g)	Phenolic OH (mmol/g)			COOH (mmol/g)	Aliphatic OH (mmol/g)	Phenolic OH (mmol/g)			COOH (mmol/g)
		C_5-substituted	Guaiacyl	*p*-hydroxyphenyl			C_5-substituted	Guaiacyl	*p*-hydroxyphenyl	
	Before cultivation					After cultivation				
CSNL	2.21	0.11	0.32	0.45	0.35					
Lignin 1	2.80	0.40	0.51	0.33	0.99	1.35	0.19	0.29	0.16	0.82
Lignin 3	1.82	0.62	0.57	0.40	0.88	2.18	0.52	0.60	0.36	0.97
Lignin 5	1.43	0.57	0.47	0.36	0.72	1.97	0.61	0.60	0.41	0.90
Lignin 7	1.58	0.85	0.52	0.42	0.80	1.85	0.53	0.57	0.43	0.96

* CSNL represents corn stover native lignin; After cultivation represents the lignin samples collected at the end of cultivation; Lignin 1 represents the fractionated lignin produced by Cases 1 in Table 1

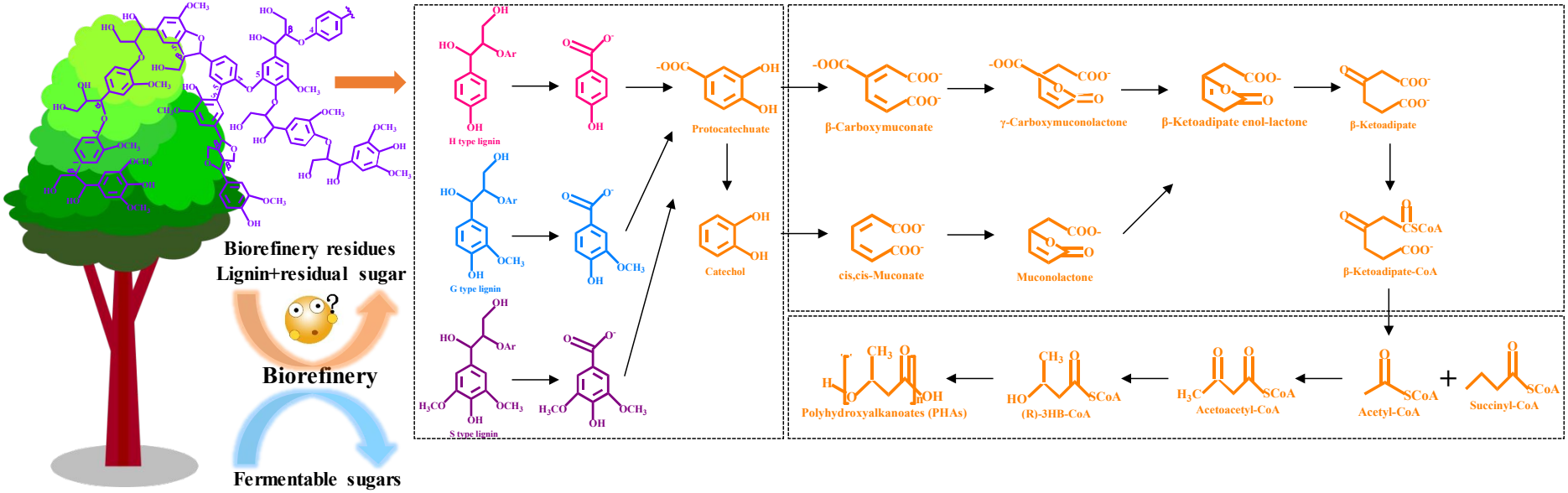
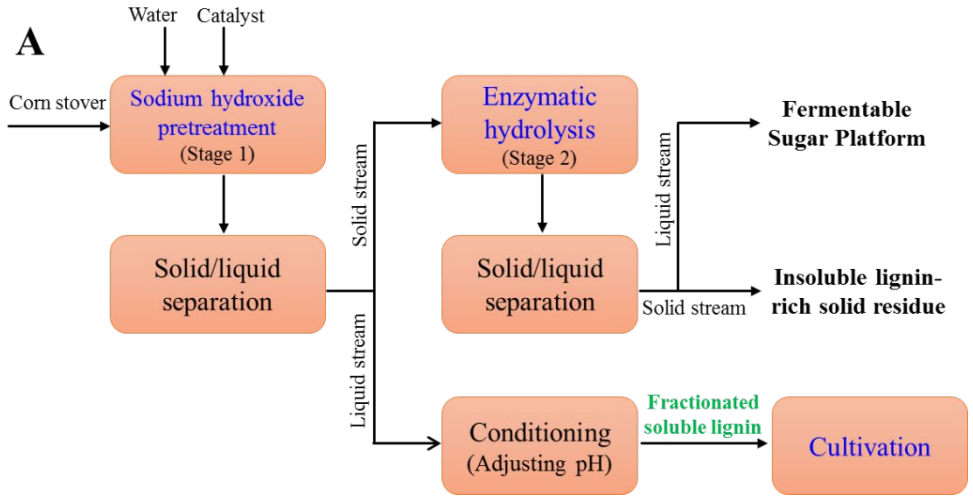


Figure 1 Biological valorization of biorefinery residues using ligninolytic bacteria in an integrated biorefinery


A
Water
Catalyst
Corn stover
Sodium hydroxide pretreatment (Stage 1)
Solid/liquid separation
Solid stream
Liquid stream
Enzymatic hydrolysis (Stage 2)
Solid/liquid separation
Liquid stream
Solid stream
Fermentable Sugar Platform
Insoluble lignin-rich solid residue
Conditioning (Adjusting pH)
Fractionated soluble lignin
Cultivation

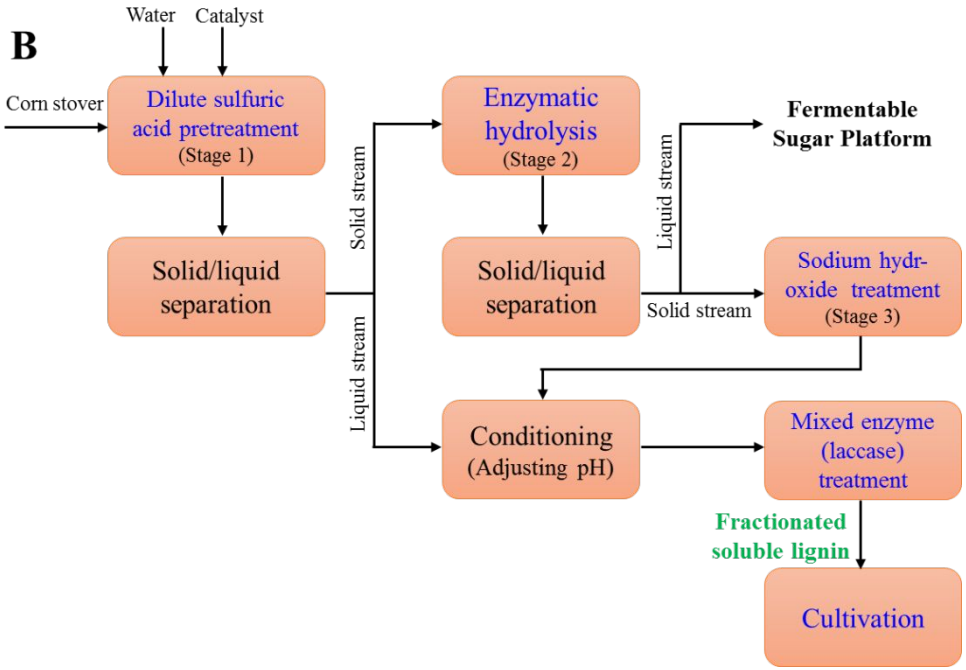


Figure 2 (A) Biorefinery using sodium hydroxide pretreatment and (B) integrated biorefinery design incorporated with lignin valorization for improving the utilization efficiency of corn stover biomass

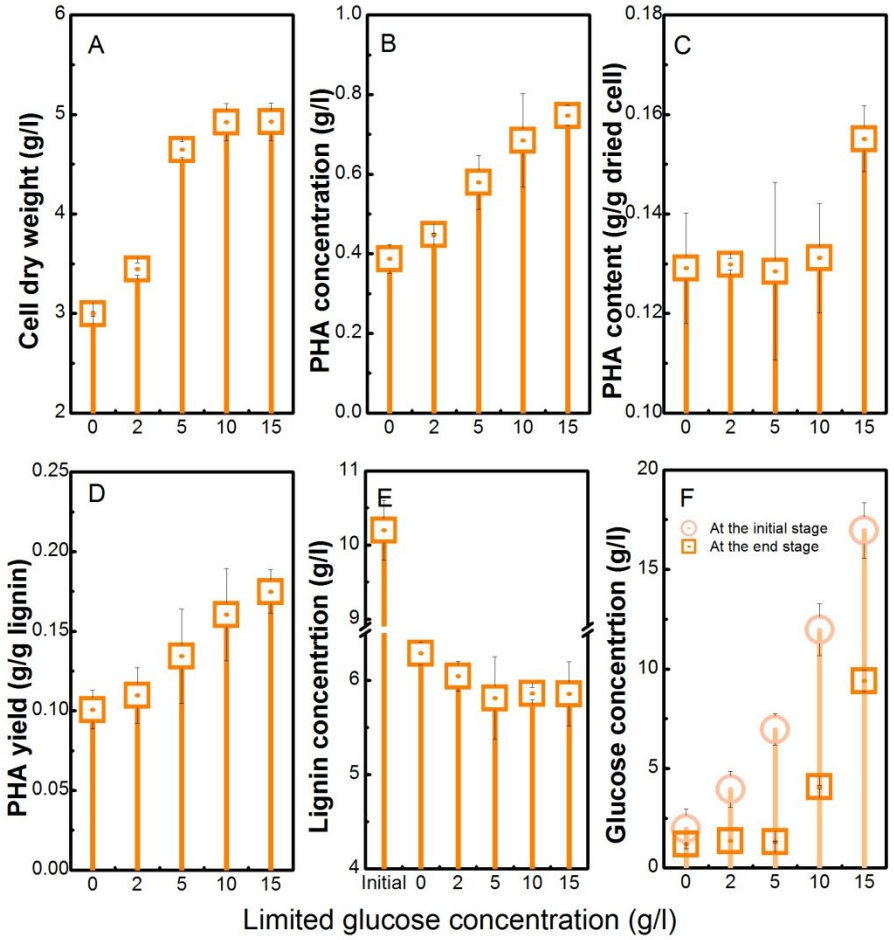

A
Cell dry weight (g/l)
B
PHA concentration (g/l)
C
PHA content (g/g dried cell)
D
PHA yield (g/g lignin)
E
Lignin concentrtion (g/l)
F
Glucose concentrtion (g/l)
At the initial stage
At the end stage
Initial
Limited glucose concentration (g/l)

Figure 3 Polyhydroxyalkanoate (PHA) cultivation using the soluble lignin stream with the addition of limited glucose as carbon sources by *P. putida* KT2440. Initial represents the lignin concentration at the initial stage of cultivation. Cultivation was conducted at pH 7.0, 28°C, and 200 rpm for 18 h.

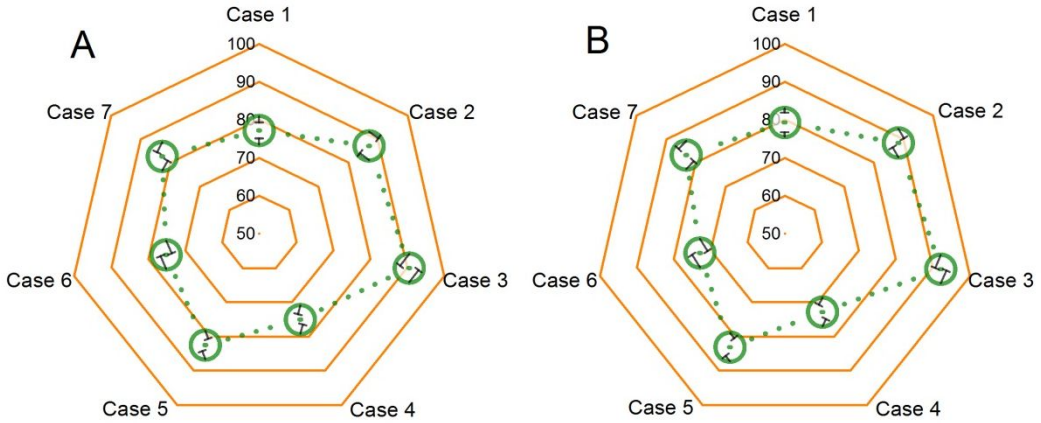


Figure 4 (A) Glucan and (B) xylan conversion in enzymatic hydrolysis of pretreated corn stover produced from each biorefinery. Biorefinery strategies are shown in Table 1

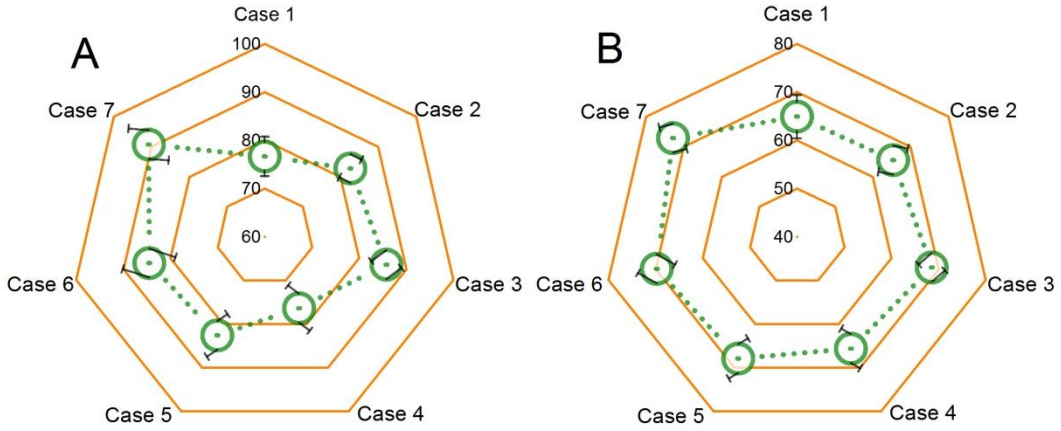


Figure 5 (A) Glucose and (B) xylose yield in the whole fractionation process of corn stover biomass. Biorefinery strategies are shown in Table 1

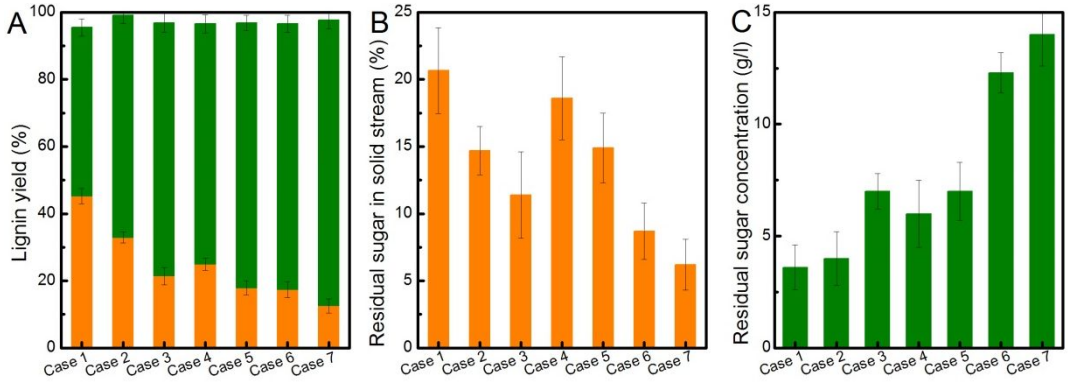


Figure 6 (A) Lignin yield in the solid (orange) and liquid (green) streams, (B) residual sugar in the solid stream, and (C) residual sugar concentration in the soluble lignin stream from the final stage of each biorefinery. Biorefinery strategies are shown in Table 1

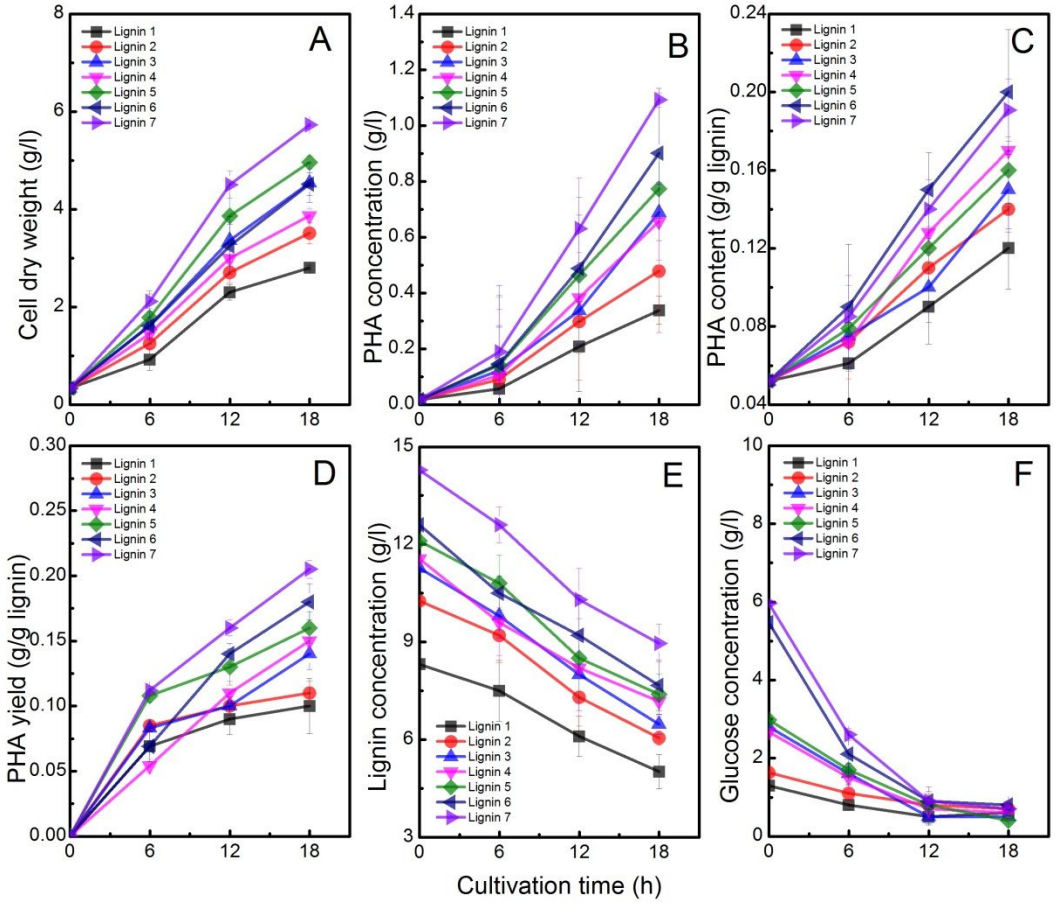


Figure 7 Cultivation time course for polyhydroxyalkanoate (PHA) production using the soluble lignin stream as carbon sources from each biorefinery by *P. putida* KT2440. Lignin 1 represents the fractionated lignin produced by Cases 1 in Table 1.

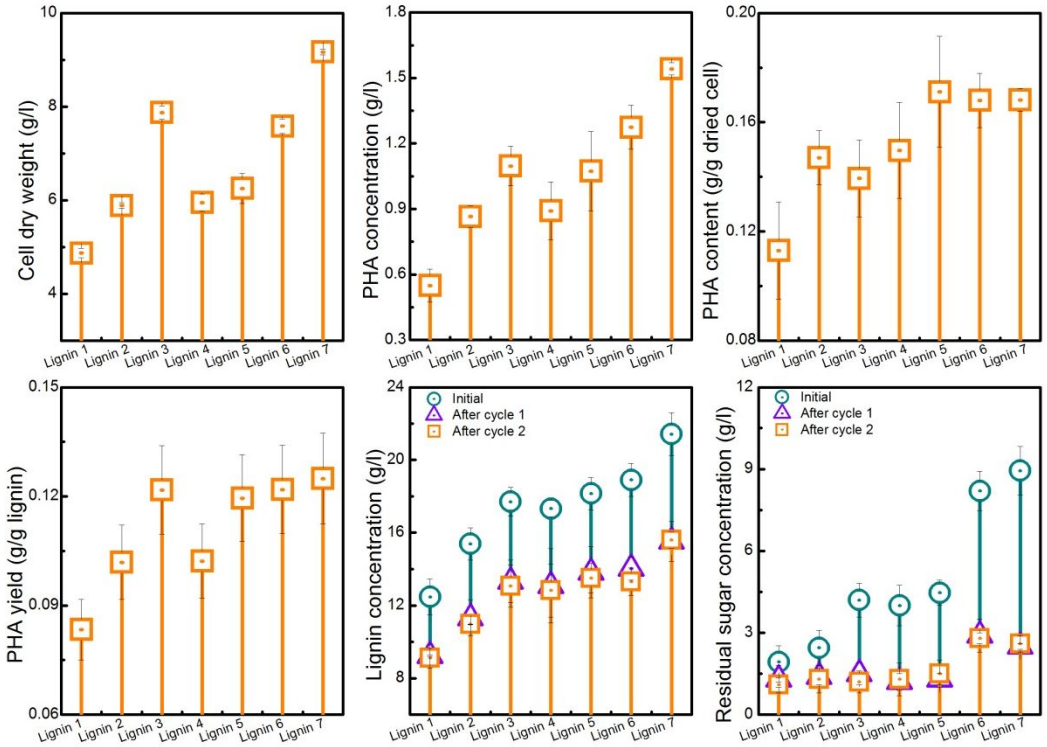


Figure 8 Polyhydroxyalkanoate (PHA) production by fed-batch cultivation of the soluble lignin stream produced from each biorefinery. Lignin 1 represents the fractionated lignin produced by Cases 1 in Table 1. Initial represents the lignin concentration at the initial stage of cultivation. After cycle 1 represents after fed-batch cultivation cycle 1.

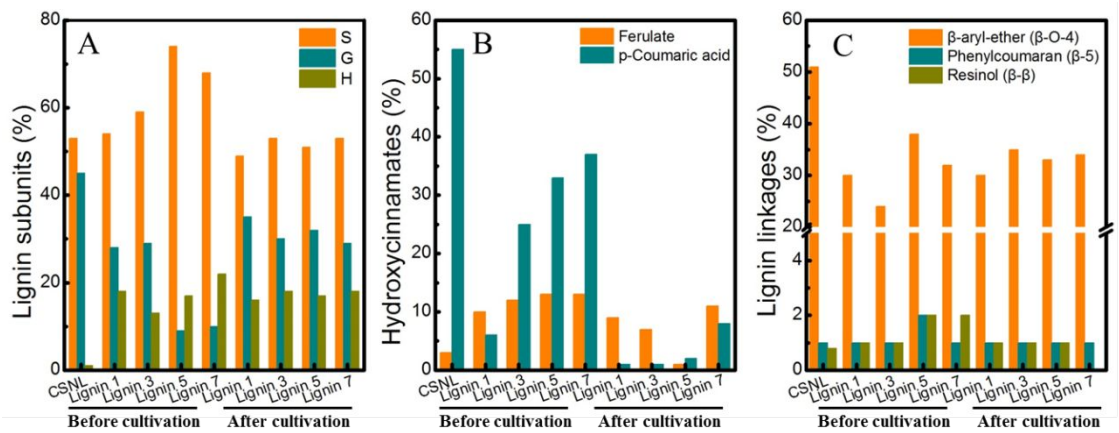


Figure 9 The lignin subunits, hydroxycinnamates and linkages of the fractionated lignin produced from each biorefinery. CSNL represents corn stover native lignin. Lignin 1 represents the fractionated lignin produced by Cases 1 in Table 1. After cultivation represents the lignin samples collected at the end of cultivation using *P. putida* KT2440