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Synergistic Enzymatic and Microbial Lignin Conversion

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Abstract

The utilization of lignin for fungible fuels and chemicals represents one of the most imminent challenges in modern biorefinery. However, bioconversion of lignin is highly challenging due to its recalcitrant nature as a phenolic heteropolymer. This study addressed the challenges by revealing the chemical and biological mechanisms for synergistic lignin degradation by a bacterial and enzymatic system, which significantly improved lignin consumption, cell growth and lipid yield. The *Rhodococcus opacus* cell growth increased exponentially in response to the level of laccase treatment, indicating the synergy between laccase and bacterial cells in lignin degradation. Other treatments like iron and hydrogen peroxide showed limited impact on cell growth. Chemical analysis of lignin under various treatments further confirmed the synergy between laccase and cells at chemical level. ^{31}P nuclear magnetic resonance (NMR) suggested that laccase, *R. opacus* cell and Fenton reaction reagents promoted the degradation of different types of lignin functional groups, elucidating the chemical basis for the synergistic effects. ^{31}P NMR further revealed that laccase treatment had the most significant impact for degrading the abundant chemical groups. The results were further confirmed by the molecular weight analysis and lignin quantification by Prussian Blue assay. The cell-laccase fermentation led to a 17-fold increase of lipid production. Overall, the study indicated that laccase and *R. opacus* can synergize to degrade lignin efficiently, likely through rapid utilization of monomers generated by laccase to promote reaction toward depolymerization. The study provided a potential path for more efficient lignin conversion and development of consolidated lignin conversion.

1. Introduction

As a main constituent of lignocellulosic biomass, lignin is the second most abundant biopolymer in the terrestrial plants.[1-4] Despite the abundance, the utilization of lignin has been particularly challenging due to its recalcitrance nature.[3] In nature, lignin is a complex aromatic heteropolymer composed of phenylpropane units cross-linked via a variety of chemically stable bonds to confer recalcitrance of plant cell wall.[5, 6] The utilization of the lignin in the biorefinery waste streams as feedstock for biofuels and bioproducts represents a unique opportunity to improve cost-effectiveness, carbon and energy efficiency of biorefineries.[3, 6] Bioconversion recently emerged as a potentially effective strategy for lignin processing.[7-9] Despite the lignin recalcitrance, many nature biomass utilization systems including white rot fungi and termites evolved capacity to degrade and utilize lignin.[6, 7, 10, 11] Among the different systems, *Rhodococcus opacus* bacteria have recently been established with the capacity to convert lignin into lipid,[8, 9, 12, 13] yet the industrial application of the platform is hindered by the low yield of target compounds. The challenge can be addressed by integrating the enzymatic and chemical depolymerization with bacterial conversion to achieve a simultaneous depolymerization and fermentation (SDF) process. It is thus critical to understand if and how the enzymatic depolymerization can be synergized with bacterial conversion. Moreover, it is also important to achieve in-depth understanding of the scientific principles, chemical, and biological mechanisms involved in lignin degradation in an enzyme-cell combined system. In this article, we aim to address these scientific questions by investigating the potential synergistic enzymatic and microbial conversion of lignin.

Lignin depolymerization is the key step to enable the bioconversion of the phenolic heteropolymer, in a way similar to saccharification in cellulose conversion.[4, 6] However, unlike cellulose with the β-1,4-glucosidic link as the chemical bond and glucose as the monomer, lignin

contains diverse aromatic monomers and various types of chemical bonds or interunit linkage such as β-O-4, α-O-4/β-5 (phenylcoumaran), β-β (resinol), dibenzodioxocin, 4-O-5, and 5-5.[14] The requirement of redox reaction to cleave these bonds further complicated the deploymerization process. Previous studies have well characterized three lignin depolymerization system including laccase-based, peroxidase-based and Fenton reaction-based systems.[6, 7, 15] Model lignin degradation organisms like white rot fungi have all three lignin depolymerization systems, yet bacteria like *Rhodococci* generally have one or two of these systems, with a lower efficiency as compared to the white rot fungi and termites. In this research, we will reverse design the mechanisms in natural biomass utilization systems by combining the laccase and Fenton reaction system to evaluate how the combination of enzyme, chemical, and cell treatment will synergize the lignin depolymerization and promote lignin utilization by bacteria.

Laccase is chosen for the enzyme-cell system due to its capacity to self-generate radicals, which distinguished laccase-based lignin depolymerization from Fenton reaction and peroxidase (Figure S1). For both Fenton reaction and peroxidase, the reactions will depend on the radicals that are often generated and recycled by other enzymatic systems. Laccase provided a potentially self-sufficient system for lignin degradation. However, the unique mechanism imposed a fundamental scientific question: how would laccase and cell synergize during the lignin utilization? In other words, considering that both polymerization and depolymerization activities exist for laccase, can the cells consume aromatic compounds generated from the lignin oxidation to promote the reaction toward depolymerization? In this article, we addressed these questions by demonstrating the enzyme-cell synergy on lignin degradation at both biological and chemical levels.

2. Materials and supplies

2.1. Lignin, strain and culture medium

Kraft lignin (catalog#370959) and laccase from *Trametes versicolor* (catalog# 51639) was purchased from Sigma-Aldrich (St. Louis, MO, USA). *Rhodococcus opacus* PD630 (DSM-44193) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig Germany). The *Rhodococcus* Minimal (RM) medium for lignin fermentation was modified from previous publications and described with details in Supplementary Material.[16]

2.2. Lignin fermentation

The seed culture was prepared by inoculating a single colony of *R. opacus* PD630 into 20 mL Tryptic Soy Broth (TSB) medium, and cultivated at 28 °C to OD_{600} 1.5. The cultured strain were harvested by centrifuging and washed twice with equal volume of RM medium without lignin. The washed cells were resuspended in 20 mL RM medium without lignin. 1 mL of resuspended cells was added to 100 mL of lignin fermentation medium. For laccase and Fenton reaction treatments, the enzyme and chemicals were added at the same time of strain inoculation. The fermentation was carried out in 250 mL flask capped with eight layers of cheesecloth and one layer of kraft paper at a shaking speed of 200 rpm to keep the fluent air circulation, which helps to maintain the oxygen in solution. The fermentation was carried out at 28 °C for 144 h.

2.3. Lignin concentration analysis by Prussian Blue assay

The lignin was completely dissolved by adjusting the pH to 12.5 and the concentration was measured by mixing the dissolved lignin with Prussian Blue reagents and reading the absorbance at 700 nm with UV/vis spectrophotometer.[17, 18] The detailed method was also presented in Supplementary Material.

2.4. Cell concentration determination

To determine the number of living cell, 100 μL of fermentation culture was serial diluted and plated on Tryptic Soy agar plate. The numbers of colonies were counted from the plates and converted to colony forming unit/mL (CFU/mL).

2.5. Total lipid extraction

Total lipid of the bacterial *R. Opacus* PD630 after lignin fermentation was extracted with chloroform-methanol using modified Folch lipid extraction method.[19] The detailed lipid extraction protocol was described in Supplementary Material.

2.6. Lignin characterization by gel permeation chromatography

The lignin gel permeation chromatography (GPC) analysis was performed after acetylation on a PSS-Polymer Standards Service (Warwick, RI, USA) GPC SECurity 1200 system, featuring Agilent HPLC 1200 components equipped with four Waters Styragel columns (HR1, HR2, HR4 and HR6) and an UV detector (270 nm) as described in our previous publications.[9]

2.7. Lignin structure analysis by nuclear magnetic resonance (NMR)

All the NMR experiments were carried out at a Bruker Avance III 400-MHz NMR spectrometer. Heteronuclear single quantum coherence (HSQC) and ^{31}P NMR measurements of lignin samples were carried out following literature methods, as described in Supplementary material.[20, 21]

3. Result and Discussion

3.1. Laccase treatment significantly promote the cell growth on lignin

Laccase treatment significantly promoted the cell growth of *R. opacus* PD630 on lignin. As shown in Fig. 1A, the laccase treatment significantly promoted the PD630 cell growth on kraft lignin as the sole carbon source. The CFU (colony-forming unit) of *R. opacus* PD630 cells after six days of growth increases exponentially in response to the activity of laccase in the enzyme-cell system. The cell growth was very slow without laccase treatment due to the relatively low initial inoculation. However, with the increased concentration of laccase, the CFU after six days of cultivation can achieve an exponential increase to 1.85×10^7/mL at 2 U/mL of laccase treatment. Considering that kraft lignin is mostly insoluble, laccase treatment might lead to the depolymerization and solubilization of lignin to provide more carbon source for *R. opacus* PD630 cells, which in turn promoted the cell growth. The result clearly indicated the synergy between laccase and cells, and such synergy was confirmed by further chemical analysis.

3.2. Fenton reaction has less synergy with cell system

As aforementioned, Fenton reaction is another type of widely studied lignin degradation mechanisms.[7] Fenton reaction is believed to play an important role for lignin deoplymerization in termite gut and wood-degrading fungus.[7, 22-24] We therefore examined if synergistic effect can be achieved for laccase treatment and Fenton reaction during bacterial lignin conversion. The classic model for Fenton reaction is that ferrous iron is oxidized by hydrogen peroxide to ferric iron, forming hydroxyl radical which could attack lignin. Considering that some iron ions may lead to the precipitation of lignin, the chelated iron ions using EDTA were also used in the Fenton reaction experiment. Taken these into considerations, different types of iron ions and H_2O_2 were combined with laccase to study their effect on lignin conversion by *R. opacus* PD630. As compared to the laccase plus cell treatment, different types of iron ions have limited effects in

promoting cell growth when combined with laccase plus *R. opacus* PD630 cell (Figure 1B). While all types of iron-laccase combination treatment increased the CFU at six days after fermentation, the impacts on cell growth is marginal as compared to that of laccase plus cell treatment. In addition, there was no significant difference observed in cell growth among different types of iron used in the treatment. Even though some synergy can be found for Fenton reaction, laccase, and cell system, the results indicated that laccase by itself has a much stronger synergy with *R. opacus* PD630 for cell growth on lignin as compared to that of Fenton reaction.

Furthermore, study of Fenton reaction alone with cells demonstrated a much more limited synergistic effect for cell growth (H_2O_2 + Fe^{2+}) as compared to that of laccase and cells. To reveal that if this limited effects of Fenton reaction on lignin conversion was caused by the radical elimination by catalase, we detected the catalase activities under different conditions. The results indicated that *R. opacus* PD630 did not express significant catalase during lignin fermentation (Figure S2). In nature, Fenton reaction depends on the quinone and other mediators, as well as the enzyme system to regenerate these mediators, to eventually reduce the ferric iron to ferrous iron for the sustainable reaction. In addition, iron catalyzed radicals are necessary for effective Fenton reaction. The relatively limited synergistic effects between laccase and Fenton reaction might be due to the lack of a sustainable radical or chemical mediator regeneration system. Moreover, it seems that the radicals generated by laccase didn't contribute significantly to the Fenton reaction. The synergy as shown by cell growth was further confirmed by the chemical analysis of lignin structure during the fermentation.

3.3. Changes of lignin molecular weight during cell-laccase co-fermentation

Comprehensive lignin characterization was carried out to determine the key factors contributing to synergistic effects between laccase and *R. opacus* PD630 cells. GPC analysis was

carried out to evaluate the changes of molecular weight under different fermentation conditions.[25] Five different conditions were compared as shown in Table 1 and Table 2, and these conditions included reference lignin sample without any bacterial or laccase treatment (no cell, Treatment I), lignin after the bacterial fermentation (cell only, Treatment II), lignin after bacterial fermentation with laccase treatment (cell + laccase, Treatment III), lignin after bacterial fermentation with laccase and ferrous iron treatment (cell + laccase + Fe^{2+}, Treatment IV) , and lignin after bacterial fermentation with laccase and Fenton reaction treatment (cell + laccase + Fe^{2+} + H_2O_2, Treatment V). These five fermentation conditions represented different combination of bacterial, enzymatic and chemical treatments, including PD630 only, PD630 with laccase enzyme, and PD630 with both laccase and Fenton reaction.

As shown in Table 1, PD630 plus laccase treatment (Treatment III) led to the most significant increase in molecular weight as compared to the cell-only (Treatment II) and no-cell reference (Treatment I). The number average molecular weight (M_n) for cell-only fermentation (Treatment II) was not significantly different from no-cell reference (Treatment I), indicating that *R. opacus* PD630 has limited capacity for lignin depolymerization. However, a significant increase in M_n and M_w was observed when adding laccase into the fermentation (Treatment III vs. Treatment II). No significant differences in M_n were found among Treatment III, IV and V, indicating limited synergy of Fenton reaction and laccase on lignin degradation. In addition, even though polydispersity index and weight average molecular weight (M_w) had relatively larger variation, the trends were the same as that of M_n, where *R. opacus* PD630 and laccase together led to the most significant increase of molecular weight. The slightly decrease of M_w for samples with Treatment V (cell, laccase, Fe^{2+}, and H_2O_2) was probably due to the effects of Fenton reaction to promote the degradation of some high molecular weight lignin. The results was

consistent with the slightly increased cell growth upon laccase, Fe^{2+} and H_2O_2 treatment as well as the functional group degradation data in the later part of the study.

The overall increase of lignin molecular weight upon cell and laccase treatment could be due to two reasons. On one side, laccase treatment might have significantly improved the usage of low molecular weight lignin, when combined with *R. opacus* PD630 fermentation. On the other side, the increase of M_w and M_n might also be due to the lignin polymerization caused by laccase. The laccase and cell synergy might have various impacts on different types of lignin. For low molecular weight lignin, the synergy between cell and laccase might have promoted the lignin degradation and subsequent consumption of degraded products. The rapid consumption of low molecular weight lignin could promote cell growth and leave high molecular weight lignin in the system. The overall effects will be both less low molecular weight lignin and potential re-arrangement of high molecular weight lignin structure. Nevertheless, an additional control experiment indicated that GPC analysis of laccase-only treated lignin showed a similar elution profile of gel permeation chromatograph as compared to the untreated control lignin (Figure S3), suggesting no considerable difference in the molecular weights of total lignin after laccase-only treatment. In addition, the increased cell growth and lignin consumption, as well as the subsequent NMR analysis all suggested that laccase and *R. opacus* PD630 synergy could have led to more efficient consumption of low molecular weight lignin. The consumption of low molecular weight lignin could result in relatively more high molecular weight lignin in the fermentation system, and the composition changes well correlated with the increased M_n and M_w.

3.4. Laccase and *R. opacus* PD630 cell synergized lignin degradation as revealed by functional group contents

In complementary to GPC analysis, ^{31}P NMR spectra of the lignin samples from the aforementioned five treatments were analyzed to evaluate how laccase, cell, and Fenton reaction impacted the lignin degradation (Table 2). The changes of hydroxyl group contents in lignin was determined by ^{31}P NMR spectra of the lignin derivatized with TMDP (2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane) as described in the Materials and supplies section. TMDP reacts with the hydroxyl groups of lignin to form phosphitylated derivatives that can be detected by ^{31}P NMR.[20] In particular, the aliphatic hydroxyl, phenolic, and carboxylic acids groups of lignin were identified based on characteristic chemical shifts[25] and then quantified by peak integration as shown in Table 2.

R. opacus PD630 cell, laccase, and Fenton reaction could all lead to lignin degradation as indicated by decreased functional group contents from ^{31}P NMR analysis. As shown in Table 2, *R. opacus* PD630 cell-only treatment (Treatment II vs. I) resulted in a substantial decrease of β-5 condensed phenolic OH and carboxylic OH groups, while other OH functional groups barely degraded. The results suggested that *R. opacus* PD630 alone could cause lignin structural changes, yet the lignin degradation capacity is very limited in terms of the types of chemical bonds and the degree of functional group reduction. With the addition of laccase into fermentation using *R. opacus* PD630 (Treatment III vs. II), the lignin had a further significant decrease in the contents of aliphatic OH and guaiacyl phenolic OH groups, the two most abundant hydroxyl groups in kraft lignin. For example, the degradation of aliphatic OH group was increased from 2.5% (Treatment II, cell-only) to 21% (Treatment III, cell + laccase). Moreover, the addition of Fenton reaction agents (Treatment IV and V) led to no further decrease of these functional groups (except for carboxylic OH group at the treatment V). As compared to the untreated control lignin, a slight decrease of aliphatic and guaiacyl phenolic OH groups was

observed in laccase-only treated lignin, as revealed by ^{31}P NMR analysis (Figure S4). Lund and Ragauskas also observed a decrease in the content of condensed and guaiacyl phenolic groups upon treatment of lignin with laccase alone.[26] In summary, the results revealed that the enhanced lignin hydroxyl groups decrease was mainly due to laccase combined *R. opacus* PD630 cell fermentation, suggesting the synergy of laccase with *R. opacus* PD630 cell treatment for the lignin degradation.

The two dimensional (2D) ^{1}H-^{13}C HSQC was another method used to understand the mechanism of lignin degradation. HSQC spectra of lignin were measured for the aforementioned treatments. Signals from guaiacyl (G) and *p*-hydroxyphenyl (H) units were readily observed in the aromatic regions (Fig. S5), confirming the lignin as a typical G/H type softwood lignin with G as the major monolignol units. Semi-quantitative analysis of HSQC spectra showed that the cell plus laccase (Treatment III) treated lignin had a slightly decrease of H unit (~4 %) and resinol subunits (~1.6 %) when compared to the control lignin sample, suggesting preferable degradation of these lignin structures in the treated lignin. In addition, the cell plus laccase treatment also resulted in a decrease of ~4.7 % in the relative abundance of end group cinnamyl alcohol. The reduction of cinnamyl alcohol can partially contribute to the decrease of aliphatic hydroxyl group content, which further supported the ^{31}P NMR results that the lignin from the cell plus laccase treatment (Treatment III) had the lowest aliphatic hydroxyl group content.

Overall, the results revealed synergistic lignin degradation by *R. opacus* PD630 cell, laccase, and Fenton reaction. Among the different treatments, the fermentation with laccase and cell together led to the significant degradation of the most abundant functional groups in lignin such as aliphatic OH and guaiacyl phenolic OH. The results indicated that laccase and *R. opacus* PD630 could synergistically promote lignin degradation, which was consistent with the increased

cell growth (Figure 1), lignin molecular weight upon laccase treatment (Table 1), and Prussian Blue assay (Figure 2A). As shown in Figure 2A, lignin degradation was significantly improved when fermented with both laccase and *R. opacus* PD630 cell.

3.5. Simultaneous Depolymerization and Fermentation (SDF) significantly improved the lipid production

It has been established that *R. opacus* PD630 fermentation of lignin produces lipid, including TAG (triacylglycerol) as the biodiesel precursor.[8] The SDF by the cell-laccase system not only promoted the cell growth and lignin degradation, but also led to significantly increased lipid production by 17 fold to 145 mg/L on insoluble kraft lignin (Figure 3B). The increase of lipid content suggests that efficiently degraded lignin monomers provide *R. opacus* cells with sufficient carbon source to accumulate lipid.

3.6. Potential mechanisms for synergy between laccase and cells

The NMR and lignin quantification analysis revealed synergistic degradation of lignin by both *R. opacus* PD630 cells and laccase with the following important features. First, the degradation of lignin by *R. opacus* PD630, laccase, and Fenton reaction were functional group specific. Unlike cellulose degradation with glucose as the single subunit, lignin is heteropolymer with diverse aromatic monomer units.[5, 6] These different monomers were further connected by various types of chemical bonds and linkages.[2] The changes of various OH functional groups as shown in Table 2 indicated that *R. opacus* PD630, laccase, and Fenton reaction each could favorably degrade different chemical structures, which might be the foundation for synergistic effects for cell-enzyme fermentation. Second, based on ^{31}P NMR analysis, laccase played an essential role for lignin degradation, because the enzyme specifically degraded both aliphatic OH and guaiacyl phenolic OH groups, the two types of most abundant hydroxyl groups in lignin.[27, 28]

Third, the strong synergy of laccase and *R. opacus* cells on lignin degradation indicated that the efficient consumption of degradation products generated by laccase promoted the depolymerization direction of the reaction. Four aspects of data were consistent to support the conclusion including the degradation of different hydroxyl functional groups, the significant improvement of cell growth upon laccase treatment, the increases in molecular weight, and the overall higher lignin consumption by the cell-laccase treatment. These features could be exploited for designing efficient conversion or modification of lignin toward various functional products.

4. Conclusion

Overall, the study unveiled an effective synergy between *R. opacus* PD630 cells and laccase for lignin degradation at both chemical processing and cell growth level. As compared to Fenton reaction, laccase treatment in the study has a much more significant impact on lignin degradation. The synergistic lignin degradation for laccase and cells was revealed at both chemical and biological level in terms of selective degradation of different functional groups and increased cell growth. The mechanistic study could enable a SDF process to significantly improve lignin utilization and cell growth. Laccase thus not only serves as a good enzyme for SDF, but also can potentially be engineered for consolidated lignin processing.

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Reference

1. A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Frederick, J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski, *Science*, 2006, **311**, 484-489.
2. W. Boerjan, J. Ralph and M. Baucher, *Annu. Rev. Plant Biol.*, 2003, **54**, 519-546.
3. A. J. Ragauskas, G. T. Beckham, M. J. Biddy, R. Chandra, F. Chen, M. F. Davis, B. H. Davison, R. A. Dixon, P. Gilna, M. Keller, P. Langan, A. K. Naskar, J. N. Saddler, T. J. Tschaplinski, G. A. Tuskan and C. E. Wyman, *Science*, 2014, **344**.
4. J. S. Yuan, D. W. Galbraith, S. Y. Dai, P. Griffin and C. N. Stewart, Jr., *Trends Plant Sci.*, **13**, 165-171.
5. Z. Xu, D. Zhang, J. Hu, X. Zhou, X. Ye, K. L. Reichel, N. R. Stewart, R. D. Syrenne, X. Yang, P. Gao, W. Shi, C. Doeppke, R. W. Sykes, J. N. Burris, J. J. Bozell, M. Z. Cheng, D. G. Hayes, N. Labbe, M. Davis, C. N. Stewart, Jr. and J. S. Yuan, *BMC Bioinformatics*, 2009, **8**, 1471-2105.
6. S. Xie, R. Syrenne, S. Sun and J. S. Yuan, *Curr. Opin. Biotechnol.*, 2014, **27**, 195-203.
7. S. Xie, X. Qin, Y. Cheng, D. Laskar, W. Qiao, S. Sun, L. H. Reyes, X. Wang, S. Y. Dai, S. E. Sattler, K. Kao, B. Yang, X. Zhang and J. S. Yuan, *Green Chem.*, 2015, **17**, 1657-1667.
8. M. Kosa and A. J. Ragauskas, *Green Chem.*, 2013, **15**, 2070-2074.
9. Z. Wei, G. Zeng, F. Huang, M. Kosa, D. Huang and A. J. Ragauskas, *Green Chem.*, 2015, **17**, 2784-2789.
10. W. Shi, R. Syrenne, J.-Z. Sun and J. S. Yuan, *Insect Sci.*, 2010, **17**, 199-219.
11. W. Shi, S. Xie, X. Chen, S. Sun, X. Zhou, L. Liu, P. Gao, N. C. Kyrpides, E. G. No and J. S. Yuan, *PLoS Genet.*, 2013, **9**, 10.
12. M. Kosa and A. Ragauskas, *Appl. Microbiol. Biotechnol.*, 2012, **93**, 891-900.
13. T. Wells, Z. Wei and A. Ragauskas, *Biomass Bioenergy*, 2015, **72**, 200-205.
14. J. Ralph, K. Lundquist, G. Brunow, F. Lu, H. Kim, P. Schatz, J. Marita, R. Hatfield, S. Ralph, J. Christensen and W. Boerjan, *Phytochem. Rev.*, 2004, **3**, 29-60.
15. D. Floudas, M. Binder, R. Riley, K. Barry, R. A. Blanchette, B. Henrissat, A. T. Martínez, R. Otillar, J. W. Spatafora, J. S. Yadav, A. Aerts, I. Benoit, A. Boyd, A. Carlson, A. Copeland, P. M. Coutinho, R. P. de Vries, P. Ferreira, K. Findley, B. Foster, J. Gaskell, D. Glotzer, P. Górecki, J. Heitman, C. Hesse, C. Hori, K. Igarashi, J. A. Jurgens, N. Kallen, P. Kersten, A. Kohler, U. Kües, T. K. A. Kumar, A. Kuo, K. LaButti, L. F. Larrondo, E. Lindquist, A. Ling, V. Lombard, S. Lucas, T. Lundell, R. Martin, D. J. McLaughlin, I. Morgenstern, E. Morin, C. Murat, L. G. Nagy, M. Nolan, R. A. Ohm, A. Patyshakuliyeva, A. Rokas, F. J. Ruiz-Dueñas, G. Sabat, A. Salamov, M. Samejima, J. Schmutz, J. C. Slot, F. St. John, J. Stenlid, H. Sun, S. Sun, K. Syed, A. Tsang, A. Wiebenga, D. Young, A. Pisabarro, D. C. Eastwood, F. Martin, D. Cullen, I. V. Grigoriev and D. S. Hibbett, *Science*, 2012, **336**, 1715-1719.
16. K. Kurosawa, P. Boccazzi, N. M. de Almeida and A. J. Sinskey, *J. Biotechnol.*, 2010, **147**, 212-218.
17. R. Budini, D. Tonelli and S. Girotti, *J. Agric. Food Chem.*, 1980, **28**, 1236-1238.
18. X. Li, E. Ximenes, Y. Kim, M. Slininger, R. Meilan, M. Ladisch and C. Chapple, *Biotechnol. Biofuels*, 2010, **3**, 1754-6834.
19. J. Folch, M. Lees and G. H. Sloane Stanley, *J. Biol. Chem.*, 1957, **226**, 497-509.
20. Y. Pu, S. Cao and A. J. Ragauskas, *Energy Environ. Scl.*, 2011, **4**, 3154-3166.
21. S. Cao, Y. Pu, M. Studer, C. Wyman and A. J. Ragauskas, *RSC Adv.*, 2012, **2**, 10925-10936.
22. A. Brune, *Nat. Rev. Micro.*, 2014, **12**, 168-180.
23. A. Vanden Wymelenberg, J. Gaskell, M. Mozuch, G. Sabat, J. Ralph, O. Skyba, S. D. Mansfield, R. A. Blanchette, D. Martinez, I. Grigoriev, P. J. Kersten and D. Cullen, *Appl. Environ. Microbiol.*, 2010, **76**, 3599-3610.

24. D. Martinez, J. Challacombe, I. Morgenstern, D. Hibbett, M. Schmoll, C. P. Kubicek, P. Ferreira, F. J. Ruiz-Duenas, A. T. Martinez, P. Kersten, K. E. Hammel, A. Vanden Wymelenberg, J. Gaskell, E. Lindquist, G. Sabat, S. S. Bondurant, L. F. Larrondo, P. Canessa, R. Vicuna, J. Yadav, H. Doddapaneni, V. Subramanian, A. G. Pisabarro, J. L. Lavin, J. A. Oguiza, E. Master, B. Henrissat, P. M. Coutinho, P. Harris, J. K. Magnuson, S. E. Baker, K. Bruno, W. Kenealy, P. J. Hoegger, U. Kues, P. Ramaiya, S. Lucas, A. Salamov, H. Shapiro, H. Tu, C. L. Chee, M. Misra, G. Xie, S. Teter, D. Yaver, T. James, M. Mokrejs, M. Pospisek, I. V. Grigoriev, T. Brettin, D. Rokhsar, R. Berka and D. Cullen, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 1954-1959.
25. H. Ben and A. J. Ragauskas, *Energ. Fuel.*, 2011, **25**, 2322-2332.
26. M. Lund and A. Ragauskas, *Appl. Microbiol. Biotechnol.*, 2001, **55**, 699-703.
27. Y. Pu, S. Anderson, L. Lucia and A. J. Ragauskas, *J. Photochem. Photobiol., A*, 2004, **163**, 215-221.
28. S. H. Ghaffar and M. Fan, *Biomass Bioenergy*, 2013, **57**, 264-279.

Figure Legend

Table 1. GPC analysis of lignin molecular weights upon different treatments.

Table 2. Decrease of lignin functional groups after different treatments.

Figure 1. The increase of cell growth in response to laccase and other treatments. A. laccase promotes *R. opacus* PD630 cells growth using lignin as carbon source. *R. opacus* PD630 showed an exponential increase of cell growth associated with laccase concentration. B. Fenton reaction has limited synergistic effect with laccase treatment to promote *R. opacus* PD630 cells growth in lignin fermentation. Except the no laccase control, all treatments contain laccase at a concentration of 1.0 U/ml in fermentation medium. Control: only the *R. opacus* PD630 cells were added to fermentation medium; H_2O_2+Fe^{2+}: *R. opacus* PD630 cells supplied with 0.2 mM $FeSO_4$ and 0.067 mM H_2O_2; Laccase: *R. opacus* PD630 cells supplied with laccase; Laccase+Fe^{2+}: *R. opacus* PD630 cells supplied with laccase, 0.2 mM $FeSO_4$ and 0.067 mM H_2O_2; Laccase+$FeCl_3$: *R. opacus* PD630 cells supplied with laccase, 0.2 mM $FeCl_3$ and 0.067 mM H_2O_2; Laccase+ NaFeEDTA: *R. opacus* PD630 cells supplied with laccase, 0.2 mM NaFeEDTA and 0.067 mM H_2O_2.

Figure 2. Lignin degradation and lipid yield of *R. opacus* PD630 with and without laccase treatment. A, Laccase treatment promoted the lignin degradation as indicated in Prussian blue assay. B, Laccase increased lipid yield from *R. opacus* PD630. For the control group, *R. opacus* PD630 cells was added to fermentation medium without laccase treatment. For laccase treatment, the cells and laccase were added to fermentation medium simultaneously.

398 Table 1. GPC analysis of lignin molecular weight upon different treatments.

Lignin Sample	M_n	M_w	Polydispersity index
I[a]	1.73×10^3	8.08×10^3	4.66
II[b]	1.67×10^3	7.58×10^3	4.55
III[c]	2.12×10^3	2.68×10^4	12.7
IV[d]	2.15×10^3	3.30×10^4	15.3
V[e]	2.12×10^3	1.90×10^4	8.98

399 [a] I No cell; [b] II Cell only; [c] III Cell + Laccase; [d] IV Cell + Laccase + Fe^{2+}; [e] V Cell + Laccase + Fe^{2+} + H_2O_2.

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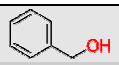
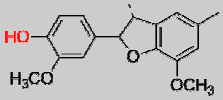
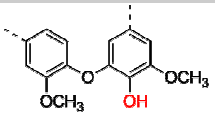
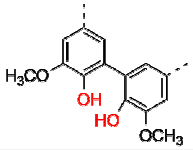
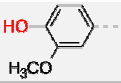
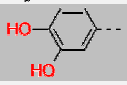
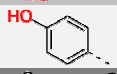
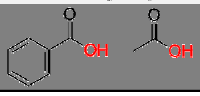
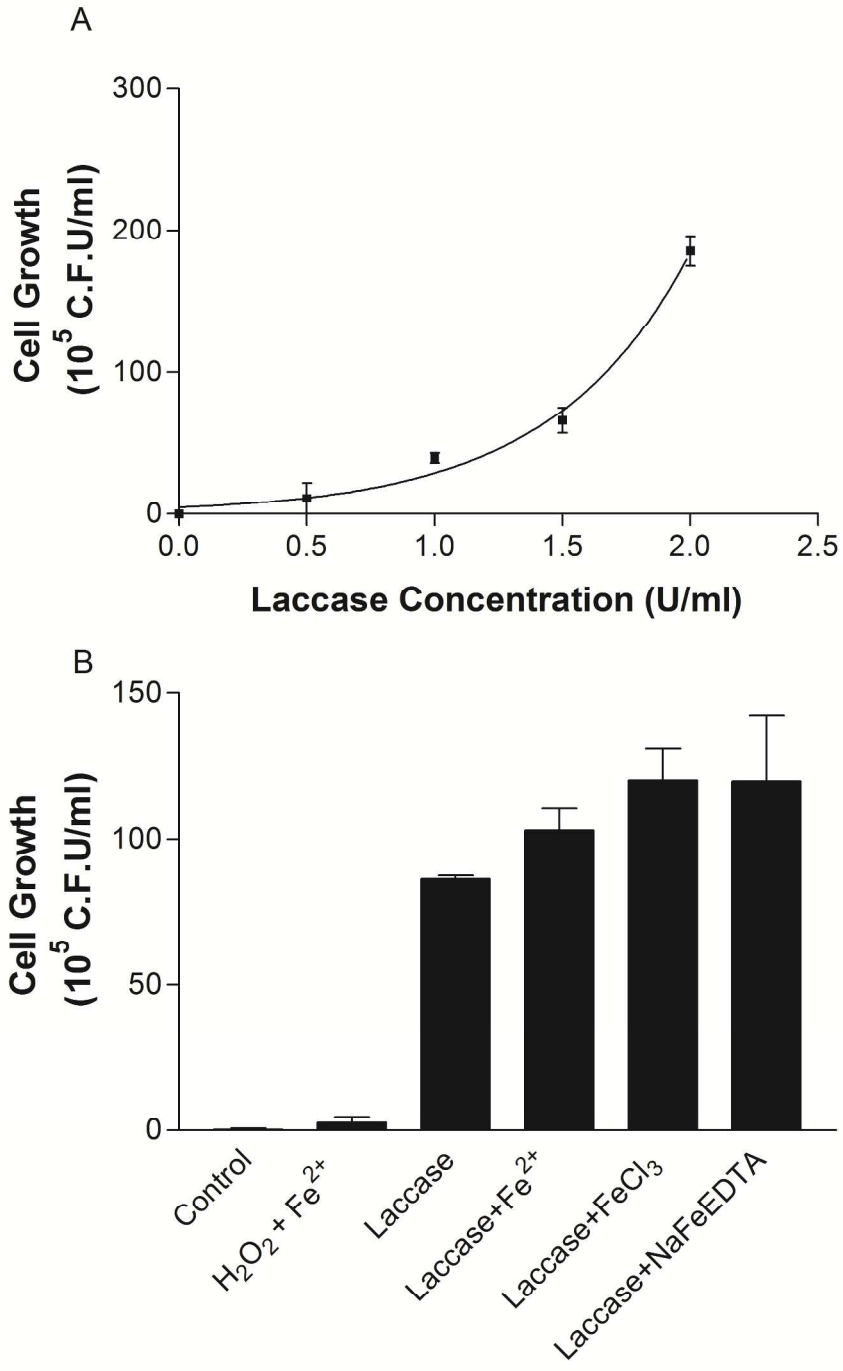
Table 2. Decrease of lignin functional groups after different treatments.

Functional Group		Integration region (ppm)	Examples	hydroxyl contents/(mmol/g lignin)				
				I[a]	II[b]	III[c]	IV[d]	V[e]
Aliphatic OH		150.0-145.2		2.38	2.32	1.88	1.98	1.99
	β-5	144.6-142.9		0.15	0.02	0.02	0.01	0.01
C_5 substituted condensed Phenolic OH	4-O-5	142.9-141.6		0.01	0.02	0.01	0.02	0.01
	5-5	141.6-140.1		0.00	0.05	0.02	0.03	0.03
Guaiacyl phenolic OH		140.1-138.8		1.32	1.40	0.98	1.00	1.02
Catechol type OH		138.8-138.2		0.04	0.02	0.01	0.02	0.02
p-hydroxy-phenyl-OH		138.2-137.3		0.08	0.06	0.02	0.03	0.03
Carboxylic acid OH		136.6-133.6		0.50	0.15	0.16	0.29	0.06

[a] I No cell; [b] II Cell only; [c] III Cell + Laccase; [d] IV Cell + Laccase + Fe^{2+}; [e] V Cell + Laccase + Fe^{2+} + H_2O_2.

415 Figure 1.



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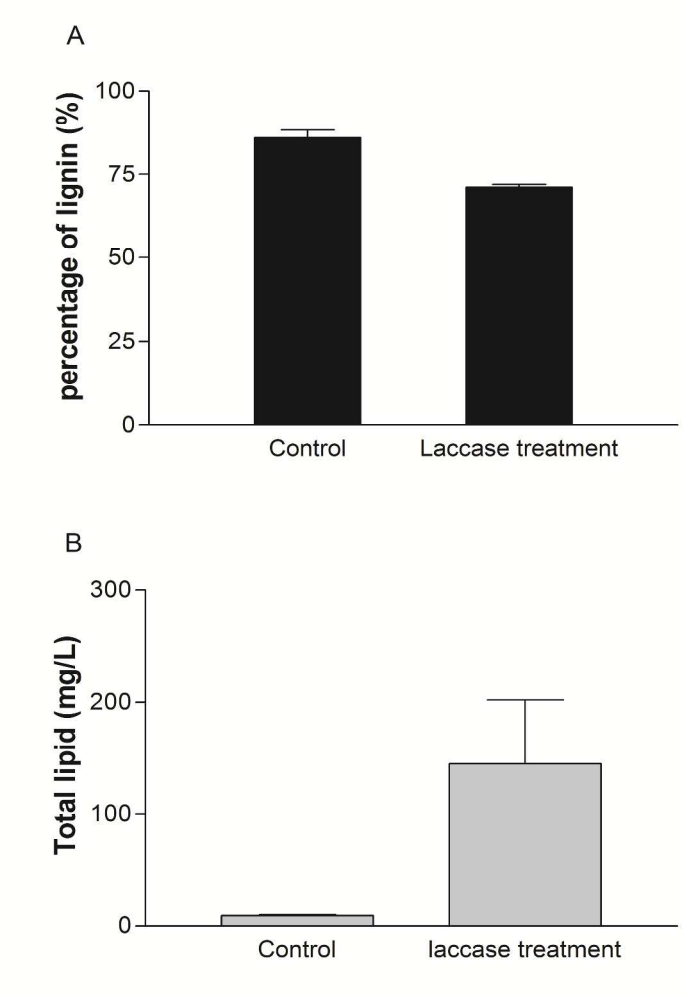
417 Figure 2.



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