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Distinct *Miscanthus* lignocellulose improves fungus secreting cellulases and xylanases for consistently enhanced biomass saccharification of diverse bioenergy crops

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ABSTRACT

Bioenergy crops provide enormous renewable biomass resources convertible for biofuel production, but lignocellulose recalcitrance fundamentally determines its enzymatic saccharification at high cost and low efficiency. In this study, total 30 diverse *Miscanthus* lignocellulose substrates were incubated with *T. reesei* strain to secret lignocellulose-degradation enzymes, and their major wall polymers features (cellulose crystallinity, hemicellulose arabinose and lignin H-monomer) were meanwhile examined with distinct impacts on the enzyme activities. Using characteristic *Miscanthus* (Msi62) de-lignin residue as inducing substrate with the *reesei* strain, this study detected that the Msi62-induced enzymes were of consistently higher enhancements on enzymatic saccharification of various lignocellulose residues examined in 17 grassy and woody bioenergy crops, particularly for the hemicellulose hydrolyses, compared to other two *reesei*-secreted cellulases and three commercial enzymes. Notably, based on SDS-gel protein separation profiling and LC-MS/MS analysis, the Msi62-induced enzymes consist of distinct cellulases (CBHI, BG, EGII) compositions and high-activity xylanases. Therefore, this study has demonstrated an applicable approach to achieve the optimal cellulases and xylanases cocktails that enable for low-costly and high-efficient enzymatic saccharification of diverse lignocellulose sources, providing a potential strategy for large-scale biofuel production in all major bioenergy crops.

1. Introduction

Lignocellulose represents the most abundant biomass resource that is renewable and convertible for biofuels and bioproducts [1,2]. Due to lignocellulose recalcitrance, however, a high-costly and low-efficient biomass enzymatic saccharification has become a critical issue restricting biofuel production at large scale [3,4]. Meanwhile, because bioenergy crops are of diverse cell wall compositions and complicated wall polymer features, it remains a technique difficulty to find out the optimal cellulases cocktails with consistently high activity for efficient enzymatic saccharification of different types of lignocellulose substrates [5].

Lignocellulose recalcitrance is principally determined by diverse plant cell wall compositions, characteristic wall polymer features and complicated wall-network styles [6]. Recently, it has been characterized that wall polymer features could distinctively affect biomass enzymatic saccharification under various physical and chemical pretreatments in different bioenergy crops examined [7,8]. For instance, the crystalline index (CrI) and degree of polymerization (DP) of cellulose are two major factors negatively accounting for biomass enzymatic hydrolysis, whereas the arabinose level and its substitution degree (reverse xylose/arabinose, Xyl/Ara) of hemicellulose could positively affect biomass enzymatic saccharification [9,10]. In addition, three monomers (S, G, H)

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proportions of lignin have been examined with distinct roles in biomass enzymatic degradation [11].

Trichoderma reesei is one of the most common microorganisms applied to secret biomass-degrading enzymes [12]. In general, the *T. reesei*-secreted cellulases consist of two major types of cellobiohydrolases and eight endoglucanases from six glycoside hydrolase families [13]. In particular, cellulases are the complexes of three different major enzymes: exoglucanase (CBH), endoglucanase (EG) and β-glucosidase (BGL), which act synergistically for complete hydrolysis of cellulose microfibrils [14,15]. In detail, cellulose microfibers are firstly cleaved by endoglucanases to release small cellulose fragments, followed by exoglucanases to produce oligosaccharides and cellobiose, and finally hydrolyzed into glucose by β-glucosidases [16]. Despite that the *T. reesei* could secret all types of cellulases and xylanases for lignocellulose degradation, it remains to explore the mixed-cellulases and xylanases that are of high activities for enzymatic hydrolyses of different sources of lignocellulose residues in major bioenergy crops.

High-yield lignocellulolytic enzyme production depends on an effective inducer for the fungi [17]. In particular, lignocellulose is an effective inductive substrate for the production of cellulases by many filamentous fungi such as *Trichoderma* spp., *Aspergillus* spp. and *Penicillium* spp [18]. However, the cellulases activity is strongly influenced by different types of lignocellulose residues such as steam-exploded corn [19], wet exploded corn stover and loblolly pine [20]. The expressions and regulations of these lignocellulolytic proteins in the secretome of *T. reesei* wild type and mutant Rut-C30 are dependent on both nature and complexity of lignocellulosic carbon sources [21]. However, although various lignocellulose residues have been applied as incubating substrates with fungi to secret multiple cellulases products, much remains unknown about the optimal lignocellulose substrate, and in particular little has been reported about wall polymer features impacts on secreted cellulases activity.

Miscanthus has been evaluated as a leading bioenergy crop, due to its high biomass yield and well adaptation to various environmental conditions [22,23]. It consists of several species with hundreds of different accessions for diverse lignocellulose residues [11]. Based on the previously-identified *Miscanthus* accessions, this study selected total 30 representative *Miscanthus* samples showing largely varied cell wall compositions and wall polymer features, and used *T. reesei* strain to incubate with both raw materials and alkali-pretreated residues of those 30 biomass samples for cellulases production *in vitro*. We then determined the filter paper activities of the secreted enzymes and sorted out the major features of three major wall polymers that distinctively affect cellulases activities. Further using two distinct *Miscanthus* samples to incubate with *T. reesei* strain, this study detected the secreted enzymes with universal enhancements for biomass enzymatic saccharification in total 17 grassy and woody bioenergy crops, providing a novel knowledge with potential simply and efficient technology for the optimal cellulases cocktails that are of high enzymatic activity and broad application in biomass degradation of all bioenergy crops.

2. Materials and methods

2.1. Biomass samples and enzymes

Total 30 *Miscanthus* accessions and other 17 biomass samples of bioenergy crops were collected from experimental fields of Huazhong Agricultural University and Hunan Agricultural University. Avicel was purchased from China National Pharmaceutical Group Co., Ltd. CTec2 was obtained from Novozyme, and the mixed-cellulases enzymes of HSB and BM were respectively purchased from Imperial Jade Biotechnology Co., Ltd. Ningxia, China, and Huaian Baimai Green Bio-energy Co., Ltd, China. β-Glucosidase, β-xylanse, endoglucanase and exoglucanase were bought from Megazyme.

2.2. Strains, media, and cultivation conditions

T. Reesei Rut-C30 (CICC 40348) was obtained from China Center of Industrial Culture Collection. The strain was grown on potato dextrose agar (PDA) at 30 °C for 7 d, and the flask cultivations conidia were harvested with ddH_2O and counted on haemocytometer. The spore germination rates were accurately examined for appropriate incubation time prior to micro fluidic analysis and sorting. The spores were collected and adjusted to a density of 6×10^6 spores/mL in liquid cellulase-inducing medium, and 500 μL of spore suspension was then incubated under 200 rpm/min shaking at 30 °C for 7 d. The Mandels-Andreotti medium contains 1.4 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.3 g/L $MgSO_4$, 0.4 g/L $CaCl_2$, 0.3 g/L urea, 1 g/L peptone, 20 g/L carbon source, 20 mL/L trace elements pH 5.8 (7.5 mg/L $FeSO_4$-$7H_2O$, 2 mg/L $MnSO_4$-H_2O, 2 mg/L $ZnSO_4$-H_2O and 3 mg/L $CoCl_2$-$2H_2O$), and the pH value was adjusted to 4.8. Liquid cultures were grown with *Miscanthus* substrates or Avicel as carbon source. Another 0.6 g *Miscanthus* powders were pretreated with 30 mL 1% (g/L) NaOH and the pretreated biomass substrates were also applied as carbon source. All experiments were performed in independent triplicates.

2.3. FPA detection and protein content

The filter paper activity (FPA) was measured as previously described [24]. About 1 mL of crude enzymes and 3 mL of citrate buffer (pH 4.8, 0.05 M) were added into a tube with 50 mg Whatman filter paper grade No 1. The reaction mixture was incubated for 60 min at 50 °C, and the enzymatic reaction was terminated by adding 2 mL DNS, followed by boiling water for 10 min. One FPA unit was defined by measuring the amount of enzyme releasing 1 μmol reducing sugar per min from Whatman filter paper grade No.1.

The protein content of the crude enzymes supernatant were determined by using Coomassie Brilliant Blue G250 dye assay [25]. The G250 dye was prepared in the solution containing ethanol (w/v; 2:1) and phosphoric acid (w/v; 1:1), and filtered through a 0.22 μm filter. The absorbance of the protein-dye complex was reading at 595 nm using UV–vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai) [26]. The diluted 1 mL protein was added with 3.0 mL G250, and the absorbance was measured 10 min later. All experiments were performed in independent triplicates.

2.4. Endoglucanases and xylanases activity assay in vitro

The substrates and enzyme preparations were dissolved in 0.05 M sodium citrate buffer (pH 4.8). Endoglucanases (EG) and xylanase (XYN) activities *in vitro* were respectively measured using carboxymethylcellulose (CMC-Na) and beechwood xylan as substrates (purchased from China National Pharmaceutical Group Co., Ltd., Shanghai yuanye Bio-Technology Co., Ltd, China) as previously described [27]. Endoglucanase activity was determined in the reaction mixture containing 0.5 mL of suitable diluted enzyme and 1 mL of 1% (W/V) CMC-Na solution in sodium citrate buffer (0.05 M, pH 4.8) at 50 °C for 30 min. Xylanase activity was measured under similar conditions above, except for the CMC-Na replaced by 1.5 mL 1% xylan solution. The reducing sugars released were determined using dinitrosalicylic acid method (DNS) as previously described [28]. About 2 mL DNS was added to stop the reaction as described

above by treating at 100 °C for 10 min. Once the reaction solution was cooled to room temperature, the absorbance was read at 540 nm. The glucose and xylose were applied as the standard sugars and the enzymes inactivated at 100 °C for 10 min were used as the controls, respectively. One enzyme activity unit (U) was defined as the amount of enzyme that liberates 1 μmol glucose or xylose per minute under the assay conditions.

2.5. Proteomic analysis

The Msi62-induced enzymes were analyzed by LC-MS/MS (Jingjie PTM BioLab Co.Ltd, Hangzhou, China; Orbitrap Elite LC-MS/MS, Thermo, USA). About 200 μg proteins were rehydrated in 10 mM dithiothreitol and incubated at 37 °C for 60 min. The alkylation was performed using iodoacetamide for 45 min under dark, and the samples were desalted and collected using a Microcon YM-10 Centrifugal Filter Unit. The obtained proteins samples were digested thoroughly using trypsin (trypsin-protein ratio of 1:50, w/w) for 16 h. The resulting peptide mixtures were lyophilized after desalting with a ZipTip C18 column and then dissolved in double-distilled H_2O. The tryptic peptides were dissolved in 0.1% formic acid (solvent A), and directly loaded onto a home-made reversed-phase analytical column (1.8 μm, 0.15 × 1,00 mm). The gradient was comprised of an increase from 4% to 18% solvent B (0.1% formic acid in 100% acetonitrile) over 182 min, 18%–90% in 5 min and holding at 90% for the last 8 min, and all were fixed at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Thermo Fisher LTQ Obitrap ETD coupled online to the UPLC. The electrospray voltage was 2.0 kV, the m/z scan range was 350–1600 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 30,000. Peptides were then selected for MS/MS using NCE setting as 35 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan was followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Liquid chromatography-MS/MS analysis data were identified by searching the *T. reesei* Rut-C30 protein sequence databases downloaded from Uniprot (http://www.uniprot.org).

2.6. Cellulose and hemicellulose extraction and monosaccharides determination

The biomass powders were applied to extract cellulose and hemicellulose by using plant cell wall fractionation procedure as previously described [29]. After removal of soluble sugars, lipid, starch and pectin from successive extractions with phosphate buffer (pH 7.0), chloroform-methanol (1:1, v/v) and dimethyl sulphoxide (DMSO)/water (9:1, v/v) and 0.5% ammonium oxalate monohydrate (AO, w/v), the remaining pellets were suspended in 4 M KOH containing $NaBH_4$ (1.0 mg/mL) as KOH-extractable hemicelluloses fraction. The remaining non-KOH-extractable pellets were dissolved in 67% H_2SO_4 (v/v) for 1 h at 25 °C as cellulose fraction. Total hemicelluloses were accounted by measuring hexoses and pentoses of the KOH-extractable hemicellulose fraction and the pentoses of the non-KOH-extractable fraction. Hexose of the cellulose fraction was detected as cellulose level. The anthrone/H_2SO_4 method [30] and orcinol/HCl method [31] were respectively used to determine hexoses and pentoses. D-glucose and D-xylose were applied to plot standard curves, and the pentose reading at 660 nm was deducted for the final hexose calculation to eliminate the interference of pentoses on the hexose reading at 620 nm. For the anthrone/H_2SO_4 assay, about 1.0 mL aqueous sample (containing 20–100 μg hexoses) was added into 0.2% anthrone (2.0 mL) dissolved in conc H_2SO_4, and incubated in a boiling water bath for 5 min. After the sample was cooled, the absorbance was read at 620 nm. For the orcinol/HCl method, about 1.0 mL aqueous sample (containing 5–40 μg pentoses) was added into 6% orcinol (134 μL) in ethanol, followed by 0.1% $FeCl_3$-$6H_2O$ (2.0 mL) in conc HCl, and incubated in a boiling water bath for 20 min. After cool at room temperature, the sample was mixed again and its absorbance was read at 660 nm. All experiments were conducted under independent triplicates.

The KOH-extractable fraction was applied to determine monosaccharide composition of hemicellulose by CG-MS as previously described [32]. Trifluoroacetic acid (TFA) and *myo*-inositol were obtained from Aladdin Reagent Inc. 1-Methylimidazole was purchased from Sigma-Aldrich Co. LLC. Acetic anhydride and acetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd. Acid hydrolysis. The polysaccharides dissolved in 2.5 mL containing 500 μL TFA were heated in a sealed tube at 120 °C in an autoclave (15 psi) for 1 h. About 100 μL (2 mg/mL) *myo*-inositol was added as the internal standard. The supernatant was dried under Nitrogen Blowing Apparatus (N-EVAP-111) to remove TFA. For the derivatisation of monosaccharides to alditoal acetates, distilled water (800 μL) and a freshly prepared solution of $NaBH_4$ (400 μL, 100 mg/mL in 6.5 M aqueous NH_3) were added to each sample, mixed well and incubated at 40 °C for 30 min. Excess $NaBH_4$ was decomposed by adding acetic acid (800 μL). Acetic anhydride (4 mL) was added to the tube and the solution mixed again, and then 1-methylimidazole (600 μL) was added. Then dichloromethane (3 ml) was added, mixed gently, centrifuged at 2,000 *g* for 10 s for phase separation. After removing the upper phase, the sample was washed with distilled water. The collected lower phase was dehydrated by adding with anhydrous sodium sulfate and stored at −20 °C for GC-MS analysis (SHIMADZU GCMS-QP2010 Plus).

GC-MS analysis used Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25um df column and ACQ Mode (SIM). The mass spectrometer was operated in the EI mode with ionization energy of 70 ev. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 m/z in 0.45s. Calibration curves of all analytes routinely yielded correlation coefficients 0.999 or better.

2.7. Lignin level and monolignols detection

Lignin content was determined by two-step acid hydrolysis method according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory [33]. The acid-insoluble lignin was determined gravimetrically as acid-insoluble residue after correction for ash, and the acid-soluble lignin was measured by UV spectroscopy. H-, G- and S-monolignols extracted by Nitrobenzene Oxidation were determined by HPLC (SHIMADZU LC-20A with a UV-detector at 280 nm). CH_3OH: H_2O: HAc (16:63:1, v/v/v) was used as mobile phase (flow rate: 1.1 mL/min), and the injection volume was 20 μL. For lignin level assay, all experiments were conducted at independent triplicates.

2.8. Detection of cellulose features (DP, CrI)

The degree of polymerization (DP) of crude cellulose was determined using viscosity method as previously described by Hu et al. [34]. About 0.2 g dry powders of biomass samples were extracted with 4 M KOH containing $NaBH_4$ (1.0 mg/mL) at 25 °C for 1 h. After centrifugation at 4000*g* for 5 min, the pellet was re-extracted with 4 M KOH, and washed with distilled water until pH 7.0. The pellet was further extracted with 10 mL 8% (w/v) $NaClO_2$ at 25 °C for 72 h ($NaClO_2$ changed every 12 h). After centrifugation, the residues were washed with distilled water until pH at 7.0 and

dried with vacuum suction filtration. The DP of crude cellulose sample was measured using the viscosity method [35] with minor modification [9]. The relative viscosity (ηrel) values were calculated using the ratio of t/t_0, where t and t_0 are the efflux times for the cellulose solution and Cuen (blank) solvent. The intrinsic viscosity was calculated by interpolation using the United States Pharmacopeia table [36]. The intrinsic viscosity values were converted to cellulose DP according to equation: $DP^{0.905} = 0.75[\eta]$, [η] is the intrinsic viscosity of the solution calculated by interpolation using the USP table.

Cellulose crystalline index (CrI) was detected by X-ray diffraction (XRD) method (Rigaku-D/MAX instrument, Uitima III, Japan) as described [37]. The dry biomass powders were applied under plateau conditions. Ni-filtered Cu-Kα radiation (λ = 0.154056 nm) generated at voltage of 40 kV and current of 18 mA and scanned at speed of 0.0197°/s from 10° to 45°. The CrI was calculated using the intensity of the 200 peak (I_{200}, θ = 22.5°) and the intensity at the minimum between the 200 and 110 peaks (I_{am}, θ = 18.5°) as the follow: $CrI = (I_{200} - I_{am}) \times 100/I_{200}$. I_{200} represents both crystalline and amorphous materials while I_{am} represents amorphous material. Technical standard errors of the CrI method were detected at ± 0.05–0.15 using five representative samples in triplicate.

2.9. Biomass pretreatment and enzymatic hydrolysis

Biomass samples were pretreated with 5 mL 0.5% NaOH (w/v) under 150 rpm shaken at 50 °C for 2h. After centrifugation at 3000*g* for 5 min, the pretreated biomass residues were respectively incubated with the 10 FPU/g of enzyme cocktails secreted by *T. Reesei* and three commercial enzymes (HSB, BM, CTec2) and the enzymatic hydrolysis reactions were conducted as previously described [37]. All experiments were performed in independent biological triplicates.

2.10. SDS-PAGE and western blot analysis

SDS-PAGE was conducted using Stain-Free precast gels (Beijing Zoman Biotechnology Co., Ltd.) according to the manufacturer's instructions. 30 μL of induced culture supernatant (no purification) was loaded into each well. 10 μL of CBH, EG and BG were loaded into each well. Protein samples were visualized with colloidal coomassie blue staining. Western blotting was performed as previously described [38]. For Western blotting, the separated proteins were transferred from the SDS-gel to PVDF membrane. The membrane was blocked 1.5 h in TBS buffer (20 mM Tris-HCl and 500 mM NaCl, pH 7.5) supplemented with 5% nonfat dry milk, rinsed with TTBS buffer (0.05% Tween-20 in TBS) for three times and incubated with primary antibody serum (CBHI and BG was used at 1:1000 dilution) at room temperature for 1 h. After washing with TTBS at three times, the membrane was incubated with secondary antibody (goat anti-rabbit secondary antibody IgG-HRP at a 1:5000) for 1 h at room temperature. The membrane was finally washed three times with TTBS and one time with TBS (200 mM Tris-HCl, 150 mM NaCl, pH 7.5). The reactions were measured by the ECL Plus Western Blotting Detection. Protein immunoblot bands were scanned with a GeneGnome XRQ (Syngene Inc., Maryland, US).

2.11. Data collection and statistical analysis

The SPSS statistical software was applied for data analysis. Prior to statistical analysis, all data were analyzed by a Kolmogorov–Smirnov test to check for normal distribution of samples. Pearson correlation analysis was performed for coefficient calculation, and Student's *t*-test was for comparison analysis.

3. Results and discussion

3.1. Diverse Miscanthus lignocellulose substrates incubated with T. reesei for secreting varied cellulases activities

Based on the previously-identified hundreds of *Miscanthus* straws [39,40], this study selected total 30 representative *Miscanthus* accessions that showed distinctive cell wall compositions in mature straws (Fig. 1A; Table S1). By comparison, the cellulose levels were varied from 27% to 42%, hemicellulose contents were ranged from 18% to 28% and lignin levels were from 21% to 31%. Meanwhile, this study determined that those *Miscanthus* accessions were of largely varied wall polymer features (Table S1). For instances, the *Miscanthus* straws showed cellulose crystalline index (CrI) values ranged from 49% to 62% and had even more varied cellulose DP values from 151 to 298 (Fig. 1B). Despite that two major monosaccharides (arabinose/Ara, xylose/Xyl) of hemicelluloses were also varied in those *Miscanthus* samples, their Xyl/Ara ratios even showed more variations from 6.1 to 15.7 (Fig. 1C). In addition, the *Miscanthus* straws remained large variations in three monomers proportions of lignin in particular on the G-monomer (Fig. 1D). Because three major wall polymers features could basically account for lignocellulose recalcitrance properties [3], the results suggested that those 30 *Miscanthus* accessions should be applicable as distinctive lignocellulose substrates for inducing cellulases production with *T. ressei* strain.

To incubate *Miscanthus* lignocellulose substrates with *T. ressei* for cellulases production, this study also generated de-lignin biomass samples by 1% NaOH extraction with those 30 *Miscanthus* straws, due to the alkali pretreatment that could remove large amounts of lignin and small hemicellulose in the *Miscanthus* samples examined [41]. Using standard filter paper activity (FPA) assay (Fig. 1E), we determined *T. ressei*-secreted cellulases activities from the incubation with raw materials and de-lignin residues of the 30 *Miscanthus* accessions (Fig. 1F; Table S2). By comparison, the raw materials showed the FPA/ml values varied from 0.09 to 0.51 with the average value at 0.31 and coefficient of variation (CV) at 40.3, whereas the de-lignin residues had the FPA/ml values from 0.40 to 0.76 with relatively higher average value of 0.54 FPA/ml and lower CV at 15.8 among total 30 *Miscanthus* accessions examined. Hence, the data demonstrated that the distinct *Miscanthus* lignocellulose substrates could induce *T. ressei* to produce cellulases with largely varied enzymatic activities in both raw materials and de-lignin residues.

3.2. Major wall polymer features of incubating substrates for distinctively affected cellulases activity

Correlation analysis has been well applied to account for wall polymer feature impacts on biomass enzymatic saccharification [42]. As those 30 *Miscanthus* samples showed large variations of lignocellulose features and cellulases activities, this study performed a correlation to sort out how the incubated lignocellulose substrates significantly affect *T. ressei* secreting enzymes activities based on the FPA assay (Fig. 2). As a result, the cellulose levels showed a significantly negative correlation with the FPA in both raw materials and de-lignin residues of 30 *Miscanthus* samples at $P < 0.01$ levels (n = 30), whereas the cellulose CrI values were negatively correlated with the raw materials, rather than the de-lignin residues (Fig. 2A). By contrast, among the monosaccharides of hemicellulose, the Ara levels could positively affect the FPA in both raw materials and de-lignin residues of *Miscanthus* samples at $P < 0.01$ levels (Fig. 2B). Despite that the Xyl levels did not show any significant correlation, the Xyl/Ara ratios had a negative correlation. Because the Xyl/Ara ratio reversely reflects the Ara substitution

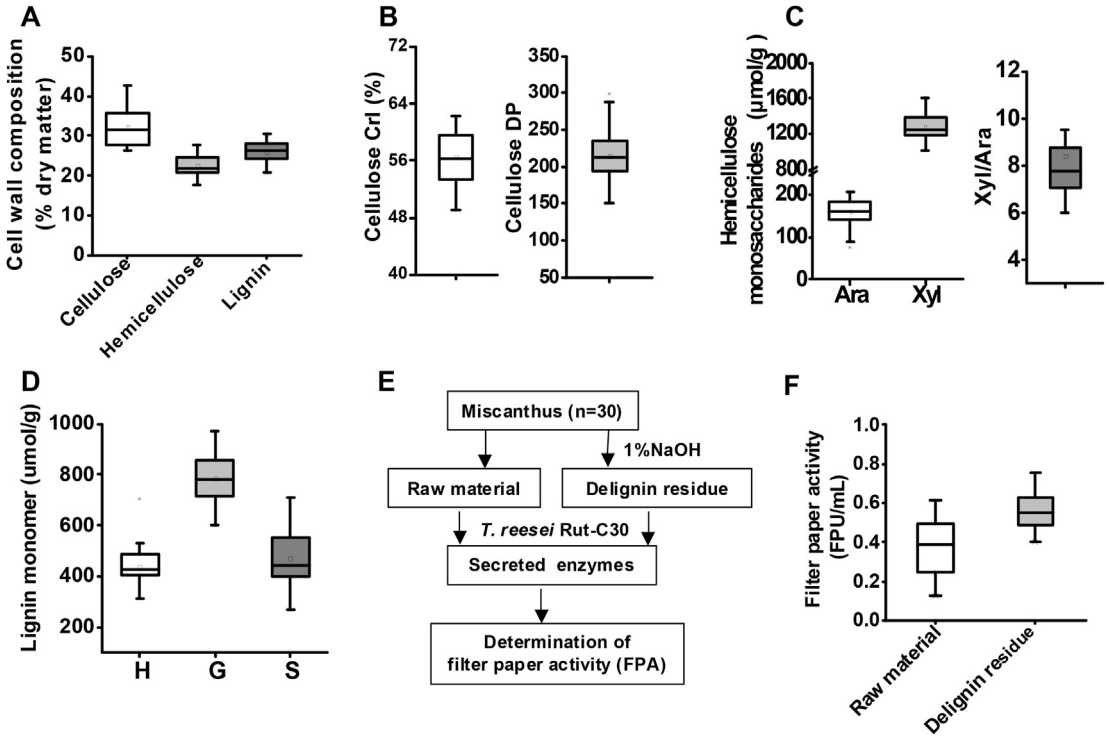

Fig. 1. Diverse cell wall compositions and wall polymer features of *Miscanthus* accessions and variation of filter paper activity of crude enzymes secreted by *T. reesei* using *Miscanthus* samples as substrates (n = 30). (A) Cell wall composition (% dry matter); (B) Cellulose crystallinity index (%) and cellulose DP; (C) Hemicellulose monosaccharides (μmol/g) and Xyl/Ara (xylose/arabinose); (D) Three lignin monomers (μmol/g); (E) Flow chart of *Miscanthus* as carbon source for *T. reesei* secreted cellulases; (F) Filter paper activity of raw material and de-lignin residue. The line and square within the box presented the median and mean values of all data, the bottom and top edges of the box indicated 25 and 75 percentiles of all data, the top and bottom bars presented maximum and minimum values of all data.

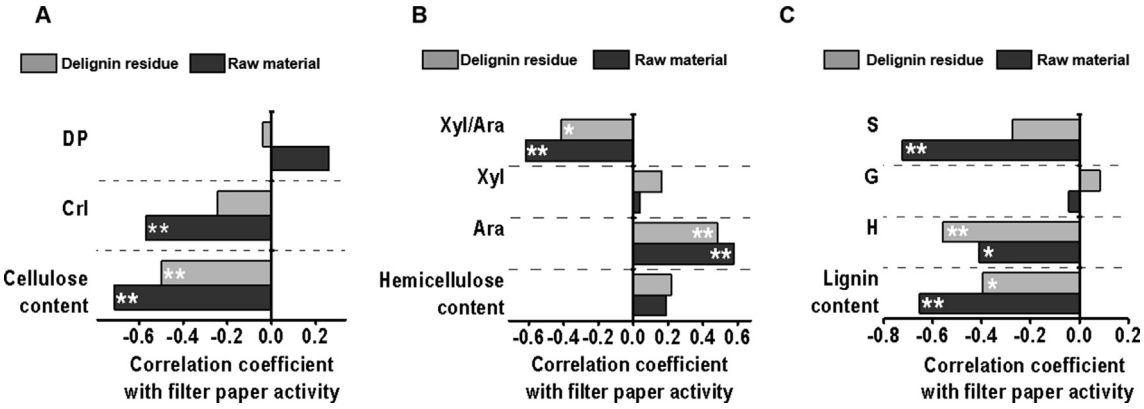

Fig. 2. Correlation analysis among wall polymer levels and features of 30 *Miscanthus* accessions and the filter paper activity of crude enzymes secreted by *T. reesei* using 30 *Miscanthus* samples as substrates. (A) Cellulose features; (B) Hemicellulose monosaccharides; (C) Lignin monomers. * and ** as significant difference between two samples of each pair by *t*-test at $P < 0.05$ and < 0.01 (n = 30).

degree of xylan in lignocellulose residues, the results indicated that the branched Ara level of lignocellulose residue could not only enhance its enzymatic saccharification as previously described [43], but it should also raise its inducing capacity for high cellulase activity secreted by *T. ressei* strain. Furthermore, this study examined that lignin levels and H-monomer proportions could negatively affect the FPA in both raw materials and de-lignin residues, but the S-monomer only had a negative impact on the raw materials, rather than the de-lignin residues (Fig. 2C), probably due to the most S-monomers extracted with the alkali pretreatment. Taken together, the results demonstrated that the incubated-lignocellulose substrate features could distinctively affect the *T. ressei* secreted cellulases activities.

3.3. Consistently enhanced enzymatic saccharification of diverse biomass residues using T. ressei-secreted cellulases

To confirm the characteristic lignocellulose substrates enabled to induce high-activity cellulases secreted by *T. ressei*, this study selected two *Miscanthus* lignocellulose substrates (Msi62, Msa05) that showed distinct cell wall compositions and wall polymers features (Table 1). Due to its relatively reduced cellulose level and CrI, lower hemicellulose Xyl/Ara ratio and less lignin level and H-monomer proportion of raw material, the Msi62 substrate could

Table 1
Wall polymers levels and features of two representative *Miscanthus* accessions and one commercial Avicel sample.

Sample		Cellulose		Hemicellulose				Lignin			
		Content (% dry mater)	CrI (%)	Content (% dry mater)	Ara (%)	Xyl (%)	Xyl/Ara	Content (% dry mater)	H (% total)	G (% total)	S (% total)
Raw material	**Msi62**	27.73 ± 0.12	46.94	25.78 ± 3.25	12.09	83.68	6.92	24.55 ± 0.74	25.43	52.66	21.90
	Msa05	38.03 ± 0.48	50.37	25.14 ± 1.25	10.37	87.29	8.42	30.08 ± 0.70	23.60	48.10	28.30
Delignin rsidue	**Msi62**	46.10 ± 1.01	48.77	26.12 ± 0.45	9.57	82.12	8.58	18.90 ± 0.69	22.61	50.38	27.01
	Msa05	43.37 ± 1.22	57.12	25.50 ± 0.46	8.42	89.27	10.60	17.85 ± 0.77	22.26	50.54	27.20
	Avicel	100.00	77.78								

induce *T. ressei* to secreted cellulases with FPA at 0.44, whereas the Msa05 only had the FPA at 0.12 (Table S2). At the de-lignin residue samples, the Msi62 accession remained slightly higher FPA value than that of the Msa05 sample. Furthermore, by performing *T. ressei* strain incubation with the de-lignin residues of Msi62 and Msa05 along with the commercial Avicel as pure cellulose control, this study collected the secreted cellulases and determined their FPA *in vitro* (Fig. 3A). Using the dosages of cellulases subjected to the same FPA levels among the incubated de-lignin Msi62, Msa05 and Avicel substrates, this work compared their biomass enzymatic saccharification by measuring hexoses, pentoses and total soluble sugars (pentose and hexoses) yields released from enzymatic hydrolyses of 0.5% NaOH pretreated residues in total 17 diverse biomass samples harvested from 11 major types of grassy and woody bioenergy crops and one lowly plant (fern). As a result, two *Miscanthus* (Msi62, Msa05) induced cellulases could lead to obviously higher hexoses yields (% cellulose) than those of the Avicel in almost all 17 biomass samples examined, and the Msi62 remained the highest average hexoses yield with the least coefficient of variation among three substrates (Msi62, Msa05, Avicel) (Fig. 3B; Table 2). Notably, three substrates induction could even cause much different pentoses yields (% hemicellulose), and the Msi62 induction showed consistently higher pentoses yields than the Msa05 did in all 17 biomass samples examined (Fig. 3C), probably due to its much lower Xyl/Ara ratio than that of the Msa05. On the other hands, the Avicel induction caused the lowest pentoses yields, which should be due to its pure cellulose with little induction of hemicellulose-degradation enzymes. Further calculation indicated that the Msi62 induction showed the highest total sugars (hexoses and pentoses) yields (% dry matter), whereas the Avicel had the

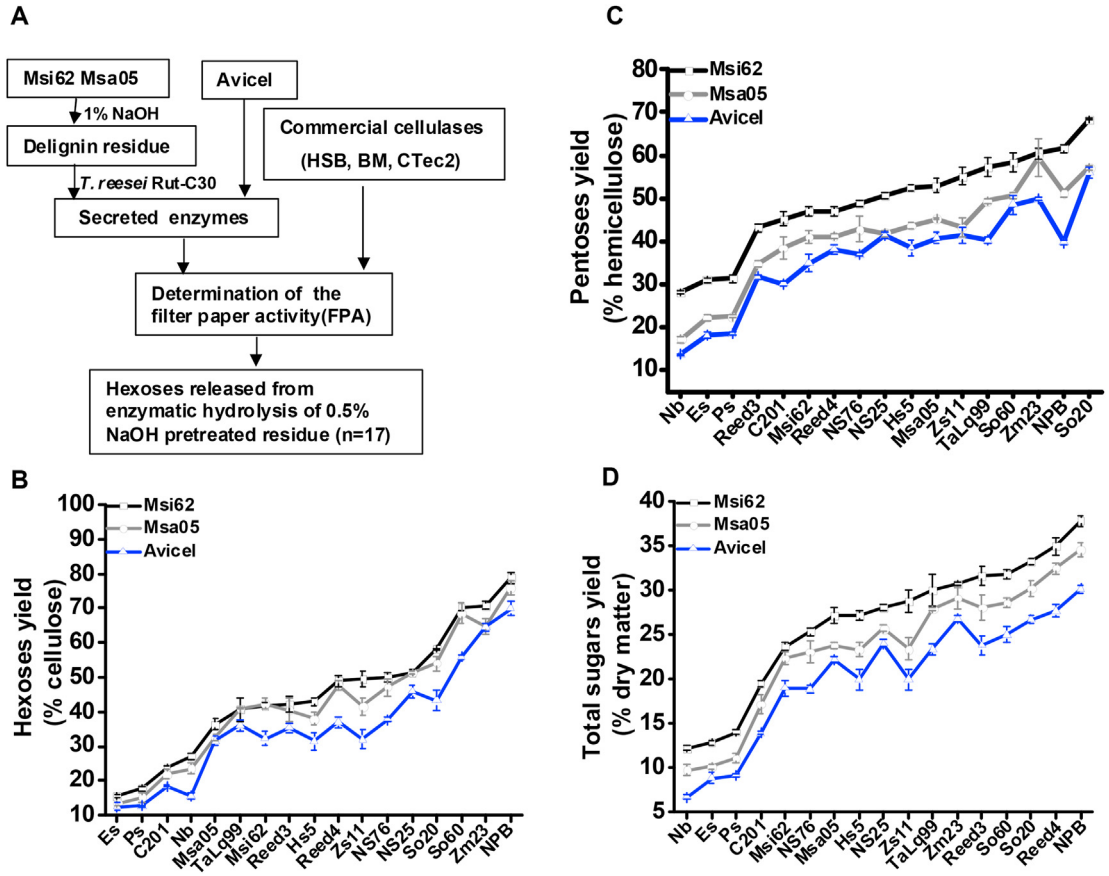

Fig. 3. Comparison of biomass saccharification using *T. reesei*-secreted enzymes. (A) Flow chart of *Miscanthus* and Avicel as inducing substrates for *T. reesei* secreting the cellulases and xylanases added into enzymatic hydrolyses of 17 biomass samples after 0.5% NaOH pretreatment, and three commercial cellulases as controls; (B), (C) and (D) presented hexoses yields, pentoses yields and total sugars (pentoses + hexoses) yields released from enzymatic hydrolyses after 0.5% NaOH pretreatment in 17 biomass samples. Data indicated mean ± SD (n = 3).

Table 2
Average sugars yields released by enzymatic hydrolyses by *T. reesei* secreted cellulases and commercial cellulases of 17 biomass samples after 0.5% NaOH pretreatment.

	Secreted enzymes			Commercial enzymes		
	Msa05	Msi62	Avicel	HSB	BM	CTec2
Hexoses (%cellulose)	42.30 ± 17.7[@] (42)[#]	**45.05 ± 18.06 (40)**	36.01 ± 16.57 (46)	34.72 ± 15.62 (45)	33.69 ± 16.06 (48)	**45.47 ± 18.47 (40)**
Pentoses (%hemicellulose)	41..28 ± 11.76 (29)	**49.31 ± 11.27 (23)**	36.36 ± 11.34 (31)	35.42 ± 12.15 (34)	36.62 ± 11.12 (30)	**41.01 ± 12.30 (30)**
Total sugars (% dry matter)	23.54 ± 7.56 (32)	**26.39 ± 7.67 (29)**	20.36 ± 6.98 (34)	19.74 ± 6.86 (35)	19.72 ± 6.69 (34)	**24.57 ± 7.55 (31)**

[@]Average sugars of 17 biomass samples from 12 bioenergy crops as shown in Table S3; [#]Coefficient of variation/CV of 17 samples was calculated subjective to the equation: (standard derivation/means) × 100%. Data indicated mean ± SD (n = 17).

lowest ones in all 17 biomass samples examined (Fig. 3D; Table 2). Hence, the results demonstrated that the optimal lignocellulose substrate (Msi62) could induce *T. ressei* strain to secret the cellulases with consistently more enhancements to the enzymatic saccharification of diverse lignocellulose residues in all grassy and woody bioenergy plants examined.

3.4. Comparable biomass saccharification among T. ressei-secreted cellulases and commercial enzymes

With respect to the Msi62 lignocellulose substrate enabled to induce high activity cellulases for consistently enhanced biomass enzymatic saccharification, this study also used three commercial cellulases (CTec2, HSB, BM) to perform enzymatic hydrolyses of those 17 biomass samples (Fig. 4; Table 2). Compared to the Msi62-induced cellulases, three commercial cellulases generally showed distinct enzymatic hydrolysis capacities among total 17 biomass samples examined. In particular, the CTec2 hydrolysis led to the average hexoses yield at 45.5% (% cellulose) similar to the Msi62 at 45.1%, whereas the HSB and BM had lower average hexoses yields of 35% and 34%, respectively (Fig. 4A). Furthermore, the Msi62-induced cellulases could cause significantly higher pentoses yields (% hemicellulose) than those of three commercial cellulases in all individual biomass samples examined (Fig. 4B), leading to their much different average pentoses yields such as 49.3% for the Msi62-induced cellulases and 35.4%–41% for three commercial cellulases (Table 2). In addition, the three commercial cellulases showed largely varied pentoses yields among total 17 biomass samples with the CV (coefficient of variation) from 30% to 34%, whereas the Msi62-induced cellulases had CV at 23%, suggesting

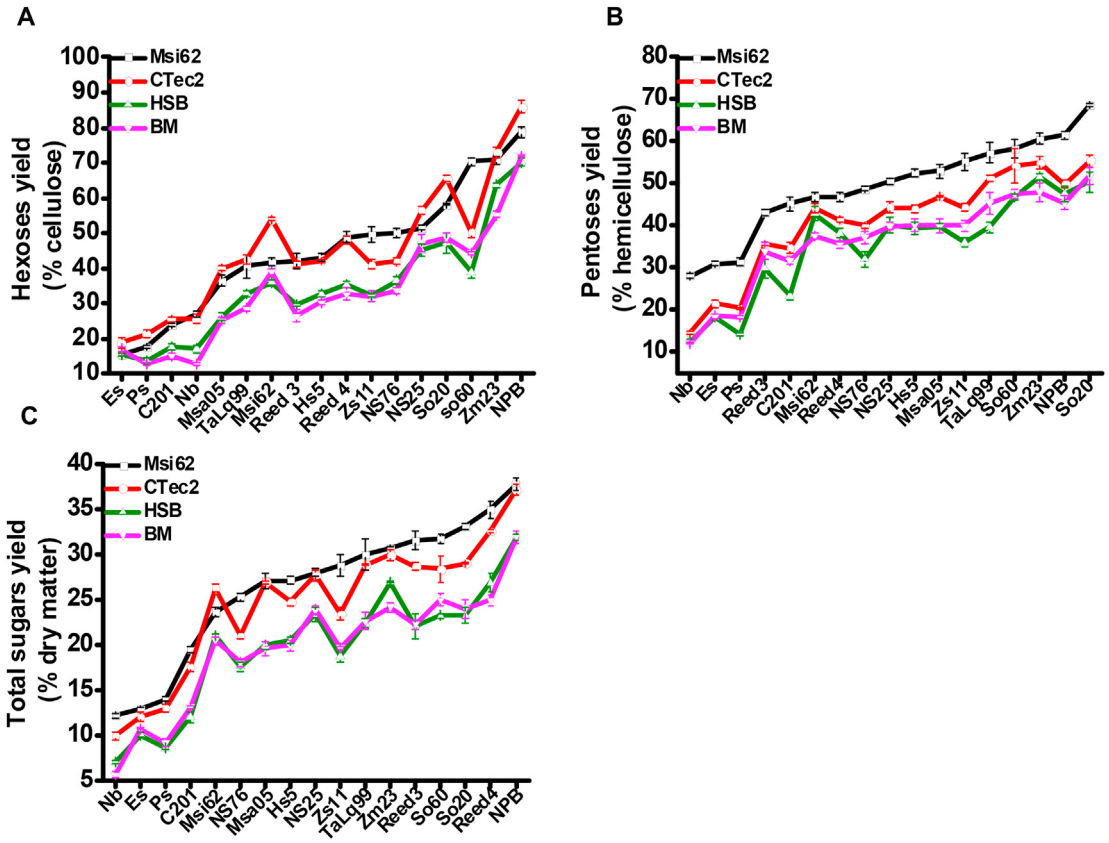

Fig. 4. Comparison of biomass enzymatic saccharification using *T. reesei*-secreted enzymes and commercial cellulases. (A), (B) and (C) presented hexoses yields, pentoses yields and total sugars yields released from enzymatic hydrolyses after 0.5% NaOH pretreatment in 17 biomass samples. Data indicated mean ± SD (n = 3).

that the Msi62-induced cellulases should be of consistent enhancements to hemicellulose hydrolyses of 17 biomass samples. Taken together, the Msi62-induced cellulases remained higher average total sugars yield (% dry matter) at 26.4% with relatively lower CV at 29%, compared to the three commercial cellulases with total sugars yields at 19.74, 19.71 and 24.57, respectively (Table 2). Therefore, even though the Msi62-induced mixed-cellulases had the enzymatic activity for cellulose hydrolyses to release hexoses yields similar to the CTec2 in all bioenergy crops examined, it should be of consistently higher enzymatic activity for hemicellulose digestions than those of three commercial enzymes.

3.5. Distinct cellulose-degradation enzymes profiling and activity among T. ressei-secreted cellulases and commercial enzymes

To understand why the *T. ressei* strain incubated with the Msi62 de-lignin substrate could secret the cellulases showing relatively higher enzymatic activities for cellulose hydrolysis, this study performed SDS-gel protein separation for the secreted-enzymes profiling (Fig. 5). Incubated with *Miscanthus* de-lignin residues (Msi62, Msa05) and commercial Avicel substrates, the *T. ressei* strain secreted total proteins to different degrees, and particularly the Msi62-induced sample showed significantly higher proteins level than those of the Msa05-and Avicel-induced samples by 22.34% and 30.35%, respectively (Fig. 5A). By comparison, all three *T. ressei*-secreted samples had relatively lower total proteins than those of two commercial enzymes (HSB, CTec2) samples. Using the proteins that were of the same FPA (1.0432 FPU) activities as described above, we observed a similar separation pattern of the induced-cellulases (EGII, CBHI, BG) on the SDS-gel among three *T. ressei*-secreted samples, which were different from two commercial enzymes samples (Fig. 5B). Then, this study conducted a classic Western blot analysis to confirm two major cellulases (CBHI, BG) using their specific antibodies (Fig. 5C). Based on the semi-quantitative survey, the Msi62-induced sample showed the CBHI and BG enzymes with the highest and the second higher levels among all samples examined (Fig. 5D), consistent with the findings that the Msi62-induced sample was of the most enhanced activities for enzymatic saccharification of lignocellulose residues detected in grassy and woody plants, compared to other *T. ressei*-secreted samples and commercial enzymes. Except the CTec2 sample displaying a very faint band for EGII enzyme, all other samples did not show any corresponding bands for the EGII, suggesting that the EGII enzyme may not be enough to be detectable in the loading samples of SDS-gel running. However, this study further performed LC-MS/MS analysis of *T. reesei*-secreted enzymes induced by Msi62 de-lignin substrate, and detected at least three EG enzymes including endo-β-1,4-glucanase I, II, IV (Table 3; Fig. S1). Furthermore, we measured that the Msi62-induced sample was of EGII activity *in vitro* using CMC-Na substrate (Fig. 5E and F), which was significantly higher than that of the Avicel-indued sample, but lower than those of two commercial enzymes (HSB, CTec2). On the other hands, despite that the CTec2 sample had the relatively low CBHI level, it was of the high BG content and EGII activity, which should be accountable for its relatively high enzymatic saccharification as described above. In addition, the LC-MS/MS analysis showed other several enzymes involved in cellulose degradation (Table 3), consistent with the other weak bands observed in five samples. Hence, the results revealed that the Msi62-induced sample should have its characteristic cellulases composition and distinct enzymes activities for high lignocellulose enzymatic saccharification in almost all bioenergy crops examined.

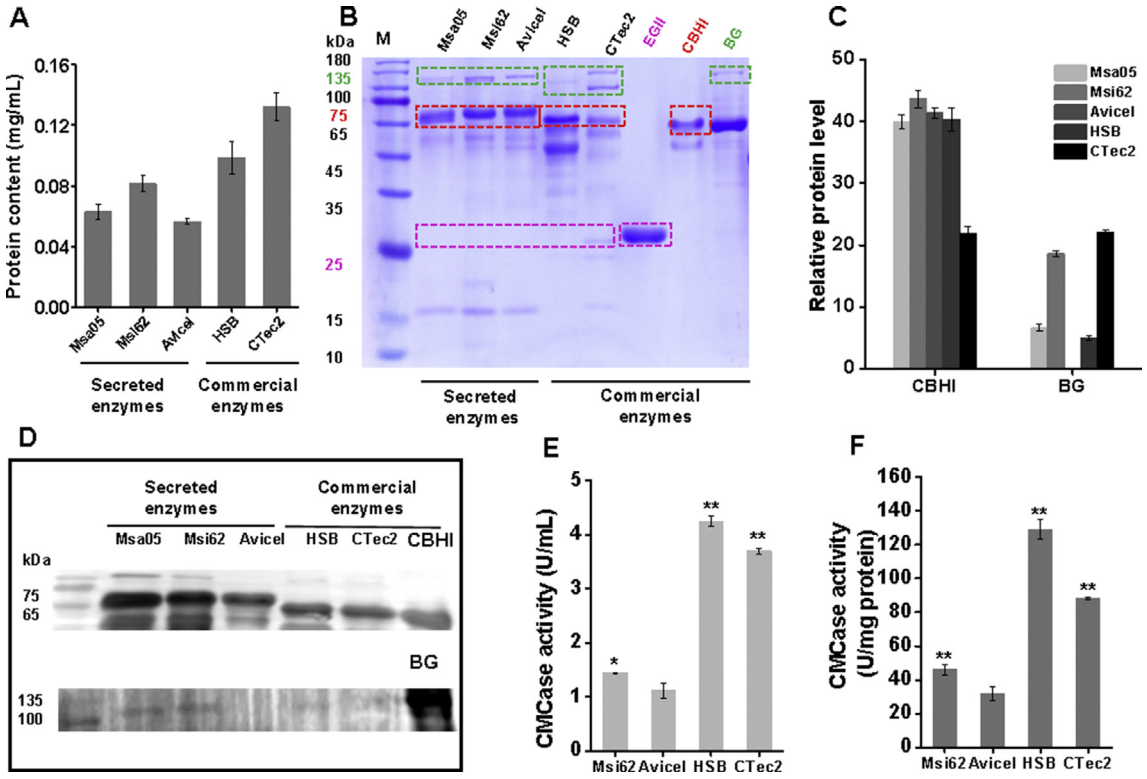

Fig. 5. Protein profiling of *T. reesei*-secreted enzymes and commercial cellulases. (A) Total protein content (mg/mL); (B) SDS-PAGE images (Green box highlighted for BG, Red for CBHI and pink for EGII); (C) Western blot analysis of (B) sample using antibodies of CBHI and BG; (D) Relative protein levels of CBHI and BG by semi-quantitative analysis. (E, F) EG II enzyme activity assay *in vitro* using CMC-Na substrate. EGII, CBHI and BG enzymes as standard markers of cellulases. Data indicated mean ± SD (n = 3).

Table 3
LC-MS/MS analysis of *T. reesei*-secreted enzymes induced by Msi62 de-lignin substrate.

Protein Name	Accession No.	Score	MW[kDa]
Cellobiohydrolase I	A0A024RXP8.1	212.88	54.1
Cellobiohydrolase II	A0A024SH76.1	37.64	49.6
Endo-β-1,4-glucanase I	A0A024SNB7.1	49.57	48.2
Endo-β-1,4-glucanase II	A0A024SH20.1	23.71	44.1
Endo-β-1,4-glucanase IV	ETS06300.1	20.09	35.5
Xyloglucanase	ETS01326.1	24.46	87.1
β-xylosidase	ETS03193.1	16.24	87.1
Endo-1,4-β-xylanase I	P36218.1	7.82	24.6
Endo-1,4-β-xylanase II	5ZKZ	11.20	20.7
Swollenin	ETR97650.1	28.84	51.5
β-1,3-glucanosyltransferase	ETR97889.1	26.75	56.5
Hypothetical protein	ETS06328.1″ title = "ncbi-p:ETS06328.1">ETS06328.1	26.31	32.9
ECM33-like protein	ETR98782.1″ title = "ncbi-p:ETR98782.1">ETR98782.1	14.80	41.8
Acetyl xylan esterase	ETR98562.1	13.08	21.9

3.6. Enhanced *T. ressei*-secreted enzyme activity for hemicellulose degradation of diverse biomass residues

As the Msi62 de-lignin substrate could induce *T. ressei* strain to secret the enzymes with consistently higher activity for hemicellulose degradation of diverse lignocellulose residues among all bioenergy crops examined (Table 2), this work also conducted SDS-gel running to test the potential enzymes involved in catalyzing hemicellulose hydrolyses (Fig. 6). Using commercial xylanase (XYN^{LNP}) as putative controls for enzymatic hydrolysis of xylan, we identified this xylanase molecular weights on the SDS-gel approximate 32 kDa, but this molecular weight band was not observed in the three *T. ressei*-secreted samples and two commercial enzymes samples (Fig. 6A). However, based on the LC-MS/MS analysis of *T. reesei*-secreted enzymes, the Msi62-induced sample exhibited at least three enzymes involved in xylan hydrolysis such as β-xylosidase, endo-1,4-β-xylanase I and endo-1,4-β- xylanase II (Table 3; Fig. S2), consistent with the previous findings that *T. reesei* could produce complicated xylanolytic enzymes such as xylanase I (XYN I), xylanase II (XYN II), and XYN III in a size range of 19, 21 and 32 kDa, etc [44]. Hence, this study attempted to predict the possible xylanases isoforms of the five samples on the SDS-gel. Notably, this study detected that the Msi62-induced sample was of much higher endoxylanases activity *in vitro* than those of other samples (Fig. 6B and C), which should explain why the Msi62-induced enzymes were of consistently higher activities for hemicellulose hydrolyses of diverse biomass residues in all grassy and woody bioenergy crops examined in this study. However, it definitely remains to confirm the structures and activities of those predicated xylanases in the future.

4. Conclusions

Using 30 diverse *Miscanthus* lignocellulose substrates to incubate with *T. reesei* strain, this study examined that their major wall polymer features distinctively affected filter paper activities of the *reesei*-secreted enzymes. In comparison, the characteristic Msi62-induced enzymes could significantly more enhance biomass saccharification, in particular on the average total sugars yield of 26.4% (% dry matter) achieved from the enzymatic hydrolyses of diverse biomass residues in 17 bioenergy crops examined, whereas other two *reesei*-secreted and three commercial enzymes showed the average sugars yields of 19.7%–24.6%. Protein profiling and LC-MS/MS analysis indicated that the Msi62-induced enzymes consist of distinct cellulases composition and high-activity xylanases. Hence, this study has provided a novel strategy for optimal enzymes production applicable for enhancing biomass enzymatic saccharification in all bioenergy crops.

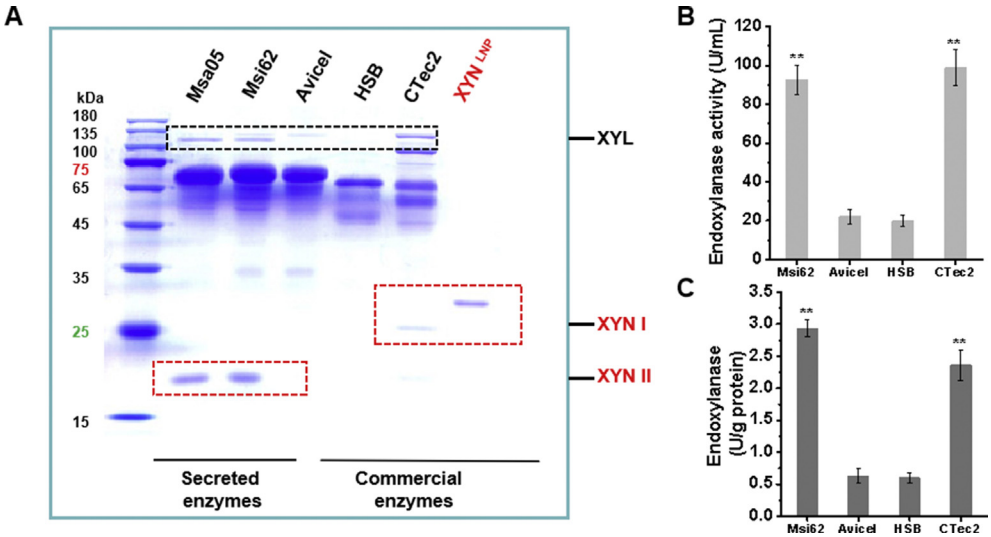

Fig. 6. Protein separation profiling and enzyme activity of *T. reesei*-secreted and commercial enzymes. (A) SDS-PAGE running (Black box highlighted for XYL, red for XYNI/XYNII) and (B, C) enzyme activity assay *in vitro* using beechwood xylan substrate. XYN^{LNP} enzymes as standard markers of xylanases. Data indicated mean ± SD (n = 3).

CRediT authorship contribution statement

Peng Liu: Investigation, Methodology, Formal analysis, Writing – original draft. **Ao Li:** Investigation, Methodology. **Youmei Wang:** Validation, Data curation. **Qiuming Cai:** Investigation, Formal analysis. **Haizhong Yu:** Formal analysis, Methodology. **Yuqi Li:** Software, Formal analysis. **Hao Peng:** Software, Methodology. **Qian Li:** Software, Methodology. **Yanting Wang:** Validation, Project administration. **Xiaoyang Wei:** Investigation. **Ran Zhang:** Editing, Formal analysis. **Yuanyuan Tu:** Project administration, Editing. **Tao Xia:** Editing, Methodology. **Liangcai Peng:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2021.04.107.

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