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Saccharification of Pumpkin Residues by Coculturing of *Trichoderma reesei* RUT-C30 and *Phanerochaete chrysosporium* Burdsall with Delayed Inoculation Timing

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ABSTRACT: *Trichoderma reesei* and *Phanerochaete chrysosporium* with different lignocellulose-degrading enzyme systems have received much attention due to their ability to biodegrade lignocellulosic biomass. However, the synergistic effect of the two fungi on lignocellulose degradation is unknown. Herein, a cocultivation of *T. reesei* RUT-C30 and *P. chrysosporium* Burdsall for biodegradation of lignocellulosic pumpkin residues (PRS) was developed to produce soluble saccharide. Results indicated that a cocultivation of the two fungi with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days produced the highest saccharide yield of 53.08% (w/w), and only 20.83% (w/w) of PRS were left after one batch of fermentation. In addition, this strategy increased the activities of secreted cellulases (endoglucanase, cellobiohydrolase, and β-glucosidase) and ligninases (lignin peroxidase and manganese peroxidase), which correlated to the increased saccharide yield. Besides, the resulting monosaccharides including glucose (1.23 mg/mL), xylose (0.13 mg/mL), arabinose (0.46 mg/mL), and fructose (0.21 mg/mL) from cocultures exhibited much higher yields than those from monoculture, which provides basal information for further fermentation research. This bioconversion of PRS into soluble sugars by cocultured fungal species provides a low cost method based on lignocellulose for potential biofuels or other bioproduct production.

KEYWORDS: *pumpkin residues, lignocellulose, biological degradation, saccharification, cocultivation*

INTRODUCTION

Lignocellulose occupies more than 60% of plant biomass on earth and is mainly composed of cellulose, hemicellulose, and lignin.[1−3] Due to its abundance and inexpensiveness, lignocellulosic biomass is an attractive starting material for bioproducts, ranging from biofuels to specialty chemicals.[4,5] In order to realize the effective conversion of lignocellulosic biomass, it is necessary to break the recalcitrant barriers of natural lignocellulose and to liberate fermentable sugars.[5] This involves pretreatment technologies that reduce the lignin content or cellulose crystallinity, increase the specific surface area of cell wall polysaccharides or the pore size of the materials, and thus accelerate enzymatic hydrolysis.[5−7] Compared to the chemical, physical, and thermal processes, biological pretreatments by microorganisms and their secreted enzymes show great potential because they are environmentally friendly and energy efficient.[8−10]

Economical and effective lignocellulolytic enzyme complexes that contain cellulases, hemicellulases, and ligninases can be prepared by substrate fermentation.[1] These lignocellulolytic enzymes are mainly from the fungi, of which *T. reesei* is being widely used as a source of cellulases and hemicellulases for hydrolysis of lignocellulosic cellulose substrates into soluble sugar components.[11] However, the lignin binds cellulose and hemicellulose, and it plays an important role in imparting rigidity and microbial or enzyme resistance to the lignocellulose degradation, bringing about some early difficulties in the lignocellulose pretreatment. To resolve this problem, white rot fungi are good choices that have received extensive attention because of their powerful lignin-degrading enzyme systems.[3,12] Specifically, a species of filamentous basidiomycete white rot fungus, *P. chrysosporium*, participates in the degradation process of lignocellulosic materials by its secreted ligninolytic enzyme complexes such as laccases, manganese peroxidases (MnP), and lignin peroxidase (LiP).[13−15] Recently, coculturing (inoculation at the same time) of fungi with similar secreted lignocellulolytic enzyme systems, for example, cellulase and hemicellulase secreting fungi *T. reesei* and *Aspergillus*, have been successfully employed in producing higher specific enzyme yields based on lignocellulosic biomass.[16−18] However, the synergistic effect of coculturing with different lignocellulolytic enzyme systems is lacking in research. Given that the *T. reesei* and *P. chrysosporium* secrete different lignocellulolytic enzymes,[11,12,15] and cocultures with high fungi diversity and multiple lignocellulose-degrading enzyme systems could enable such cultures to utilize mixtures of substrates with various compositions.[17] This raised the question of whether proper operating conditions of this two fungi coculturing can be reached to enhance individual lignocellulolytic enzyme production for lignocellulose degradation. Inoculation timing may be an important operating condition for coculturing in view of their different metabo-

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lisms;[11,15] however, there is rare related literature reported. Thus, it is worth investigating their coculturing upon changing inoculation timing for lignocellulose saccharification, as well as the underlying mechanism the secreted enzymes are involved in.

The pumpkin residues (PRS), a kind of agricultural byproducts, that were generated during the water extraction of the soluble pumpkin polysaccharide[19] are one lignocellulosic biomass without utilization. The PRS are promising lignocellulosic materials for fermentable sugar production. Toward this objective, a coculturing of *T. reesei* RUT-C30 and *P. chrysosporium* Burdsall based on PRS biomass was developed to enhance lignocellulose degradation for saccharification. Relevant lignocellulose-degrading enzymes were also analyzed to explore the underlying mechanism for the increased yield of saccharides. Moreover, the monosaccharide distributions of the resulting sugars were detected, providing basal information for potential fuels or other bioproduct production.

MATERIALS AND METHODS

Pumpkins, Strains, and Chemicals. Fresh lady godiva pumpkin (*Cucurbitaceae pepo lady godiva*) fruits at the commercially mature stage were purchased from a local commercial market in Beijing, China. PRS used in this study were obtained as follows. First, sliced fresh pumpkins (500 g) and deionized water (2×10^3 mL) were mixed and pulped using a Kau yang mill, and the pumpkin seeds were discarded. Second, the resulting mixtures were centrifuged for 15 min at 2000*g* to precipitate insoluble PRS. Third, to make sure the remaining sugar was washed off, the precipitates were washed three times with 200 mL of deionized water, and each time was followed by one step of centrifugation (2000*g*, 15 min). The resulting precipitates were lyophilized and ground to pass through a 100 mesh sieve (0.45 mm), and 4.45 g of PRS (dry mass) was obtained from 500 g of fresh pumpkins. The powdery PRS was then stored at room temperature prior to use.

The strains of *T. reesei* RUT-C30 and *P. chrysosporium* Burdsall CGMCC 5.776 were obtained from Agricultural Culture Collection of China and China General Microbiological Cultural Collection Center, Beijing, respectively. Both strains were preserved in mineral oil at 4 °C before use. *T. reesei* RUT-C30 was activated by culturing on potato dextrose agar (PDA) slant in a chemostat at 30 °C for 4 days until sporulation occurred; *P. chrysosporium* Burdsall was cultured under the same condition as that of *T. reesei* RUT-C30 but grew for 7 days.[20] The prepared PDA medium was autoclaved at 121 °C for 20 min before use. All reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) and of analytical grade.

Medium. The preculture medium was prepared as follows (one flask): 3.0 g/L glucose, 1.0 g/L peptone, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.0032 g/L $MnSO_4{\cdot}H_2O$. The PRS fermentation medium was prepared as follows (one flask): 6.0 g/L powdery PRS, 2.0 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 0.4 g/L $MgSO_4$, 0.5 g/L $CaCl_2$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.0032 g/L $MnSO_4{\cdot}H_2O$, 2 mL/L Tween-80. For both media, the initial pH value was adjusted to 5.0 by adding citrate buffer solution (0.5 M). Finally, the media were sterilized by autoclaving at 121 °C for 20 min before fermentation.

Fermentation Conditions. Cultivation was performed in two stages: seed growth and saccharides production. For the seed growth stage, *T. reesei* RUT-C30 from the PDA culture was inoculated into a 250 mL Erlenmeyer flask with 50 mL of preculture medium. Each flask was placed in a shaking incubator at 30 °C and 170 rpm for 36 h to obtain an amount of biomass (about 1×10^6 spores/mL) for later degradation.[21] *P. chrysosporium* Burdsall was precultured for 4 days to obtain a satisfactory biomass (about 5×10^5 spores/mL) under the same conditions as *T. reesei* RUT-C30 cultivation.

At the saccharide production stage, two culturing types were performed: monoculture and cocultures. Monoculture was conducted by inoculating 1 mL of seed cultures (2×10^5 spores/mL) of *T. reesei* RUT-C30 or *P. chrysosporium* Burdsall into a 250 mL Erlenmeyer flask with 100 mL of PRS medium (0.6 g of powdery PRS), followed by 7 days of incubation in a shaking incubator at 170 rpm, 30 °C. For the cocultures, different inoculation sequencing led to two absolutely different coculturing types. First, 1 mL of seed cultures (1×10^5 spores/mL) of *T. reesei* RUT-C30 was inoculated into a 250 mL Erlenmeyer flask with 100 mL of PRS medium (0.6 g of powdery PRS), and they were incubated in a shaking incubator at 170 rpm for 7 days, 30 °C. Then, 1 mL of *P. chrysosporium* Burdsall seed culture (1×10^5 spores/mL) was inoculated into the above medium (*T. reesei* RUT-C30 growing in) with different delayed time (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 days) after *T. reesei* RUT-C30 inoculation. The second type of cocultures just changed the inoculation sequence of the two fungi but followed the same cultivation conditions as the first.

Determination of Inoculation Amount of Two Fungi. The effect of different inoculation amount of the two fungi coculturing on saccharides production was investigated. Each concentration of the seed cultures of *T. reesei* RUT-C30 or *P. chrysosporium* was set as 1×10^5 spores/mL, and each inoculation amount (mL) of the two fungi was both set ranging from 0.5 to 2.0 mL (0.5, 1.0, and 2.0 mL). Thus, there are 9 groups, namely, (0.5 + 0.5), (0.5 + 1.0), (0.5 + 2.0), (1.0 + 0.5), (1.0 + 1.0), (1.0 + 2.0), (2.0 + 0.5), (2.0 + 1.0), and (2.0 + 2.0) mL of two fungi for cocultivation. Here, the two numbers in the bracket represent the inoculation volume (mL) of *T. reesei* RUT-C30 or *P. chrysosporium*, respectively. The fermentation process followed the same procedure as described in "Fermentation Conditions".

Preparation of Saccharides from Monoculture or Cocultures of PRS. The saccharides yield in the fermentation broth was detected every other day (8 times including the zeroth day) during 7 days. Briefly, the fermentation broth of monoculture or cocultures was centrifuged for 15 min at 6000*g* to obtain the supernatant mixtures. Then the supernatant was deproteinized with SEVAG reagent.[22] After that, the resultant supernatant was mixed with three volumes of ethanol (95%) and kept standing overnight at 4 °C.[19] Finally, the precipitates from the ethanol dispersion were collected by centrifugation at 6000*g* for 15 min and were dissolved in distilled water. The resulting isolates were lyophilized to obtain saccharide powder.

Analytical Methods. Contents of cellulose and hemicellulose in PRS were determined by the reported method.[23] The lignin content was determined according to the "National renewable energy laboratory (NREL): Laboratory Analytical Procedure (LAP) (Version 2006)". Each component (cellulose, hemicellulose, and lignin) content in PRS before degradation was as follows (percentage in dry biomass, w/w): cellulose 44.2%, hemicelluloses 18.7%, and lignin 9.0%. After degradation, the fermentation broth of monoculture or cocultures was centrifuged for 15 min at 6000*g* to obtained the precipitates; followed by three times washing by deionized water (500 mL), and each time was followed by one step of centrifugation (6000*g*, 15 min) to make sure the fungi and the remaining sugar were removed. The resulting precipitates, thus the remaining lignocellulosic residue, were collected and lyophilized for composition analysis according to the same methods as that of PRS. Component degradation rate (percentage of degradation, w/w) were calculated based on the percent ratio of each component weight loss after biodegradation to the component content in initial PRS, and it is defined as eq 1.

$$\begin{aligned}&\text{Degradation rate (\%)}\\&= \{[\text{Component content}_{\text{PRS}}\,(\text{g})\\&- \text{Component content}_{\text{remaining residue}}\,(\text{g})]\\&/[\text{Component content}_{\text{PRS}}\,(\text{g})]\} \times 100\%\end{aligned} \quad (1)$$

where the Component $\text{content}_{\text{PRS}}$ is the content of cellulose, or hemicellulose, or lignin in initial PRS and Component $\text{content}_{\text{remaining residue}}$ is the content of cellulose, or hemicellulose, or lignin in the remaining PRS after degradation. All samples were analyzed in triplicate.

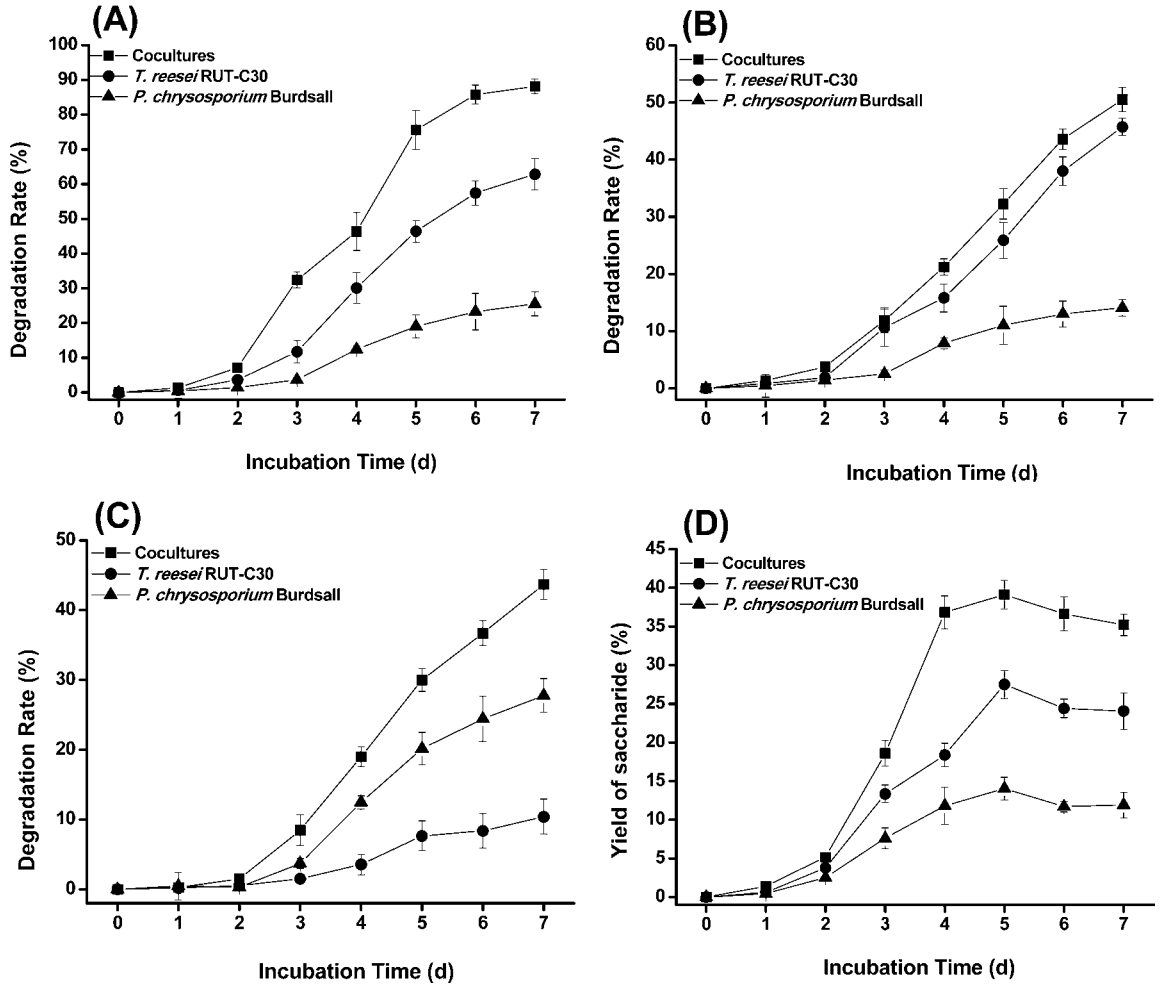

Figure 1. Degradation rates of (A) cellulose, (B) hemicellulose, and (C) lignin in lignocellulosic PRS, and (D) the resulting saccharide in the fermentation broth of monoculture of *P. chrysosporium* Burdsall, monoculture of *T. reesei* RUT-C30, and cocultures of the two fungi (simultaneously inoculated).

Saccharide content released from biodegradation of PRS was determined using Phenol-sulfuric acid method with glucose as a standard.[24] The yield of saccharides was calculated as according to the following eq 2.

$$\text{Yield (\%)} = \{[\text{Saccharide content (g)}] / [\text{PRS content (g)} \times 0.629]\} \times 100\% \quad (2)$$

where 0.629 is the ratio of the sum of cellulose and hemicellulose content in PRS (w/w). All samples were analyzed in triplicate.

Enzyme Analysis. Lignocellulolytic enzymes obtained from monoculture of *T. reesei* RUT-C30, *P. chrysosporium* Burdsall, and cocultures were analyzed in supernatants throughout the experiment every 1.0 day. Cellulase activities of endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (βG) were measured according to the procedures of Cai et al.[25] Assays for hemicellulase, including xylanase,[26] pectinase,[27] and mannase,[28] they were all developed at 50 °C. Ligninolytic enzymes LiP, MnP, and laccase were analyzed as follows. The LiP assay was based on the oxidation of 32 μM Azure B and 100 μM H_2O_2 in 50 mM sodium tartrate buffer (pH 4.5).[29] MnP assay was conducted in solution containing 100 μM $MnSO_4$ and 100 μM H_2O_2 in 0.1 mM sodium tartrate buffer at pH 5.0.[30] Laccase activity was assayed by detecting the oxidation of 5 mM 2, 2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) in a 50 mM acetate buffer sodium at pH 5.0.[31] Cellulase, hemicellulase, and lignase activities were all expressed as international units (U) and were defined as 1 μmol of product formed per minute under the assay conditions. The final activity is the mean value of three measurements.

Analysis of Monosaccharide Composition. Monosaccharide composition was analyzed by high performance liquid chromatography (HPLC) (KNAUER series, Germany) with a refractive index detector and a Shodex SP 0810 Pb^{2+} carbohydrate column (8 mm ×300 mm). The column oven temperature was 80 °C. Specially, the mobile phase was ultrapure water and the flow rate was 1.0 mL/min. The prepared saccharide (50 mg) was hydrolyzed with 2 mL of 2 M trichloroacetic acid for 6 h at 100 °C in a sealed glasstube. After hydrolyzation, the monosaccharides were dried (60 °C) in the fume cupboard. Sample and standards, prepared in solution (5 mg/mL), were filtered through 0.45 μm membranes and the injection volume was 20 μL. Glucose, galactose, arabinose, fructose, mannose, and xylose were used as monosaccharide composition standards.

RESULTS AND DISCUSSION

Biodegradation of Lignocellulosic PRS and Saccharide Production. Degradation rates of lignocellulose component and the resulting saccharide yield in the fermentation broth of *P. chrysosporium* Burdsall, *T. reesei* RUT-C30 and cocultures (simultaneous inoculation) were shown respectively in Figure 1. As cultivation time increased upon 7 days of fermentation, the maximum degradation rates of cellulose were 25.49, 62.84, and 88.14%, respectively, by monoculture of *P. chrysosporium* Burdsall, *T. reesei* RUT-C30 and cocultures (Figure 1A). Similar results of the highest degradation rates of hemicellulose and lignin were obtained, that is 14.09, 45.73, and

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50.54% for hemicellulose (Figure 1B), and 27.77, 10.40, and 43.68% for lignin (Figure 1C) by monoculture of *P. chrysosporium* Burdsall, *T. reesei* RUT-C30 and cocultures, respectively. In short, the cocultures of *P. chrysosporium* Burdsall and *T. reesei* RUT-C30 (simultaneous inoculation) revealed the best lignocellulose-degrading ability toward cellulose and lignin among the three cultivation types, although no significant difference was observed in hemicellulose-degrading rate as compared to that of monoculture of *T. reesei* RUT-C30.

The fermentation and degradation of lignocellulosic PRS by the fungi was also a simultaneous saccharification process as shown in Figure 1D. The yields of saccharide increased gradually, and the maximum value during the 7 days of fermentation ranged to 14.04%, 27.50%, and 39.12% by monoculture of *P. chrysosporium* Burdsall, *T. reesei* RUT-C30 and cocultures (simultaneous inoculation), respectively. Saccharide yields of the three cultivation types all peaked at fifth day, and then they decreased, a consequence of saccharification of lignocellulosic PRS and saccharide consumption for fungi growth.

It is well-known that cellulose is a linear polymer of glucose linked through β-1, 4-linkages, and it is usually arranged in microcrystalline structures and complexed with hemicellulose and lignin, which complicates its hydrolysis.[32,33] In order to resolve this problem, lignocellulose needs to be hydrolyzed into soluble carbohydrates by various technologies. In the present case, a coculturing of *P. chrysosporium* Burdsall and *T. reesei* RUT-C30 for PRS fermentation rather than monoculture remarkably increased degradation rates of cellulose and lignin (Figure 1A and C), which involved in the pretreatment strategy based on removal or hydrolysis of lignin and reduction in cellulose crystallinity.[4,34] This coculturing biological treatment also represented a new fermentation type where these two fungi respectively involved in the degradation of cellulose and lignin. However, this raised the question of why this coculturing reached the highest degradation ratio of PRS and saccharide yield. In view of their different metabolisms, effects of different inoculation timing of two fungi on PRS degradation and enzyme activity changes are worthy of investigation.

Determination of Inoculation Timing of Cocultivation. Before investigating the effect of inoculation timing of cocultivation on saccharide yield, the inoculation amount of the two fungi was studied (Figure 2). It showed that the group 1.0 mL of *T. reesei* RUT-C30 (1×10^5 spores/mL) cocultured with 1.0 mL of *P. chrysosporium* Burdsall (1×10^5 spores/mL) revealed the highest saccharide yield (39.12%); less or more inoculation amount all decreased the yield to different extents (Figure 2). Results indicated that inoculation amount is an important factor affecting saccharide yield. Lower fungal biomass inoculation may influence the enzyme production, resulting in the lower saccharide yield, while increasing inoculation amount also brought about a lower saccharide production, which was probably due to the higher saccharide consumption for fungi growth. Thus, we choose 1 mL of *T. reesei* RUT-C30 and 1 mL of *P. chrysosporium* Burdsall for cocultivation.

In order to gain a better understanding of the synergistic effect of cocultivation timing on saccharides production, different delayed time (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 d) of *P. chrysosporium* Burdsall or *T. reesei* RUT-C30 inoculation for fermentation of PRS was performed, and the resulting saccharide yields are shown in Figure 3. Upon 7 day

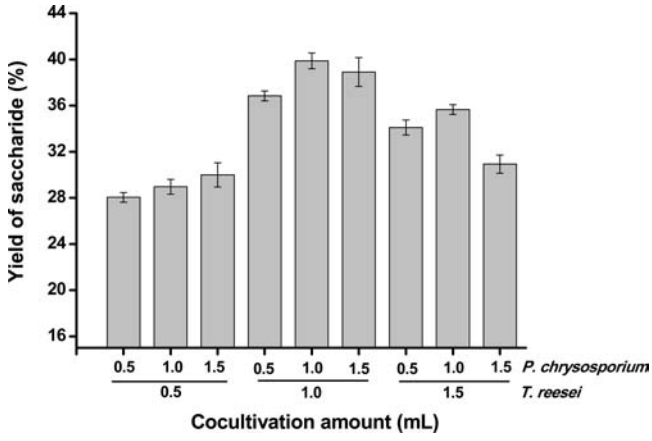

Figure 2. Yield of saccharides in cocultivation influenced by different inoculation amount (mL) of *T. reesei* RUT-C30 and *P. chrysosporium* Burdsall. The concentrations of *T. reesei* RUT-C30 and *P. chrysosporium* Burdsall inoculation are both set as 1×10^5 spores/mL.

cocultivation with *T. reesei* RUT-C30 inoculation delayed for 0 day, it reached to the highest saccharide yield of 39.12% after which the yield gradually decreased (Figure 3A). On the other hand, the type with *P. chrysosporium* Burdsall inoculation delayed time for 1.5 days yielded the highest value of 53.08% (Figure 3B). These results suggested that the coculturing with different delayed time inoculation of *P. chrysosporium* Burdsall or *T. reesei* RUT-C30 after another significantly influenced the lignocellulose degradation. Specially, the saccharide yield from cocultivation with *P. chrysosporium* Burdsall inoculation delayed for 1.5 day increased 3.77- and 1.93-fold compared with that from monocultivation of *P. chrysosporium* Burdsall and *T. reesei* RUT-C30, respectively. In addition, the final lignocellulose component changes and saccharide production before and after this kind of coculturing are shown in Table 1. Results indicated that when 0.6 g of PRS (6.0 g/L, 100 mL media in a 250 mL flask) was performed for cocultivation, about 0.2 g of soluble saccharide was produced and only 20.83% of pumpkin residues remained after one batch of fermentation. The final degradation ratios of cellulose, hemicellulose and lignin were 93.2, 58.1, and 64.8%, respectively (Table 1).

This finding was reminiscent of the enhanced glucan degradation by the cocultures of *Aspergillus niger* and *T. reesei* Rut-C30 as a result of the enhanced cellulase production.[21] However, it is worth mentioning that the substrates and the coculture fungi used in the present work are different from above study. *P. chrysosporium* Burdsall, rather than *Aspergillus niger* cocultured with *T. reesei* Rut-C30 probably lead to distinct lignocellulosic enzymes for enhanced PRS degradation. Therefore, the complex enzyme mixtures throughout the cocultivation reflecting the synergy effect of this kind of coculturing were further studied below.

Enzyme Analysis throughout the Coculturing Process. The activities of secreted lignocellulose-degrading enzymes, including cellulases (EG, CBH, and βG), hemicellulases (xylanase, pectinase, and mannase), and ligninases (LiP, MnP, and laccase), were studied during the entire fermentation periods (Figure 4). As shown, only marginal cellulose and hemicellulose activities were observed during the whole fermentation periods in the monoculture of *P. chrysosporium* Burdsall. In contrast, cocultivation with *P. chrysosporium* Burdsall inoculation delayed time for 1.5 d remarkably improved EG, CBH and βG activities to 3.39 U/ml

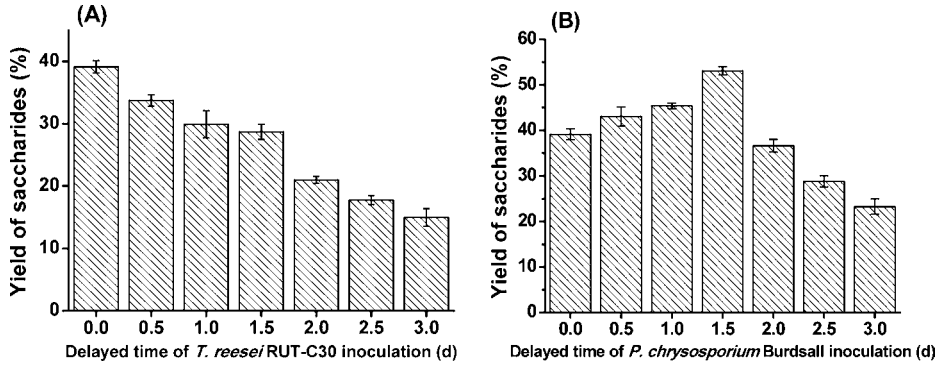

Figure 3. Yield of saccharides in cocultivation influenced by different delayed time of (A) *T. reesei* RUT-C30 and (B) *P. chrysosporium* Burdsall inoculation after another.

Table 1. Component Changes in Lignocellulosic PRS and the Saccharide Production by Coculturing with *P. chrysosporium* Burdsall Inoculation Delayed for 1.5 Days by One Batch of Fermentation

	composition in PRS (100 mL of PRS media after one batch of fermentation)				
fermentation time (d)	total weight (g)	cellulose (g)	hemicellulose (g)	lignin (g)	saccharide production (g)
0	0.6	0.265 ± 0.021	0.112 ± 0.020	0.054 ± 0.011	—[a]
7	0.125 ± 0.014	0.018 ± 0.002	0.047 ± 0.006	0.019 ± 0.003	0.201 ± 0.012

[a]Value of saccharide content that was hardly detected in 0th day fermentation.

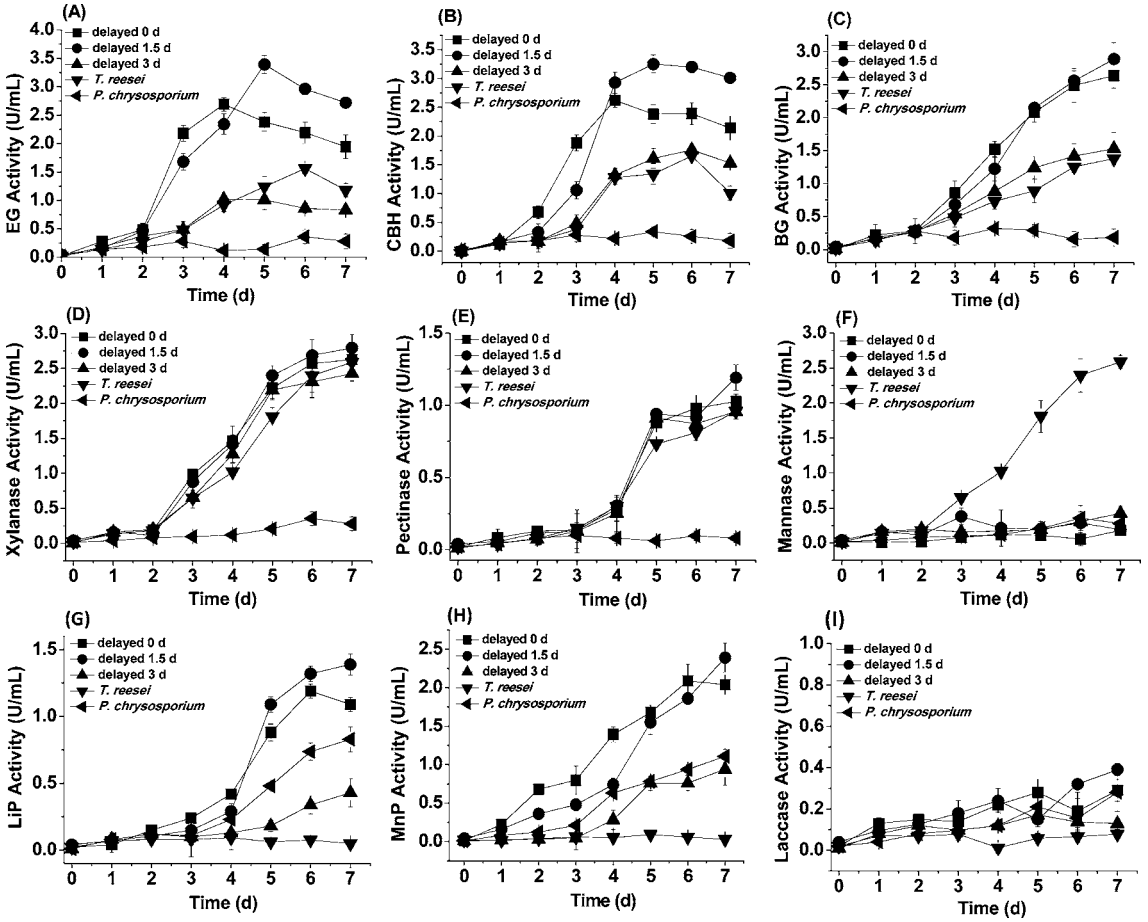

Figure 4. Time courses of activities of (A–C) cellulases, (D–F) hemicellulases, and (G–I) ligninases influenced by cocultivation with different delayed time of *P. chrysosporium* Burdsall inoculation after *T. reesei* RUT-C30.

(day 5) and 3.25 U/mL (day 5) and 2.89 U/ml (day 7), respectively; whereas inoculation delayed for 3 d played a negative effect on the these activities and 0 d delay joined the middle value (Figure 4A–C). Thus, this synergetic effect of coculturing by a delayed inoculation of *P. chrysosporium* Burdsall for 1.5 d gave an obvious advantage over

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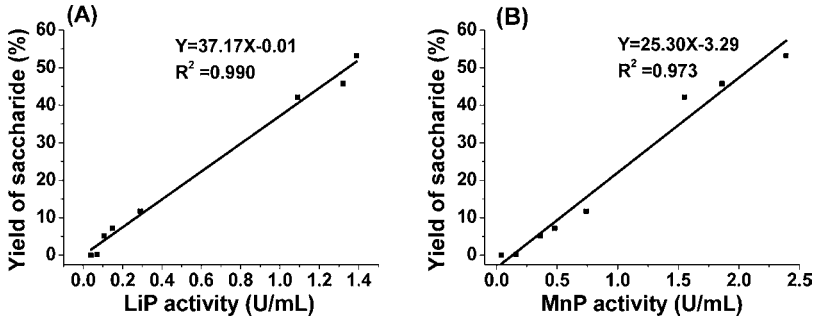

Figure 5. Correlations between saccharide production and ligninase activities (A, LiP, and B, MnP) as exhibited in the cocultivation of two fungi with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days.

monoculturing for improving EG, CBH, and βG activities. As for hemicellulase, including xylanase and pectinase, coculturing with a delayed inoculation of *P. chrysosporium* initiated no remarkable changes as compared to the control (monoculture of *T. reesei*) (Figure 4D and E); meanwhile, this kind of coculturing significantly inhibited the mannase activity (Figure 4F). Similar to cellulases, an inoculation of *P. chrysosporium* delayed for 0 and 1.5 days both improved the activities of ligninases, that is LiP (1.09 U/mL and 1.39 U/mL) and MnP (2.04 U/mL and 2.39 U/mL), respectively. However, the maximum values were deferred as the delayed time increased (Figure 4G and H). With regard to the laccase activity, only negligible enzymic activities were detected in all fermentation types for PRS degradation (Figure 4I), exhibiting a low laccase secretion ability of the current fermentation type.

Within the three main polymers in lignocellulose, lignin is a highly branched macromolecule composed of several types of aromatic acids that has an important role in preventing lignocellulosic materials against biological attack.[32] Thus, the majority of lignocellulosic pretreatment strategies were then focused on the removal or degradation of lignin.[35] As shown in Figure 5, a good correlation could be observed between ligninase activities and saccharide production as exhibited in the cocultivation with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days. Hence, there is a possibility that the increased ligninase activities coordinately promoted the biodegradation of lignocellulosic PRS. In addition, only coculturing with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days produced the highest cellulose activities of EG, CBH, and βG, which could lower the highly recalcitrant of crystalline cellulose. The higher CBH activity could even alleviate the inhibited effect on cellulose degradation caused by cellobiose.[36] These beneficial aspects, that is the enhanced cellulase and ligninase activities, together resulted in the improved lignocellulose degradation. This outcome is similar to the result of the solid substrate fermentation where *Trichoderma reesei* and *Aspergillus niger* were used to produce high activities of endoglucanase and β-glucosidase.[37] We inferred that the delayed inoculation could regulate metabolic capabilities of both *P. chrysosporium* Burdsall and *T. reesei* RUT-C30, especially in terms of the increased cellulase and ligninase activities for degrading cellulose and lignin. Consequently, this result also explains why coculturing with *P. chrysosporium* Burdsall delayed inoculation induced the highest saccharide yield.

Monosaccharide Composition from Different Fermentation Cultures. The monosaccharide composition of the resulting sugars after fermentation is helpful to provide information and guidelines for usage of pumpkin residues, for example, its potential fermentation for biofuel or other chemical production. A comparison of the monosaccharide composition in cocultures with that in monoculture was conducted to deeply understand the synergetic effect of cocultivation on saccharide production. As shown in Figure 6,

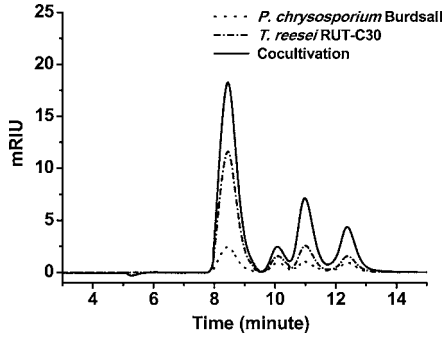

Figure 6. Distributions of monosaccharide composition in the monoculture of *P. chrysosporium* Burdsall, monoculture of *T. reesei* RUT-C30, and cocultures of two fungi with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days.

three cultivation types all produced four species of monosaccharides including glucose, xylose, arabinose, and fructose, which reflect the lignocellulose component distribution and relative enzyme activity that involved in the PRS hydrolysis. Specifically, the coculturing with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days produced the highest monosaccharide contents: glucose (1.23 mg/mL), xylose (0.13 mg/mL), arabinose (0.46 mg/mL), and fructose (0.21 mg/mL) among the three cultivation types, while the yields of these four sugars from monoculture of *P. chrysosporium* Burdsall revealed the lowest values. This is not surprising because the cocultivation of the two fungi with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days remarkably improved EG, CBH, and βG activities (Figure 4A–C), which was responsible for degradation of cellulose for sugar production.[33] On the other hand, this kind of coculturing also significantly improved the activities of ligninases (laccase and LiP), which promote the degradation of the lignin as well as the decrease of the rigidity and microbial or enzyme resistance of the cellulose and hemicellulose.[1,38] Thus, the outcome of monosaccharide content from different fermentation broths was a reflection of lignocellulose-degrading enzyme activities in cocultures or monoculture.

In the present work, we developed a cocultivation of *T. reesei* RUT-C30 and *P. chrysosporium* Burdsall for biodegradation of

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lignocellulosic PRS producing soluble saccharide. Results demonstrated that cocultivation of the two fungi with *P. chrysosporium* Burdsall inoculation delayed for 1.5 days produced remarkably higher saccharide yield compared with that from monoculture of *P. chrysosporium* Burdsall or *T. reesei* RUT-C30, respectively. This attempt for the biodegradation of lignocellulosic agricultural wastes by cocultures of two fungi would be of low chemical and energy use, and it also is an environmentally friendly, controllable, and effective approach. This bioconversion of PRS into soluble sugars by cocultured fungal species provides a low cost method based on lignocellulose for potential biofuels or other bioproduct production.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

T. reesei, *Trichoderma reesei*; *P. chrysosporium*, *Phanerochaete chrysosporium*; PRS, pumpkin residues; PDA, potato dextrose agar; EG, endoglucanase; CBH, cellobiohydrolase; βG, β-glucosidase; LiP, Lignin peroxidase; MnP, manganese peroxidase; HPLC, high performance lipid chromatography

REFERENCES

(1) Tengerdy, R. P.; Szakacs, G. Bioconversion of lignocellulose in solid substrate fermentation. *Biochem. Eng. J.* **2003**, *13*, 169−179.

(2) El-Zawawy, W. K.; Ibrahim, M. M.; Abdel-Fattah, Y. R.; Soliman, N. A.; Mahmoud, M. M. Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production. *Carbohyd. Polym.* **2011**, *84*, 865−871.

(3) Wan, C.; Li, Y. Microbial delignification of corn stover by ceriporiopsis subvermispora for improving cellulose digestibility. *Enzyme Microb. Tech.* **2010**, *47*, 31−36.

(4) Fu, D.; Mazza, G.; Tamaki, Y. Lignin extraction from straw by ionic liquids and enzymatic hydrolysis of the cellulosic residues. *J. Agric. Food Chem.* **2010**, *58*, 2915−2922.

(5) Xu, C.; Ma, F.; Zhang, X. Lignocellulose degradation and enzyme production by irpex lacteus CD2 during solid-state fermentation of corn stover. *J. Biosci. Bioeng.* **2009**, *108*, 372−375.

(6) Hendriks, A. T. W. M.; Zeeman, G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **2009**, *100*, 10−18.

(7) Binod, P.; Satyanagalakshmi, K.; Sindhu, R.; Janu, K. U.; Sukumaran, R. K.; Pandey, A. Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. *Renew. Energ.* **2012**, *37*, 109−116.

(8) Itoh, H.; Wada, M.; Honda, Y.; Kuwahara, M.; Watanabe, T. Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. *J. Biotechnol.* **2003**, *103*, 273−280.

(9) Laser, M.; Schulman, D.; Allen, S. G.; Lichwa, J.; Antal, M. J.; Lynd, L. R. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. *Bioresour. Technol.* **2002**, *81*, 33−44.

(10) Chandra, R. P.; Bura, R.; Mabee, W. E.; Berlin, A.; Pan, X.; Saddler, J. N. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics. *Adv. Biochem. Eng. Biotechnol.* **2007**, *108*, 67−93.

(11) Montenecourt, B. S.; Eveleigh, D. E. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. *Adv. Chem. Ser.* **1979**, *181*, 289−301.

(12) Tuor, U.; Winterhalter, K.; Fiechter, A. Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. *J. Biotechnol.* **1995**, *41*, 1−17.

(13) Burdsall, H. H. J. Taxonomy of industrially important white-rot fungi. In *Environmentally Friendly Technologies for the Pulp and Paper Industry*; Young, R. A., Akhtar, M. J., Eds.; Wiley: New York, 1998; pp 259−272.

(14) Ramírez, D. A.; Muñoz, S. V.; Atehortua, L.; Michel, F. C., Jr. Effects of different wavelengths of light on lignin peroxidase production by the white-rot fungi *Phanerochaete chrysosporium* grown in submerged cultures. *Bioresour. Technol.* **2010**, *101*, 9213−9220.

(15) Reid, I. D.; Chao, E. E.; Dawson, P. Lignin degradation by *Phanerochaete chrysosporoum* in agitated cultures. *Can. J. Microbiol.* **1985**, *31*, 88−90.

(16) Ahamed, A.; Vermette, P. Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA as fed batch in a stirred tank bioreactor. *Biochem. Eng. J.* **2008**, *42*, 41−46.

(17) Castillo, M. R.; Gutierrez-Correa, M.; Linden, J. C.; Tengerdy, R. P. Mixed culture solid substrate fermentation for cellulolytic enzyme production. *Biotechnol. Lett.* **1994**, *16*, 967−972.

(18) Duenas, R.; Tengerdy, R. P.; Gutierrez-Correa, M. Cellulase production by mixed fungal solid substrate fermentation of sugar cane bagasse. *World J. Microbiol. Biotechnol.* **1995**, *11*, 333−337.

(19) Li, Q.; Fu, C.; Ren, Y.; Hu, G.; Cai, T. Effects of protein-bound polysaccharide isolated from pumpkin on insulin in diabetic rats. *Plant Food Hum. Nurt.* **2005**, *60*, 1−4.

(20) Falih, A. M. Influence of heavy-metals toxicity on the growth of *Phanerochaete Chrysosporium*. *Bioresour. Technol.* **1997**, *60*, 87−90.

(21) Fang, H.; Zhao, C.; Song, X. Y. Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: Response surface methodology or using cellulase from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02. *Bioresour. Technol.* **2010**, *101*, 4111−4119.

(22) Staub, A. M. Removal of proteins: Sevag method. *Methods Carbohydr. Chem.* **1965**, *5*, 5−6.

(23) Van Soest, P. J.; Wine, R. H. The determination of lignin and cellulose in acid detergent fiber with permanganate. *J. Assoc. Off. Anal. Chem.* **1968**, *51*, 780−785.

(24) Dubois, M.; Gilles, K. A.; Hamilton, J. K. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

(25) Cai, Y. J.; Chapman, S. J.; Buswell, J. A.; Chang, S. T. Production and distribution of endoglucanase, cellobiohydrolase, and beta-glucosidase components of the cellulolytic system of volvariella volvacea, the edible straw mushroom. *Appl. Environ. Microbiol.* **1999**, *65*, 553−559.

(26) Machuca, A.; Ferraz, A. Hydrolytic and oxidative enzymes produced by white- and brown-rot fungi during Eucalyptus grandis decay in solid medium. *Enzyme Microb. Technol.* **2001**, *29*, 386−391.

(27) Wilkins, M. R.; Widmer, W. W.; Grohmann, K.; Cameron, R. G. Hydrolysis of grapefruit peel waste with cellulase and pectinase enzymes. *Bioresour. Technol.* **2007**, *98*, 1596−1601.

(28) Khalil, A. I. Production and characterization of cellulolytic and xylanolytic enzymes from the ligninolytic white-rot fungus *Phanerochaete chrysosporium* grown on sugarcane bagasse. *World J. Microbiol. Biotechnol.* **2002**, *18*, 753−759.

(29) Archibald, F. S. A new assay for lignin-type peroxidases employing the dye azure B. *Appl. Environ. Microbiol.* **1992**, *58*, 3110−3116.
(30) Camarero, S.; Sarkar, S.; Ruiz, D. F.; Martinez, M. J.; Martinez, A. T. Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. *J. Biol. Chem.* **1999**, *274*, 10324−10330.
(31) Hakala, T. K.; Lundell, T.; Galkin, S.; Maijala, P.; Kalkkinen, N.; Hatakka, A. Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus *Physisporinus rivulosus* grown on spruce wood chips. *Enzyme Microb. Technol.* **2005**, *36*, 461−468.
(32) Leonowicz, A.; Matuszewska, A.; Luterek, J.; Ziegenhagen, D.; Wojtas-Wasilewska, M.; Cho, N. S.; Hofrichter, M.; Rogalski, J. Biodegradation of lignin by white rot fungi. *Fungal Genet. Biol.* **1999**, *27*, 175−185.
(33) Tomme, P.; Warren, R. A.; Gilkes, N. R. Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol.* **1995**, *37*, 1−81.
(34) Malherbe, S.; Cloete, T. E. Lignocellulose degradation: fundamentals and applications. *Rev. Environ. Sci. Biotechnol.* **2002**, *1*, 105−114.
(35) Xie, Y. M.; Yasuda, S.; Wu, H.; Liu, H. B. Analysis of the structure of lignin−carbohydrate complexes by the specific C-13 tracer method. *J. Wood Sci.* **2000**, *46*, 130−136.
(36) Juhász, T.; Szengyel, Z.; Réczeyb, K.; Siika-Aho, M.; Viikari, L. Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. *Process Biochem.* **2005**, *40*, 3519−3525.
(37) Gutierrez-Correa, M.; Portal, L.; Moreno, P.; Tengerdy, R. P. Mixed culture solid substrate fermentation of *Trichoderma reesei* with *Aspergillus niger* on sugar cane bagasse. *Bioresour. Technol.* **1999**, *68*, 173−178.
(38) Asiegbu, F. O.; Paterson, A.; Smith, D. E. The effects of co-fungal cultures and supplementation with carbohydrate adjuncts on lignin biodegradation and substrate digestibility. *World J. Microbiol. Biotechnol.* **1996**, *12*, 273−274.