Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Simultaneous Conversion of All Cell Wall Components by Oleaginous Fungus without Chemi-physical Pretreatment

Shangxian Xie[ab], Xing Qin[b], Yanbing Cheng[b], Dhrubojyoti Laskar[c], Weichuan Qiao[b], Su Sun[b], Luis H. Reyes[d], Xin Wang[b], Susie Y. Dai[e], Scott E. Sattler[f], Katy Kao[d], Bin Yang[c], Xiaoyu Zhan[a,*], and Joshua S. Yuan[ab,*]

[a] School of Life Science & Technology, Huazhong University of Science and Technology, Wuhan, 430074, P.R. China

[b] Synthetic and Systems Biology Innovation Hub, Department of Plant Pathology and Microbiology, Institute for Plant Genomics and Biotechnology, Texas A & M University, College Station, TX, 77843, USA

[c] Center for Bioproducts and Bioenergy, Washington State University, Richland, WA, 99354, USA

[d] Department of Chemical Engineering, Texas A&M University, College Station, TX, 77843, USA

[e] Office of Texas State Chemist, Texas AgriLife Research, College Station, TX 77843, USA Grain,

[f] Grain, Forage, and Bioenergy Research Unit, USDA-ARS, Lincoln, NE, 68583, USA

* To whom correspondence should be addressed: Joshua Yuan: syuan@tamu.edu and Xiaoyu Zhang: zhangxiaoyu@mail.hust.edu.cn;

Abstract

Lignin utilization during biomass conversion has been a major challenge for lignocellulosic biofuel. In particular, the conversion of lignin along with carbohydrate for fungible fuels and chemicals will both improve the overall carbon efficiency and reduce the need for chemical pretreatments. However, few biomass-converting microorganisms have the capacity to degrade all cell wall components including lignin, cellulose, and hemicellulose. We hereby evaluated a unique oleaginous fungus strain *Cunninghamella echinulata* FR3 for its capacity to degrade lignin during biomass conversion to lipid, and the potential to carry out consolidated fermentation without chemical pretreatment, especially when combined with sorghum (*Sorghum bicolor*) *bmr* mutants with reduced lignin content. The study clearly showed that lignin was consumed together with carbohydrate during biomass conversion for all sorghum samples, which indicates this organism has the potential for biomass conversion without chemical pretreatment. Even though dilute acid pretreatment of biomass resulted in more weight loss during fungal fermentation than untreated biomass, the lipid yields were comparable for untreated *bmr6/bmr12* double mutant and dilute acid-pretreated wild-type biomass samples. The mechanisms for lignin degradation in oleaginous fungi were further elucidated through transcriptomics and chemical analysis. The studies showed that in *C. echinulata* FR3, Fenton Reaction may play an important role in lignin degradation. This discovery is among the first to show that a mechanism for lignin degradation similar to ones found in white and brown rot basidiomycetous fungi exists in an oleaginous fungus. This study suggests that oleaginous fungus such as *C. echinulata* FR3 can be employed for complete biomass utilization in a consolidated platform without chemical pretreatment, or can be used to convert lignin waste into lipids.

Introduction

Lignin modification and utilization are essential for sustainable and cost-effective production of fuels and chemicals from lignocellulosic biomass. Traditional approach for biomass conversion follows three steps including pretreatment, saccharification, and fermentation[1, 2] A major goal of pretreatment is to disrupt lignin structure to make cellulose more accessible to saccharification.[1, 3] However, most of chemical or physical pretreatment strategies require high energy input and hash conditions such as high temperature and/or extreme pH, which often also resulted in toxic waste. Biological pretreatment, on the other side, can be carried out in milder conditions and be more environmentally friendly. However, it is still challenging to apply biological pretreatment for biofuel production due to the long reaction time. Lignin modification on feedstock thus emerged as an alternative strategy for reducing recalcitrance and developing cost-effective bioconversion with simplified pretreatment.[4, 5]

Traditionally, lignin modification was extensively studied in model plants such as *Medicago*, *Arabidopsis*, and *Nicotiana*.[4, 6, 7] Recently, significant advances have been made in the improvement of grasses, including sorghum and switchgrass, as biofuel feedstocks.[8-10] However, most research on the lignin modification of grasses has been carried out via genetic modification, which creates regulatory issues for commercial implementation. In contrast, a unique set of chemically mutagenized lignin modification *brown midrib* (*bmr)* mutants were developed in sorghum as major assets in determining how modifications to the lignin biosynthesis pathway impact biomass conversion.[11, 12] The biomass from *bmr6* or *bmr12* plants was significantly reduced in lignin content and altered in lignin composition in relative to wild-type biomass. Each mutant showed increased saccharification and ethanol conversion efficiency for cellulose.[13] When combined in a double mutant, the effects were additive for reducing lignin content and

increasing conversion efficiency.[14] Despite the previous advances in ethanol platforms, few studies focused on how lignin modification will impact the oleaginous fungus conversion of biomass into lipid. Also unknown was whether feedstock lignin modification could promote *C. echinulata* FR3 conversion of biomass without chemi-physical pretreatment.

Recently, lipid production from biomass using oleaginous microbe has been explored.[15, 16] Many studies have reported oleaginous fungi, including *Mortierella isabellina* and *Mucor circinelloides*, could produce lipid from lignocellulolytic hydrolysates.[15, 16] Recent researches also showed that oleaginous bacteria, such as *Rhodococcus opacus* PD630, may have the potential capacity to use lignin as sole carbon resource for lipid production.[17] In addition, oleaginous yeast can also be used for converting biomass hydrolysates[18]. Even though the overall conversion rate of carbohydrates-to-lipid is much lower than that of ethanol, the oleaginous fungus-based platform has at least three major advantages. First, from a broader biodiesel industry perspective, lignocellulosic biomass provides a reliable feedstock for the significant amount of lipid needed for biodiesel refineries. Second and more importantly, oleaginous fungal species have been widely used for biotransformation of aromatic compounds, suggesting the possibility of using these species to utilize lignin along with carbohydrates to biofuel products.[19, 20] For example, we identified a fast-growing oleaginous fungus *C. echinulata* FR3 with the capacity to accumulate more than 40% of dry cell weight in lipids when grown on glucose. Previous studies indicated that other *C. echinulata* strains could transform complex aromatic compounds.[19] However, despite these promising findings, the particular strain has not been used to convert lignocellulosic biomass, and neither has oleaginous fungus been evaluated for lignin degradation capacity during biomass conversion. Third and the most importantly, considering the potential to utilize lignin, this unique oleaginous strain may be used to convert biomass directly

without chemi-physical pretreatment. Even though different conversion platforms were developed to process different compositions of plant cell wall, few platforms can actually convert lignin together with carbohydrate into fungible fuels and products via biological processes. The study aims to offer an alternative approach where a simple biological system could convert all the composition of plant cell wall into biofuel product without additional pretreatment process.

Therefore, we will evaluate the possibility of such system with three aspects of studies. **First**, we evaluated whether the oleaginous fungus *C. echinulata* FR3 can convert lignin while processing carbohydrate. **Second**, we investigated if lignin modification of feedstock can be integrated with this oleaginous fungus strain to improve efficiency of biomass conversion without chemical pretreatment. **Third**, we further investigated the mechanisms for lignin utilization by oleaginous fungi using transcriptomics and chemical analysis. The discovery indicated that oleaginous fungus like FR3 can be exploited for complete biomass utilization in a consolidated platform without chemical pretreatment, or can be enhanced to convert lignin waste into lipid. These novel oleaginous fungus-based platforms could have profound impacts on energy and environmental sustainability when implemented.

2. Material and Supplies

2.1. Microorganism maintenance and spore inoculum preparation

C echinulata FR3 was isolated by Key Laboratory of Molecular Biophysics at Huazhong University of Science and Technology, and identified by China General Microbiological Culture Collection Center in Beijing. The strain was maintained at 4℃ on potato dextrose agar (PDA) slant. To prepare the spore inoculum, the fungal mycelium was incubated on PDA in 250mL

Erlenmeyer flasks at 28℃ for 5 days to form spores. The spores were then collected by washing the mycelium with 10mL sterile water and transferred to 100mL of potato dextrose broth (PDB) medium in 250mL Erlenmeyer flask with a final spore concentration of 3×10^7 spores/L. The inoculation was cultivated at 28℃ with a shaking speed of 150rpm for 36h.

2.2. Sorghum biomass

The signal- and double- mutant sorghum stocks were developed as previously described.[21] The wild-type and mutant lines of sorghum were grown in randomized block design at University of Nebraska Agricultural Research and Development fields near Mead, NE in 2010. The samples consisted of bulk biomass harvested from 4 plots. The grain heads were removed and sorghum lignocellulosic tissue (leaves and stalks) were dried at 50℃ in air-blowing oven for 3 days. The tissue was ground using a Wiley® mill (2-mm screen; Arthur H. Thomas Co., Philadelphia, PA).

2.3. Dilute-acid pretreatment

The dilute-acid pretreatment of sorghum was carried out by mixing with diluted sulfuric acid to a final concentration of 1.5% (w/w) at a solid loading of 1:17 (w/v). The mixture was maintained at 121℃ for 1h. After the reaction, the samples were neutralized to pH 7.0 with 2M sodium hydroxide.

2.4. Enzymatic hydrolysis and fungal conversion

For untreated sorghum stover enzymatic hydrolysis, samples were adjusted with distilled water to a solid loading of 1:17 (w/v) and autoclaved at 121℃ for 20 min with liquid cycle. Different cellulase enzyme (Sigma, C2730) loading (3.0, 4.5 and 6.0 FPU/g sorghum) were used for hydrolysis at 28℃ on a rotary shaker incubator at 150rpm for 48h. For the acid-pretreated wild-type sorghum, the cellulase was directly added to the neutralized sample with an enzyme

loading of 4.5 FPU/g sorghum biomass. The hydrolysis reactions were conducted as aforementioned. After hydrolysis, the pre-cultured *C. echinulata* FR3 were inoculated at a 5% (v/v) fungal biomass loading, and cultured on a rotary shaker incubator at 28℃ and 150rpm.

2.5. Sorghum biomass and mycelium biomass evaluation

The sorghum and mycelium biomass from the fermentation were harvested every 72-hour period (0, 3, 6 and 9 days) by centrifugation. The fungal mycelium pellets were separated from the sorghum biomass manually, and both the sorghum and fungal mycelium were washed with distilled water twice and dried at 60℃ for three days to a constant weight. After drying, the weight of both sorghum and fungal mycelium was measured and recorded.

2.6. Measurement of reducing sugar, cellulase and xylanase activity

The cultivation supernatant was collected by centrifugation to measure reducing sugar concentration, cellulase, and xylanase activities. Reducing sugar was measured with the DNS (3,5-dinitrosalicylic acid) reagent using the absorbance at 540 nm with a glucose standard curve.[22] The cellulase activity in the medium was assayed based on the 'filter-paper-unit (FPU)' following the Laboratory Analytical Procedure (LAP 006) from the National Renewable Energy Laboratory (NREL). The xylanase activity was assayed using xylan as the substrate to measure the release of reducing sugar by DNS method by reaction of 30 min at 37℃ and pH 4.8. One unit of xylanase activity was calculated as 1 μmole of reducing sugar from xylan per min at the reaction condition.

2.7. Lipid production evaluation

The extraction and composition analysis of total lipid were achieved by GC/MS (gas chromatograph/mass spectrometry) according the protocol described in our previous study.[23] The total lipid of the fungal mycelium was extracted in the form of fatty acid methyl ester (FAME)

with sulfuric acid–methanol method and collected in pre-weighted tubes. The tube with extracted FAME was further dried to a constant weight and then be weighted to derive the lipid yield.

The lipid composition was analyzed by GC/MS using an Agilent 7890 GC (Agilent Technologies, Santa Clara, CA) coupled with an Agilent 5975 mass spectrometer. An HP-5MSI column was used. The injection port was kept at 280 °C and the MS transfer line was set to 100 °C. The GC oven temperature was programmed as follows: initial with 40 °C (0.5 min), increase to 110 °C at 5 °C/min, and then increase from 110 to 300 °C at 20 °C/min for a total run time of 24 min. The raw chromatography and mass spectra data were processed with software Enhanced ChemStation (Agilent Technologies, Santa Clara, CA), and the quantity of the specific lipid molecules was analyzed by the peak area.

2.8. Compositional analysis

Structural carbohydrates (cellulose and hemicellulose), lignin, and ash content of sorghum stovers were determined according to Laboratory Analytical Procedure from the National Renewable Energy Laboratory (NREL).[24] Carbohydrate concentrations were analyzed using high-performance liquid chromatography (HPLC 1260 Infinity; Agilent Technologies, CA). HPLC analyses were carried out with an Aminex HPX-87P Column (300 mm by 7.8 mm; Bio-Rad Laboratories, CA) with HPLC grade water as mobile phase and a flow rate of 0.6 mL/min at 85°C using RI detector. The lignin, cellulose and hemicellulose degradation efficiencies were calculated as following:

Lignin (cellulose, hemicellulose) lost (%)

$$= 100 - \frac{lignin\ (cellulose,hemicellulose) content\ in\ converted\ sorghum \times final\ weight\ of\ sorghum\ after\ conversion}{lignin\ (cellulose,hemicellulose) content\ in\ raw\ sorghum \times inicial\ weight\ of\ raw\ sorghum}$$

2.9. Solid state 13C CP/MAS NMR analysis

The carbon-13 cross-polarization (CP) magic angle spinning (MAS) (^{13}C CP/MAS) Solid-state NMR was carried out to confirm the compositional and chemical changes of the structural components for sorghum biomass under different treatments. The control biomass tissues (250 mg) and samples after conversion were individually packed in 5-mm pencil type rotor and the spectra were recorded under identical acquisition parameters. The solid state ^{13}C CP/MAS analysis were carried out at 100 MHz on a Bruker Avance 400 spectrometer, (NMR center, Washington State University), equipped with a Chemagnetics double resonance probe. A contact time of 0.5 ms, proton field ca. 40 kHz during CP and data acquisition, relaxation delay of 4 s and spinning speed of 5 kHz were applied to obtain the 13C CP/MAS spectra. All the corresponding 13C CP/MAS spectra were derived from 17,500 scans, with the chemical shifts given in δ ppm. The integrals for each resonance and/or chemical shift values arising from the cell wall components of fungal converted biomass were normalized with reference to their corresponding control spectra for semi-quantitative analysis.

2.10. Transcriptomics analysis

The total RNA was extracted from *C. echinulata* FR3 grown on sorghum for 6 days with Qiagen RNeasy Plant Mini Kit. The transcriptomics sequencing of the fungal samples were performed using Illumina HiSeq 2500 by Institute of Plant Genomics and Biotechnology, Texas A&M University. The resulting reads were assembled with Trinity.[25] To further reduce the redundancy of the assembled transcripts, sequence clustering package CD-HIT were used to cluster similar sequence that meet similarity threshold >0.9 into unigene clusters.[26] Unigenes were then functionally annotated based on UniProtKB (http://www.uniprot.org/) database by the package of ncbi-blast-2.2.28+ with the threshold of $e < 10^{-6}$. To quantify the expression level of unigenes, sequencing reads were mapped to the unigene sequences. Gene expression abundance

was calculated as normalized mapping read counts per million total mapping reads per kb of the gene length. The assembled unigenes were also aligned to CAZy database based on sequence similarity to carry out the lignocellulolysic degradation functional analysis through the Bioenergy Science Center web service (http://mothra.ornl.gov/cgi-bin/cat/cat_v2.cgi?tab=ORTHOLOGS) with E-value threads $\leq 10^{-6}$ and Pfam domains bit score threshold ≥ 100. The phylogenetic analysis was based on PhyMl though Phylogeny.fr web server. [27, 28]

2.11. Determination of iron-reducing activity

Iron-reducing activity was determined based on formation of ferrozine-Fe^{2+} complex.[22] 2ml of cultivation supernatants collected by centrifugation were mixed with 0.5ml $FeCl_3$ (1.2mM) and 0.5ml ferrozine (15mM), and immediately measure the absorbance change at 562 nm with UV-Visible spectrometer for 3min. 1 unite (A/min) of iron-reducing activity was defined as the rate of absorbance increase at 562nm per minute.

3. Results and Discussion

3.1 *C. echinulata* FR3 (FR3) can degrade all cell wall components including cellulose, hemicellulose, and lignin

Biomass composition analysis confirmed the reduced lignin content in the *bmr* lines (Table S1).[13, 29] *C. echinulata* FR3 fermentation led to the degradation of all cell wall structural components including lignin. Relatively lower cellulase loadings (3.0, 4.5 and 6.0 FPU/g sorghum) were used for saccharification (Figure S1), because oleaginous fungi can secrete cellulases for saccharification.[16, 23] Both the *bmr6* and the *bmr6/bmr12* double mutants allowed higher scarification efficiency than that of wild-type (Figure S1). The sorghum biomass hydrolyzed with 4.5 FPU/g enzyme load was then subjected to *C. echinulata* FR3 fermentation

to evaluate the degradation of different cell wall components. As shown in Figure 1A, weight loss was observed for cellulose, hemicellulose and lignin during the first six days of fermentation. The weight loss reached a plateau at day 6 to day 9 for most of the samples. In general, a higher percentage of weight loss was achieved for all mutants as compared to wild-type (Figure 1). In particular, more significant lignin weight loss was found for both *bmr12* and *bmr6/bmr12* double mutants. For example, the lignin weight loss for double mutant and wide-type was 46% and 31%, respectively (Figure 1B). More significant cellulose weight loss was observed for both *bmr6* and the *bmr6/bmr12* double mutants (Figure 1C). The fermentation of *bmr6* mutant led to the most significant hemicellulose weight loss (Figure 1D). The significant degradation of cell wall lignin in *C. echinulata* FR3 is noteworthy as the phenomena is normally common to Basidiomycetes, but not to other genera of fungi. As an oleaginous fungus, the capacity to degrade lignin over 30% offers *C. echinulata* FR3 new opportunities of utilization of multiple cell wall components for useful products and the bioconversion without chemical pretreatment.

3.2. Lignin modification promoted biomass conversion by *C. enchinulata* FR3

The biomass weight loss correlated well with the growth of *C. enchinulata* FR3. In general, *C. enchinulata* FR3 grew faster and accumulated more fungal biomass on *bmr* mutants, which was consistent with the greater sorghum biomass weight loss in *bmr* mutants (Figure 2A). The fungal cell growth reached plateau phase at Day 6, when the sorghum biomass weight loss also turned to plateau phase. Among the three mutant lines of sorghum, FR3 grown on *bmr6/bmr12* double mutant and *bmr12* mutant accumulated higher cell biomass as compared to that of *bmr6* and wild-type (Figure 2A).

The weight loss and fungal growth also correlated with lipid yield. The lipid yield for all fermentation experiments increased during the first six days and decreased on Day 9 (Figure 2B).

The result could be explained by the fact that both fungal growth and sorghum biomass weight loss reached plateau at Day 6. The fungus might start catabolizing lipids after Day 6, because the reduced sugar also reached plateau phase at Day 6 (Figure S2). Day 6 thus represented the optimal time to harvest and compare lipid yields among different lines. Among the three *bmr* lines, both *bmr6* and *bmr6/bmr12* double mutants yielded more lipid during fermentation. The fermentation of *bmr6/bmr12* led to the most significant increase in lipid production.

In order to further elucidate the mechanisms for biomass degradation, we analyzed reduced sugar and enzyme activities in the fermentation system. As aforementioned, the level of reduced sugar dropped rapidly during the first six days of fermentation and reached a plateau at Day 6 to Day 9 (Figure S2). The enzyme activity reached peak level at Day 3 (Figure 2C and D). Considering that the exogenous cellulase was added two days before Day 0, the result clearly indicated that endogenous cellulase played an important role in the biomass degradation and fungal cell growth. The results in enzyme and reducing sugar analysis were consistent with the fungal growth and lipid yield. Besides the cellulose and hemicellulose, detailed analysis of lignin content was carried out using NMR spectroscopy to further confirm lignin degradation.

3.3.NMR analysis confirmed the degradation of lignin along with carbohydrates

In order to further confirm that *C. enchinulata* FR3 could convert lignin along with the carbohydrates, the solid-state NMR was carried out to analyze sorghum biomass using the conventional CP/MAS (cross-polarization-magic angle spinning) method.[30] The analysis was focused on the structural changes of the wild-type and *bmr6/bmr12* double mutant sorghum after 6-days fungal conversion as compared to the reference samples without fermentation. The normalized ^{13}C CP/MAS NMR spectrums were depicted in Figure S3 and Figure S4 and the most predominant assignments for each resonance peak were listed in Table S2.[31, 32] The

acquired spectra mainly contained chemical resonance values analogous to carbohydrate region (60-100 ppm) and aromatic region (100-162 ppm). In addition, the chemical shift resonances of carbonyl and carboxyl group (160-200 ppm), methoxyl group (52-55 ppm) and carbon in etherified and/or non-etherified region (132-152 ppm) were also observed. The comparison of ^{13}C CP/MAS NMR spectra among different samples revealed several features.

First, comparison of aromatic carboxyl resonances (165-175 ppm) indicated the significant differences in the lignin derived aromatic region (Figure S4A). The results suggested that FR3 conversion of sorghum biomass resulted in lignin utilization, confirming the lignin weight loss data. Second, the ^{13}C CP/MAS NMR analysis showed reduced intensity in the aliphatic carbonyl/carboxyl group resonances (175- 185 ppm) for both wild-type and *bmr6/bmr12* mutant after conversion by *C. enchinulata* FR3. The result strongly indicated the removal of hemicelluloses and side chain alterations in lignin macromolecular assembly,[33] which could result from cell wall deconstruction by *C. enchinulata* FR3 during the 6-days conversion process. Third, in contrast to the aforementioned decreases, a slight increase in the intensity at chemical shift values of 60-90 ppm (carbohydrate region) was observed in the biomass samples after fungal conversion. Such increase was due to the relatively more rapid reduction in the content of aromatic lignin and aliphatic carbon. The result suggested that the lignin degradation was more rapid than cellulose degradation during the six days of conversion by *C. enchinulata* FR3. The result was consistent with composition analysis. The quantification of resonances with reference to the control ^{13}C CP/MAS NMR spectra was also carried out under normalized condition to determine the contents (in %) of cell wall components in the corresponding samples (Table 1). The results further confirmed the degradation of all cell wall components including cellulose, hemicellulose and lignin by *C. enchinulata* FR3 conversion. In addition, Table 1 further

confirmed more rapid degradation of lignin, as the aromatic contents in wild-type and *bmr6/bmr12* mutant after conversion were reduce to 12.1% and 13.0% respectively, as compared to 16.8% and 18.6% in the untreated controls. Overall, the biomass composition analysis and ^{13}C CP/MAS NMR analysis were highly consistent to demonstrate that *C. enchinulata* FR3 could degrade all the plant cell wall composition including lignin, cellulose, and hemicellulose.

3.4. Similar lipid yield can be achieved for fermentation of *bmr* mutant and acid-pretreated wild-type biomass

The consumption of lignin by oleaginous fungus suggested that *C. echinulata* FR3 conversion of sorghum biomass might alter lignin content and structure, in a process similar to biological pretreatment by white rot fungus. We therefore hypothesized that biomass conversion might be carried out without chemical pretreatment, if biomass has a relatively low recalcitrance. We further compared the fungal growth and lipid yield of *C. echinulata* FR3 grown on acid-pretreated wild-type and non-pretreated *bmr6/bmr12* double mutant sorghum stover after 6 days (Figure 3A). Even though acid-pretreated wild-type sorghum led to a much higher weight loss (65.5%) than non-pretreated *bmr6/bmr12* sorghum (Figure 3B), no significant difference on the cell growth and lipid production were found between the two types samples (Figure 3A).The greater weight lose might be caused by the degradation of hemicellulose by sulfate acid during the pretreatment (Figure 3C). The GC/MS analysis also revealed that the lipid profile of *C. echinulata* FR3 has no significant difference between the two conditions (Figure S5). It is well established that various inhibitors and toxins could be generated during the dilute acid pretreatment to inhibit microbial growth.[34, 35] The inhibitors and toxins might also inhibit the cell growth and cause low lipid yield during biomass conversion by oleaginous fungus *C. echinulata* FR3.

3.5.Transcriptomics analysis revealed lignin and biomass degradation mechanism in FR3

Even though some filamentous fungi could degrade the lignocellulosic biomass, few studies indicated that any non-basidiomycetous filamentous fungi could degrade lignin. In particular, the lignin degradation capacity in oleaginous fungi might be exploited for broad applications including complete biomass utilization, consolidated biomass processing, and lignin conversion to lipid. The development of these new platforms will depend on in-depth understanding of biomass and lignin degradation mechanisms in *C. echinulata* FR3. Transcriptomics analysis of FR3 grown on the wild-type and *bmr6/bmr12* mutant was carried out to further explore the mechanisms for biomass, in particular, lignin degradation.

The result revealed that *C. echinulata* FR3 synergized extracellular lignocellulolytic enzymes and radical systems for biomass degradation. The transcriptomics analysis revealed three features. First, FR3 had cellulase enzymes, but not in a balanced combination to achieve maximized cellulose and hemicellulose degradation (Figure 4A). The analysis based on CAZy database revealed that cellulose and hemicellulose degradation enzymes were expressed, which was consistent with the cellulase and xylanase activity detected. Among the 17312 predicated gene models, 502 belonged to different CAZy families (bit score $\geq$ 100), including 131 glycoside hydrolases (GH) and 55 carbohydrate esterases (CE) (Figure S6). Despite the prevalence of GH enzymes, only 2 endoglucanase, 2 beta-1,4-glucosidase and 1 exoglucanase were identified. Among these cellulolytic enzymes, only CeFR966 encoding endoglucanase (GH9) was expressed at a high level (Figure 4A). The result indicated that *C. echinulata* FR3 had carbohydrate degradation capacity, yet the capacity is limited as compared to most of other cellulose degradation fungi.

Second, an alternative extracellular lignolytic enzyme system was identified in *C. echinulata* FR3 (Figure 4B). The enzyme system is slightly different from the classic lignolytic enzyme system based on peroxidases and laccases in white rot fungi.[36] Several gene models were annotated to be multiple copper oxidases, and one of them, CeFR1943, encoded a laccase-like multiple copper oxidases (LMCO) with a secretion signal peptide sequence. Phylogenetic analysis of this LMCO with classic laccases from fungi (ascomycetes and basidiomycetes) and plants revealed that the enzyme was related to basidiomycete fungal laccases known for strong lignin degradation capacity. The results indicated that the LMCO might involve in lignin depolymerization in *C. echinulata* FR3. Meanwhile, another gene CeFR715 encoded aromatic peroxygenase (APO) with predicted secretive signal peptides. Extracellular APOs were a novel group of heme-thiolate enzymes characterized for lignin degradation by catalyzing H_2O_2-dependent oxidative cleavage of diverse ethers in aromatic substrates.[37-39] The transcriptomics analysis thus suggested that LMCO and APO might be the main extracellular ligninolytic enzymes for *C. echinulata* FR3.

Third, a strong radical generation system was identified for *C. echinulata* FR3 and the radicals can synergize either with the aforementioned oxidative enzymes or with the iron-quinone system for Fenton reaction. Many enzymes contributing to extracellular H_2O_2 generation were discovered from the transcriptomics analysis (Figure 4B). Copper radical oxidase (CRO) and GMC oxidoreductase were two major groups involved with extracellular H_2O_2 generation for Fenton system in most studied fungi system.[40-42] Unlike the basidiomycetes expressing several CROs, only one CRO, CeFR251, was identified in *C. echinulata* FR3 encoding galactose oxidase with predicted secretive signal peptides. Moreover, this gene was expressed in a very high level as compared to many other genes (Figure 4B). At the meanwhile, several GMC

oxidoreductase-type genes were identified encoding long-chain-alochol oxidase. These resulted indicated that *C. echinulata* FR3 thus had a system to generate extracellular H_2O_2.

Fourth, the identification of iron reduction related proteins further suggested that the radical generation synergized with Fenton reaction (Figure 4B). Besides the extracellular enzymes, wood degradation by basidiomycetes often involved extracellular low molecular weight oxidants, particularly Fenton reaction generated hydroxyl radicals.[40, 43-45] The transcriptomics analysis suggested that *C. echinulata* FR3 also had the similar Fenton reaction-based radical system for lignin depolymerization. Several gene models (CeFR296, CeFR2458, CeFR 2745 and CeFR8451) were identified as putative quinone reductases (QRD) and were expressed in a relative high level. Redox cycling of secreted quinones was considered as a driving force for Fenton reaction in wood-degrading fungus.[46, 47] The expression of these putative QRD in coordination with quinate permeases (CeFR7596 and CeFR10458) for the biosynthesis and transport of quinones might enable an efficient extracellular Fenton system. In addition to the quinone-based iron reduction system, transmembrane ferric reductase (CeFR10940) was also found expressed with possible Fe^{3+} reduction function.

Overall, the transcriptomics analysis revealed a unique lignocellulose degradation system in *C. echinulata* FR3 by synergizing extracellular lignocellulolytic enzymes and hydroxyl radicals (Figure 5). Such system has enabled *C. echinulata* FR3 to become one of few known strains outside of basidiomycetes with an appreciable lignin degradation capacity. During biomass degradation, *C. echinulata* FR3 could express extracellular H_2O_2-generation related oxidoreductases (mainly galactose oxidase) and ferric reduction related proteins (QRD and ferric reductase) to mediate the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$). The generated hydroxyl radicals from Fenton-based system could both depolymerize polysaccharides and lignin

to make the lignocellulose more accessible for lignocellulolytic enzymes.[40, 44, 48] For lignin depolymerization, extracellular APO and LMCO could synergize with hydroxyl radical to carry out efficient lignin depolymerization in a way different from basidiomycetes. For cellulose and hemicellulose degradation, the enzymatic cellulose degradation pattern of *C. echinulata* FR3 was very similar to many of brown rot fungi, in which endoglucanases were highly expressed whilst cellobiohydrolases were poorly expressed or even absent during the cellulose degradation.[49] After the lignin depolymerization process, the released small molecular aromatic compounds could be transported into the cell for complete aromatic compound catabolism. Pathway analysis revealed two central aromatic catabolism pathways involving the ring-cleaving of homogentisate and 3-hydroxyanthranilate (Figure 4C).[50] Numerous cytochrome P450s (~50 genes) were also discovered in *C. echinulata* FR3, indicating that cytochrome P450s may involve in degradation/conversion of the lignin derived metabolites into central aromatic intermediates for complete degradation as previously reported.[51, 52]

3.6.Enzyme assay further confirms that Fenton reaction is one of the mechanisms for lignin degradation in *C. echinulata* FR3

In order to further verify the role of radicals and Fenton reaction in lignin degradation, iron-reducing activity was measured as an indicator for hydroxyl radical levels. The hydroxyl radical level is often impacted by iron reducing capacity, which is important in Fenton reaction. As shown in Figure 6, the iron-reducing activity significantly increased in the *C. echinulata* FR3 grown on biomass as compared to that on PDB medium. No significant difference was observed for iron reducing activity of fungus grown on wild-type and *bmr6/bmr12* mutant sorghum. The significant induction of iron-reducing activity throughout the biomass fermentation process

indicated that the induced Fenton reaction system might play an important role in lignin and biomass degradation, confirming the transcriptomics data.

3.7. Perspectives on oleaginous fungus with strong lignin degradation capacity

Overall, the biomass composition, chemical, and genomic data all suggested that oleaginous fungus *C. echinulata* FR3 has a unique capacity to efficiently utilize lignin. The discovery of an oleaginous fungus with strong lignin degradation capacity could be significant from several perspectives. First, even though lignin degradation by fungi has been studied extensively in basidiomycetes, few studies indicated that the capacity exists in filamentous fungi beyond basidiomycetes. From a scientific perspective, the genomic and molecular mechanisms for lignin degradation in *C. echinulata* FR3 and its relatedness to the mechanisms in basidiomycetes indicated that additional species evolved lignin degradation capacity in a convergent way using different mechanisms. From the application perspective, the oleaginous fungus with lignin degradation capacity will bridge an important limitation as white rot fungus has not been known, to date, to produce a fungible product from biomass. Second, the lignin degradation capacity in oleaginous fungi could enable a highly consolidated platform for biomass conversion, where chemi-physical pretreatment might not be necessary. The combination of lignin modification with fungal fermentation has already reached similar performance in terms of lipid yield (Figure 3). Such capacity can be further enhanced by genetic modification or condition optimization to achieve a more consolidated process. However, it should be pointed out that the limitation of FR3 fermentation is the relatively weak cellulose and hemicellulose degradation capacity. Third, the strong lignin degradation capacity also indicated it is possible for FR3 to be utilized for the conversion of lignin. A fungus-based lipid production platform is often robust and more amenable to small to mid-size operations. The lignin utilization capacity and potential for

consolidated processing will enable these strengths. The utilization of lignin modification mutants may further enhance the advantages. Further engineering of FR3 and optimization of fermentation conditions are necessary to enable such platforms.

4. Conclusions

In summary, we have revealed that the oleaginous fungi *C. echinulata* FR3 can accumulate high levels of lipid by degrading all the component of plant cell wall including lignin, cellulose, and hemicellulose. The biomass composition analysis and NMR-based structure analysis indicated *C. echinulata* FR3 degraded lignin with higher efficiency than cellulose/hemicellulose. Further transcriptomics studies and biochemical studies revealed that lignocellulose degradation in *C. echinulata* FR3 involved both extracellular lignocelulolytic enzymes and Fenton-system-mediated hydroxyl radicals. In addition, lignin modification in plant feedstock could improve the lignocellulose-to-lipid conversion efficiency by *C. echinulata* FR3. In an extreme case, the lignin-modified sorghum feedstock could be used for *C. echinulata* FR3 fermentation to yield similar amount of lipid as compared to pretreated wild-type plants. The study thus suggested that combining feedstock lignin modification with oleaginous fungal fermentation might deliver a cost-efficient bioconversion platform without chemical pretreatment. Considering the unique and strong lignin degradation capacity, the oleaginous fungus *C. echinulata* FR3 could serve as the base strain for further genetic engineering to achieve a sustainable and economic lignocellulose-to-biodiesel platform.

5. Acknowledgement

The work in China was supported by a grant from the National High-tech Research and Development Program of China (Grant No. 2011AA100904). The work in United States was supported by the U.S. DOE (Department of Energy) EERE (Energy Efficiency and Renewable

459 Energy) BETO (Bioenergy Technology Office) Grant (No. DE-EE0006112) to JSY and BY. The
460 research is also supported by Texas A&M Agrilife Research's biofuel initiative to JSY.

461

Reference

1. L. R. Lynd, M. S. Laser, D. Bransby, B. E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. D. McMillan, J. Sheehan and C. E. Wyman, *Nat. Biotechnol.*, 2008, **26**, 169-172.
2. B. Yang and C. E. Wyman, *Biotechnol. Bioeng.*, 2004, **86**, 88-95.
3. S.-Y. Ding, Y.-S. Liu, Y. Zeng, M. E. Himmel, J. O. Baker and E. A. Bayer, *Science*, 2012, **338**, 1055-1060.
4. F. Chen and R. A. Dixon, *Nat. Biotechnol.*, 2007, **25**, 759-761.
5. J. S. Yuan, K. H. Tiller, H. Al-Ahmad, N. R. Stewart and C. N. Stewart, Jr., *Trends Plant Sci.*, 2008, **13**, 421-429.
6. R. Franke, C. M. McMichael, K. Meyer, A. M. Shirley, J. C. Cusumano and C. Chapple, *Plant J.*, 2000, **22**, 223-234.
7. F. Chen, M. S. Srinivasa Reddy, S. Temple, L. Jackson, G. Shadle and R. A. Dixon, *Plant J.*, 2006, **48**, 113-124.
8. C. Fu, J. R. Mielenz, X. Xiao, Y. Ge, C. Y. Hamilton, M. Rodriguez, Jr., F. Chen, M. Foston, A. Ragauskas, J. Bouton, R. A. Dixon and Z. Y. Wang, *Proc. Natl. Acad. Sci. U S A*, 2011, **108**, 3803-3808.
9. A. Saballos, W. Vermerris, L. Rivera and G. Ejeta, *BioEnergy Research*, 2008, **1**, 193-204.
10. X. Li, J. K. Weng and C. Chapple, *Plant J.*, 2008, **54**, 569-581.
11. K. S. Porter, J. D. Axtell, V. L. Lechtenberg and V. F. Colenbrander, *Crop Sci.*, 1978, **18**, 205-208.
12. Z. Xin, M. L. Wang, N. A. Barkley, G. Burow, C. Franks, G. Pederson and J. Burke, *BMC Plant Biol.*, 2008, **8**, 103.
13. N. A. Palmer, S. E. Sattler, A. J. Saathoff, D. Funnell, J. F. Pedersen and G. Sarath, *Planta*, 2008, **229**, 115-127.
14. B. S. Dien, G. Sarath, J. F. Pedersen, S. E. Sattler, H. Chen, D. L. Funnell-Harris, N. N. Nichols and M. A. Cotta, *BioEnergy Research*, 2009, **2**, 153–164.
15. J. Zeng, Y. Zheng, X. Yu, L. Yu, D. Gao and S. Chen, *Bioresour. Technol.*, 2013, **128**, 385-391.
16. H. Wei, W. Wang, J. M. Yarbrough, J. O. Baker, L. Laurens, S. Van Wychen, X. Chen, L. E. Taylor, II, Q. Xu, M. E. Himmel and M. Zhang, *PLoS One*, 2013, **8**, e71068.
17. M. Kosa and A. J. Ragauskas, *Green Chemistry*, 2013, **15**, 2070-2074.
18. C. Hu, X. Zhao, J. Zhao, S. Wu and Z. K. Zhao, *Bioresour. Technol.*, 2009, **100**, 4843-4847.
19. T. J. Cutright, *Int. Biodeterior. Biodegrad.*, 1995, **35**, 397-408.
20. D. Müncnerová and J. Augustin, *Bioresour. Technol.*, 1994, **48**, 97-106.
21. J. F. Pedersen, J. J. Toy, D. L. Funnell, S. E. Sattler, A. L. Oliver and R. A. Grant, *J. Plant Reg.*, 2008, **2**, 258-262.
22. W. Du, H. Yu, L. Song, J. Zhang, C. Weng, F. Ma and X. Zhang, *Biotechnol. Biofuels*, 2011, **4**, 1754-6834.
23. S. Xie, S. Sun, S. Y. Dai and J. S.Yuan, *Algal Research*, 2013, **2**, 28-33.
24. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker, *Laboratory analytical procedure*, 2008.

25. M. G. Grabherr, B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman and A. Regev, *Nat. Biotechnol.*, 2011, **29**, 644-652.
26. W. Li and A. Godzik, *Bioinformatics*, 2006, **22**, 1658-1659.
27. A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J. F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J. M. Claverie and O. Gascuel, *Nucleic. Acids. Res.*, 2008, **36**, 19.
28. S. Guindon, J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk and O. Gascuel, *Syst. Biol.*, 2010, **59**, 307-321.
29. B. Dien, G. Sarath, J. Pedersen, S. Sattler, H. Chen, D. Funnell-Harris, N. Nichols and M. Cotta, *BioEnergy Research*, 2009, **2**, 153-164.
30. G. J. Leary, K. R. Morgan, R. H. Newman, B. Samuelsson and U. Westermark, in *hfsg*, 1986, vol. 40, p. 221.
31. X. F. Sun, R. Sun, P. Fowler and M. S. Baird, *J. Agric. Food Chem.*, 2005, **53**, 860-870.
32. G. Gilardi, L. Abis and A. E. G. Cass, *Enzyme Microb. Technol.*, 1995, **17**, 268-275.
33. D. Singh, J. Zeng, D. D. Laskar, L. Deobald, W. C. Hiscox and S. Chen, *Biomass Bioenergy*, 2011, **35**, 1030-1040.
34. J. Zhang, Z. Zhu, X. Wang, N. Wang, W. Wang and J. Bao, *Biotechnol. Biofuels*, 2010, **3**, 1754-6834.
35. S. I. Mussatto and I. C. Roberto, *Bioresour. Technol.*, 2004, **93**, 1-10.
36. 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.
37. C. Liers, T. Arnstadt, R. Ullrich and M. Hofrichter, *FEMS Microbiol. Ecol.*, 2011, **78**, 91-102.
38. M. J. Pecyna, R. Ullrich, B. Bittner, A. Clemens, K. Scheibner, R. Schubert and M. Hofrichter, *Appl. Microbiol. Biotechnol.*, 2009, **84**, 885-897.
39. M. Kinne, M. Poraj-Kobielska, S. A. Ralph, R. Ullrich, M. Hofrichter and K. E. Hammel, *J. Biol. Chem*, 2009, **284**, 29343-29349.
40. P. Kersten and D. Cullen, *Fungal Genet. Biol.*, 2007, **44**, 77-87.
41. A. Vanden Wymelenberg, G. Sabat, M. Mozuch, P. J. Kersten, D. Cullen and R. A. Blanchette, *Appl. Environ. Microbiol.*, 2006, **72**, 4871-4877.
42. C. O. Rohr, L. N. Levin, A. N. Mentaberry and S. A. Wirth, *PLoS One*, 2013, **8**, e81033.
43. 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.

44. 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.
45. C. Hori, J. Gaskell, K. Igarashi, M. Samejima, D. S. Hibbett, B. Henrissat and D. Cullen, *Mycologia*, 2013.
46. F. Guillen, C. Munoz, V. Gomez-Toribio, A. T. Martinez and M. Jesus Martinez, *Appl. Environ. Microbiol.*, 2000, **66**, 170-175.
47. J. Jensen, Kenneth A., Z. C. Ryan, A. Vanden Wymelenberg, D. Cullen and K. E. Hammel, *Appl. Environ. Microbiol.*, 2002, **68**, 2699-2703.
48. S. Xie, R. Syrenne, S. Sun and J. S. Yuan, *Curr. Opin. Biotechnol.*, 2014, **27**, 195-203.
49. D. C. Eastwood, D. Floudas, M. Binder, A. Majcherczyk, P. Schneider, A. Aerts, F. O. Asiegbu, S. E. Baker, K. Barry, M. Bendiksby, M. Blumentritt, P. M. Coutinho, D. Cullen, R. P. de Vries, A. Gathman, B. Goodell, B. Henrissat, K. Ihrmark, H. Kauserud, A. Kohler, K. LaButti, A. Lapidus, J. L. Lavin, Y.-H. Lee, E. Lindquist, W. Lilly, S. Lucas, E. Morin, C. Murat, J. A. Oguiza, J. Park, A. G. Pisabarro, R. Riley, A. Rosling, A. Salamov, O. Schmidt, J. Schmutz, I. Skrede, J. Stenlid, A. Wiebenga, X. Xie, U. Kües, D. S. Hibbett, D. Hoffmeister, N. Högberg, F. Martin, I. V. Grigoriev and S. C. Watkinson, *Science*, 2011, **333**, 762-765.
50. G. Fuchs, M. Boll and J. Heider, *Nat. Rev. Microbiol.*, 2011, **9**, 803-816.
51. D. Martinez, L. F. Larrondo, N. Putnam, M. D. Gelpke, K. Huang, J. Chapman, K. G. Helfenbein, P. Ramaiya, J. C. Detter, F. Larimer, P. M. Coutinho, B. Henrissat, R. Berka, D. Cullen and D. Rokhsar, *Nat. Biotechnol.*, 2004, **22**, 695-700.
52. H. Doddapaneni, V. Subramanian, B. Fu and D. Cullen, *Fungal Genet. Biol.*, 2013, **55**, 22-31.

Figure Legend:

Table 1. Semi-quantitative analysis of cell wall components by ^{13}C CPMAS NMR analysis. WT-raw: wild-type sorghum without fungal conversion; WT-6 days: wild-type sorghum after 6-days fungal conversion; b6b12-raw: *bmr6/bmr12* double mutant sorghum without fungal conversion; b6b12-6 days: *bmr6/bmr12* double mutant sorghum after 6-days fungal conversion.

Figure 1. Percentage of weight loss for total sorghum straw (A), lignin (B), cellulose (C) and hemicellulose (D) during the conversion of wild type (solid square), *bmr*6 mutant (solid triangle), *bmr12* mutant (open square) and *bmr*6/*bmr*12 double mutant (open triangle) sorghum biomass by *C. echinulata* FR3. The percentages of weight loss as compared to the original composition in raw sorghum biomass were recorded to represent the composition degradation efficiencies.

Figure 2. Mycelium growth (A) and lipid accumulation (B) of *C. echinulata* FR3 grown in sorghum biomass. FPU activity (C) and xylanase activity (D) of the cultivation supernatant variations during the conversion of wild type (solid square), *bmr*6 mutant (solid triangle), *bmr12* mutant (open square)and *bmr*6/*bmr*12 double mutant (open triangle) sorghum biomass by *C. echinulata* FR3. All assays were carried out in triplicates.

Figure 3. (A) Comparison of mycelium growth and lipid accumulation of *C. echinulata* FR3 grown on acid-pretreated wild type (WT) sorghum straw and non-pretreated *bmr*6/*bmr*12 double mutant (b6b12) sorghum straw for 6 days. (B) Comparison of total sorghum weight and lignin lost during the conversion of acid-pretreated wild-type and non-pretreated double mutant sorghum straw by *C. echinulata* FR3 after 6 days. (C) Comparison of cellulose and hemicellulose degradation during the conversion of acid-pretreated wild-type and non-pretreated double mutant sorghum straws by *C. echinulata* FR3 after 6 days.

Figure 4. Transcriptomic analysis to reveal the lignocellulose degradation mechanisms in *C. echinulata* FR3. (A) Expression abundance of selected cellulases and hemicellulases. (B) Expression abundance of selected extracellular lignolytic enzymes and Fenton system related enzymes. (C) Central aromatic compound catabolism pathway in *C. echinulata* FR3. HGD, homogentisate 1,2-dioxygenase; maiA, maleylacetoacetate isomerase; FAH, fumarylacetoacetase; HAAO, 3-hydroxyanthranilate 3,4-dioxygenase; ACMSD, aminocarboxymuconate-semialdehyde decarboxylase.

Figure 5. An integrated model to elucidate the lignocellulose degradation mechanisms in *C. echinulata* FR3. LMCO, laccase-like multiple copper oxidase; APO, aromatic peroxygenase; CYP, Cytochrome P450; Cel, cellulase; Hem, hemicellulase; QRD, quinone reductase; FeR, ferric reductase; GAO, galactose oxidase; GMC, GMC oxidoreductase; QUT, quinate permeases; ADE, aromatic compound degradation enzymes.

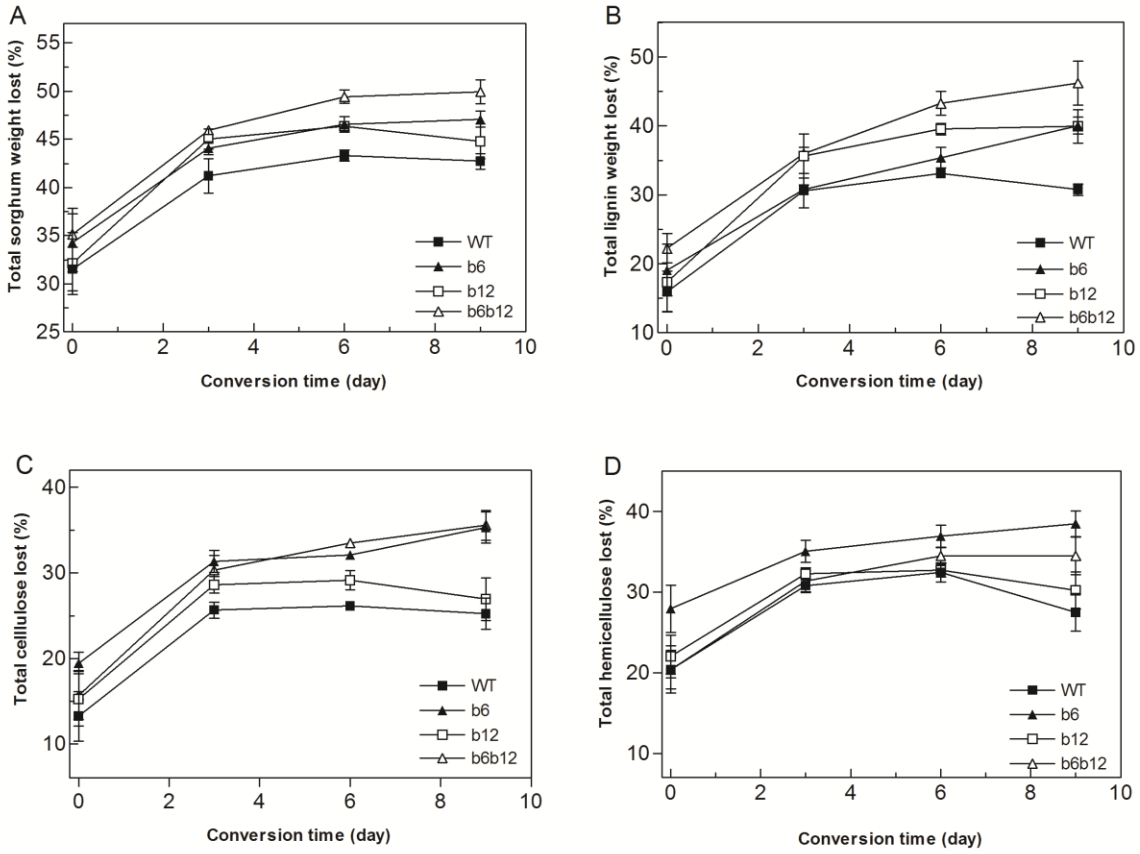
Figure 6. Measurement of iron-reducing activity of the cultivation supernatant of *C. echinulata* FR3 grown on wild-type (solid triangle) sorghum biomass, *bmr6/bmr12* double mutant (solid square) sorghum biomass, and PDB (solid inverted triangle).

620

Biomass samples	Carbohydrate content (%)	Aromatic content (%)	Methoxyl group (%)	Aliphatic carbonyl/ carboxyl group (%)	Aliphatic carbon content (%)	Carbohydrate/ Aromatic ratio
WT-raw	66.7	16.8	3.3	3.7	6.5	4
WT-6 days	75.4	12.1	2.5	2.7	4.1	6.2
b6b12-raw	63.4	18.6	3.2	5.3	6.2	3.4
b6b12-6 days	73.4	13.0	2.4	3.7	3.9	5.7

621

622 Table 1

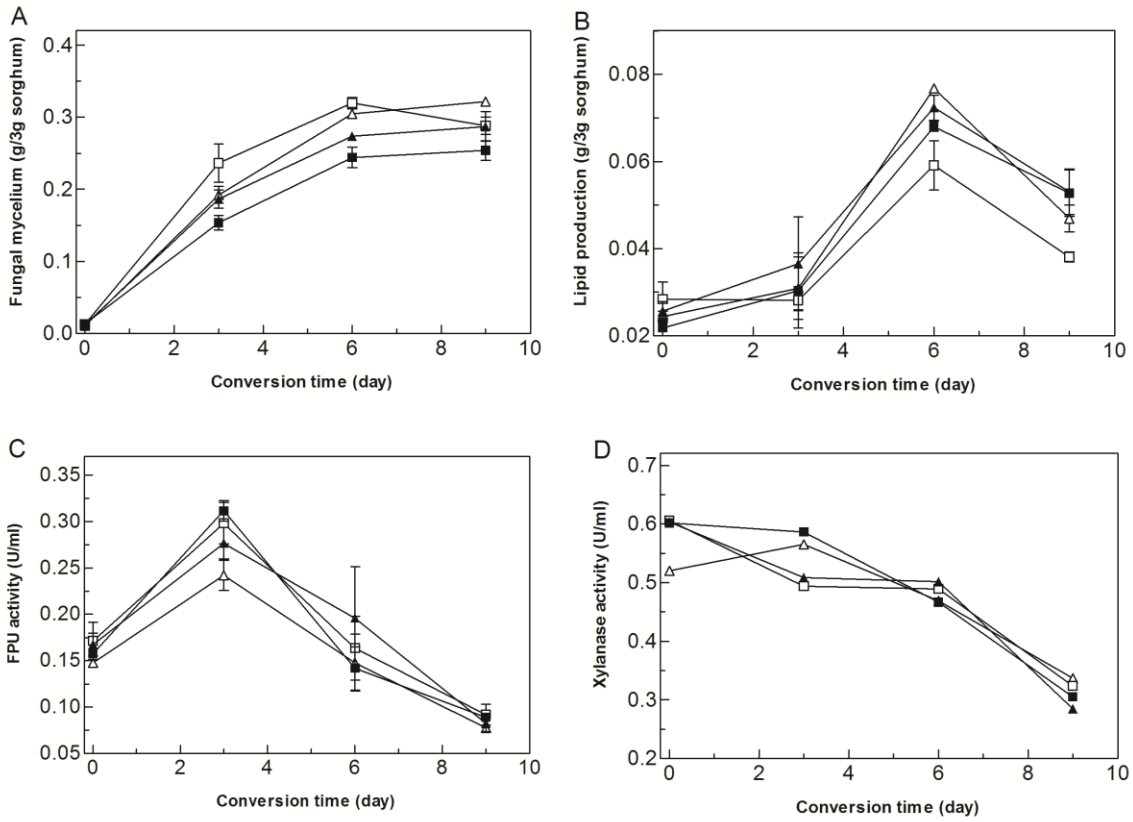


623

624 Figure 1

625

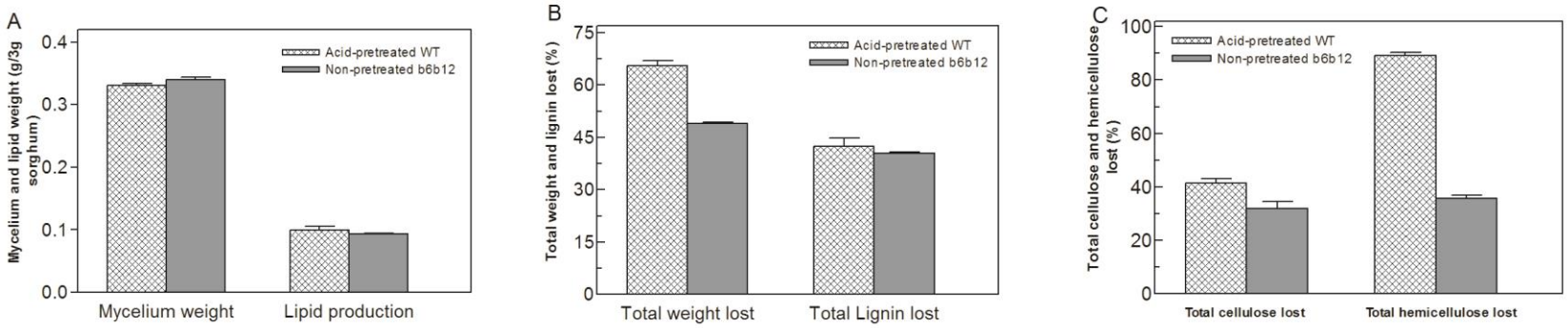
626



627

628 Figure 2

629



630

631 Figure 3

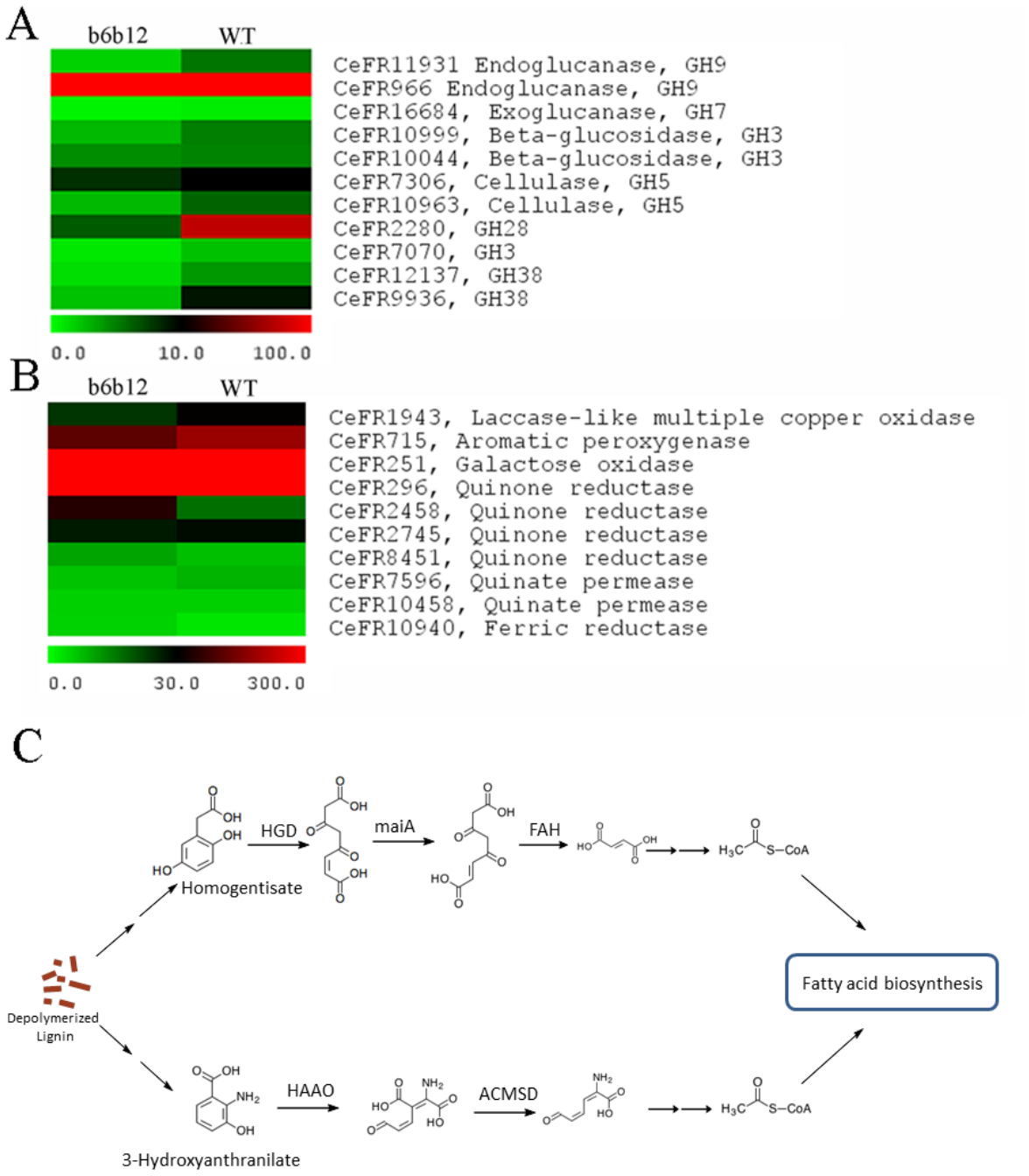


Figure 4

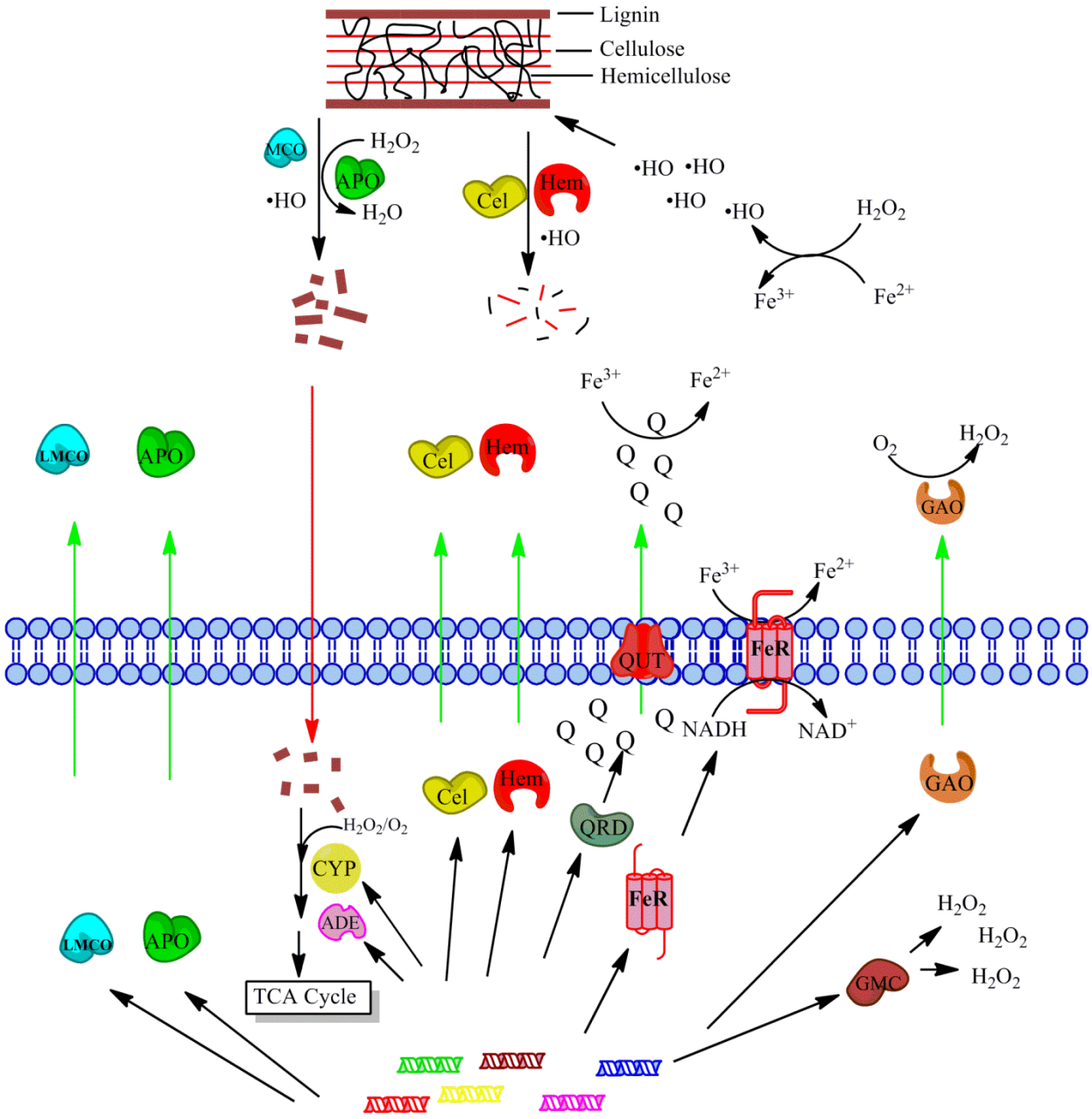
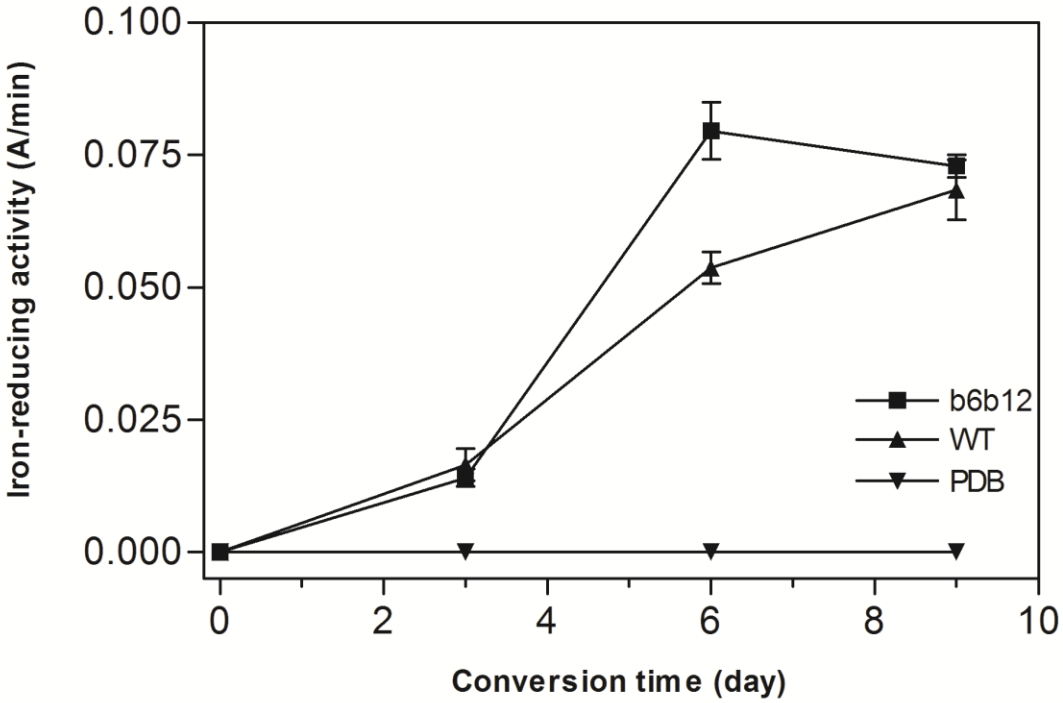
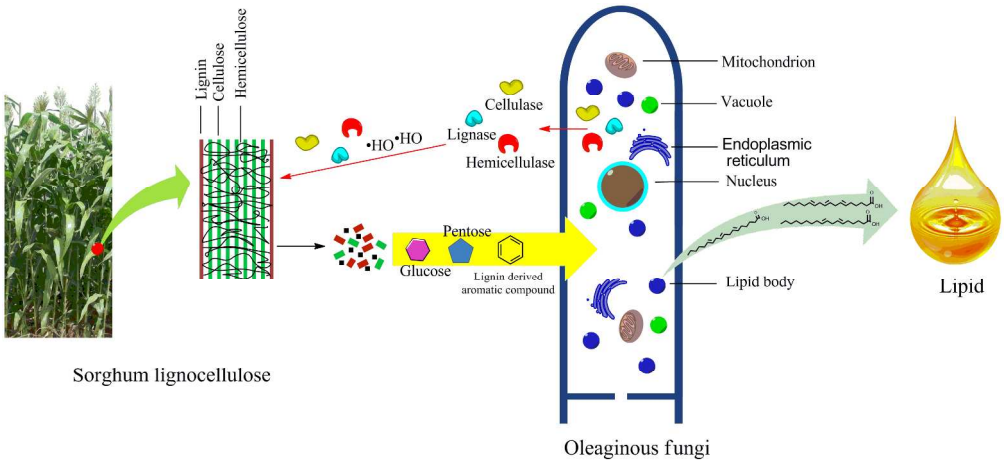


Figure 5



638

639 Figure 6



Biomass conversion of all cell wall components can be achieved by oleaginous fungus C. echinulata FR3. The strain represents one of the rare non-basidiomycetes with a strong lignin degradation machinery and capacity to convert all cell wall components to a fungible product, lipid. The unique lignin degradation capacity enables a consolidated platform for converting all cell wall components potentially without chemi-physical pretreatment.
271x124mm (300 x 300 DPI)