Efficient crude multi-enzyme produced by *Trichoderma reesei* using corncob for hydrolysis of lignocellulose

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Abstract To improve the efficiency of enzymatic saccharification for lignocellulose, an efficient crude multi-enzyme was produced by *Trichoderma reesei* using corncob, a low cost inducer. Expression of *cbh1*, *bgl1*, *egl1*, *xyn1* and positive regulator *xyl1* induced by corncob increased significantly compared to that by cellulose. After 120 h induction by corncob, enzymatic activities on filter, CMC, β -glucose and xylan increased 86.5, 46.9, 120.9 and 291.2% compared to those induced by cellulose, and the concentration of secreted protein increased by 120.8%. FPase: β -glucosidase and FPase:xylanase values in crude multi-enzyme I (ECI, induced by corncob) were higher than that in crude multi-enzyme II (ECII, induced by cellulose). Under the same hydrolysis conditions, the volume dosage of ECI was only half of ECII, but ECI still showed a maximum of 12.5 and 33.4% higher than ECII in the total reducing sugar and glucose yield in lignocellulose hydrolysis. Corncob could be a candidate for low cost production of multi-enzyme for efficient lignocellulose degradation, and this work could guide the genetic modification of *T. reesei* to obtain efficient multi-enzyme for lignocellulose hydrolysis.

Keywords Multi-enzyme · Corncob · Cellulose · Lignocellulose · *Trichoderma reesei*

Introduction

Lignocellulosic biomass is a potential renewable resource to produce biofuels and other bioproducts by microorganism fermentation (Jurgens et al. 2012; Sun and Cheng 2002). Lignocellulolytic enzymes, mainly including cellulase and hemicellulase, act synergistically to convert lignocellulosic polysaccharides into fermentable sugars (Payne et al. 2015). Among the microbes capable of producing lignocellulolytic enzymes, filamentous fungus *Trichoderma reesei* is most widely used for industrial production and academic research of cellulase and hemicellulase (Peterson and Nevalainen 2012). *T. reesei* mutants could produce over 100 g/L extracellular proteins using pure cellulose as the carbon source (Ouyang et al. 2006). However, high cost for producing lignocellulolytic enzymes and their low efficiency largely hinder the industrial utilization (Merino and Cherry 2007).

The enzyme yield and composition of the crude multi-enzyme produced by *T. reesei* are greatly influenced by different inducers. Cellulose is considered as an effective inducer for cellulase production by *T. reesei* because some soluble inducers such as cellobiose, sophorose, lactose and sorbose are too expensive to be used in industrial production. Meanwhile, the synthesis of most of the hemicellulases such as D-xylose, L-arabinose, and xylan can be induced by cellulose (Herold et al. 2013). It is reported that adding xylan to cellulose medium could lead to much production of hemicellulases and cellulase, which suggested that complex substrate is better than single inducer in the production of lignocellulolytic enzymes (Liao et al. 2014). During the induction process, these enzymes are indispensable, collaborative and coordinately regulated by positive regulators XYR1 and ACE2, and repressor ACE1 (Portnoy et al. 2011; Ling et al. 2009).

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Recent studies indicated that lignocellulose as an inducer had attracted increasing attention because of low cost, renewability, abundance in nature and capable of inducing a wide variety of enzymes. For example, higher cellulolytic activities on pretreated agricultural waste corncob were obtained than on pure cellulose (Xia and Shen 2004); a diverse enzyme system including several novel lignocellulolytic enzymes were produced on lignocellulosic biomass by quantitative proteomic analysis (Adav et al. 2012). However, the detailed induction mechanism by lignocellulose in *T. reesei* is very complicated and systematic studies on the gene expression and regulation of the main enzymes and transcription factors as well as the saccharification performance of the crude multi-enzyme are insufficient by far.

In this study, induction of the main lignocellulolytic enzymes in *T. reesei* Rut C30 by corncob and pure cellulose was systematically analyzed from the levels of transcription, enzyme activity and protein. The compositions of the crude multi-enzymes produced by corncob and cellulose and their saccharification performances were studied and compared with a commercial *T. reesei* cellulase product Celluclast 1.5L (sigma C2730).

Materials and methods

Strains and cultivations

T. reesei Rut C30 (ATCC 56765) was used in this study. The spores on a PDA plate culture at 28 °C for 7–8 days were prepared into a suspension with a concentration of 10^7 – 10^8 spores/mL. 1 mL spore suspension was transferred into 50 mL of basal medium containing Mandels' salt solution with 0.3% (w/v) glucose as the carbon source (Mandels and Reese 1960), and incubated at 28 °C, 180 rpm for 24 h to obtain enough biomass for the induction experiment.

The obtained mycelia in the preculture was filtered and washed twice with 0.9% sterile physiological saline solution and transferred into the induction medium with 1% (w/v) corncob (80 mesh, sourced from local farmer in the suburb of Tianjin, China) or pure cellulose (Sigma, USA) as the sole carbon source and 0.25 strength of the Mandels' salt solution buffered to pH 4.8 with 50 mM citrate buffer. The final inoculum concentration of the mycelium was 2.0 mg dry weight/mL. The induction experiments were carried out at 26 °C, 200 rpm.

RNA extraction and real-time quantitative PCR (qPCR)

The mycelia harvested at different induction times (0, 9, 18 and 36 h) were immediately frozen and ground to a fine

powder in liquid nitrogen. Total RNA was extracted using RNeasy pure plant kit (TIANGEN, China) according to the protocol. The quality and concentration of the extracted RNA were determined at 260 nm using a nucleic acid spectrometer (Implen, Germany). Approximately 500 ng total RNA was treated with gDNA Eraser to remove genomic DNA and subjected to reverse transcription using a Prime Script™ RT reagent kit (TaKaRa, China), which contained a blend of oligo (dT) and random hexamer primers.

qPCRs were performed in a real-time PCR system (A3500, Promega). The reaction system with a total volume of 20 µl contained 2 µl template cDNA, 10 µl SYBR® Premix Ex Taq II, 0.4 µl ROX Reference Dye 50×, 0.4 µM forward primers, 0.4 µM reverse primers and nuclease-free water. The reaction conditions were 30 s of initial denaturation at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Primers used in qPCRs were listed in supplementary Table 1. The actin encoding gene (*act1*) was used as a reference for quantification. The expressions of the selected genes at 9 h induced by corncob were defined as 1. All samples were analyzed at least in two independent experiments with three replicates in each run.

Protein profiles analysis using zymography and LTQ-MS/MS

The concentration of extracellular protein was measured by Bradford protein assay using bovine serum albumin as the standard (Bradford 1976). Zymograms of the secreted enzymes at different time induced by corncob and cellulose were analyzed by SDS-PAGE according to Laemmli's procedure (Laemmli 1970). Prior to loading on polyacrylamide gel, 30 µL protein samples with 10 µL 4× loading buffer was boiled for 5 min for denaturation. Then 20 µL supernatant (about 100 µg protein) was loaded onto a 10% polyacrylamide gel for electrophoresis.

To identify the proteins, the protein bands of interest were excised and in-gel digested with trypsin according to Liu et al. (2013). The analysis was carried out using an Agilent 1100 nanoLC system coupled with a hybrid linear ion trap-7 T Fourier transform ion cyclotron resonance mass spectrometer, LTQ-FT MS (Thermo, USA). The spray voltage was set at 1.8 kV. All MS and MS2 spectra were acquired in data-dependent mode, and the mass spectrometer was set to a full scan MS followed by ten data-dependent MS/MS scans. For data processing, all MS/MS spectra were searched against a *T. reesei* protein database using ProteinDiscoverer software (version 1.4, Thermo Scientific). Trypsin was chosen as the proteolytic enzyme, and up to two missed cleavages were allowed. Carbamidomethyl (Cys) was set as the fixed modification, and oxidation (Met) was set as the variable modification.

Table 1 Specific enzyme activities produced by *T. reesei* on corncob and cellulose

Enzyme activity	ECI ^a	ECII ^a
Protein content (mg/mL) ^b	0.53 ± 0.03	0.24 ± 0.01
FPase (U/mg) ^c	1.30 ± 0.09	1.54 ± 0.08
CMCase (U/mg) ^d	53.66 ± 0.12	80.63 ± 0.15
β-glucosidase (U/mg)	0.30 ± 0.01	0.29 ± 0.01
Xylanase (U/mg)	206.15 ± 0.23	116.38 ± 0.18
FPase:β-glucosidase	3.71 ± 0.03	4.39 ± 0.06
FPase:xylanase	0.0063 ± 0.0009	0.0132 ± 0.0012

^a ECI and ECII were induced by corncob and cellulose for 120 h, respectively

^b Protein content in the culture supernatant at the end of fermentation for 120 h

^c FPase, filter paper activity

^d CMCase, activity on carboxymethyl cellulose

The mass tolerance of the precursor ion and fragmentations was set to 20 ppm and 0.8 Da, respectively, and the peptide false discovery rate (FDR) was set to 1%.

Lignocellulolytic enzyme activity assay

The filter paper (FPase), β-glucosidase and carboxymethyl cellulose (CMCase) activities were determined by the method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose 1987). Xylanase activity was determined using dinitrosalicylic acid (DNS) method with 1% w/v birchwood xylan as the substrate as described by Bailey et al. (1992) with slight modifications. After incubated at 50 °C for 5 min, the reaction was stopped by adding 3 mL DNS followed by boiling for 5 min, and the concentration of reducing sugar was determined. Xylanase activity was defined as that one unit was the enzyme amount when one micro mole of reducing sugar (expressed as xylose) released per minute.

Enzymatic hydrolysis of pretreated lignocellulosic biomass

Corn stover was sourced from local farmer in the suburb of Tianjin, China. The air-dried corn stover was manually cut into pieces, ground in a mill, sieved by 80-mesh screen, and pretreated with NaOH solution as described earlier (Sun and Cheng 2002; Li et al. 2012). Steam-pretreated corn stover and acid-pretreated corncob were kindly provided by Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences. The compositions of the pretreated lignocellulosic biomass were determined according to the National Renewable Energy Laboratory protocol (Sluiter et al. 2008), and the contents of cellulose,

hemicellulose and lignin were as follows: alkali pretreated corn straw (58.89, 23.88 and 2.74%), steam-pretreated corn stover (51.6, 10.71 and 27.6%) and acid-pretreated corncob (49.8, 1.85 and 18.48%).

The substrate concentration in the enzymatic hydrolysis was 20 g/L in citrate buffer (0.05 M, pH 5.0). The crude multi-enzymes induced by corncob and cellulose as well as commercial Celluclast 1.5L were added, respectively, to ensure the enzyme loading at 8 FPU/g substrate. The total volume of the reaction system was controlled at 30 mL, and the reaction was carried out at 50 °C, 160 rpm. After 48 h, hydrolysis was stopped by boiling the sample for 5 min, followed by centrifugation for 10 min at 7000×g. The concentrations of reducing sugar and monosaccharides in the supernatant were measured.

The concentration of the reducing sugar was measured by the DNS method (Miller 1959), and the contents of monosaccharides in the hydrolysate were quantified by high performance liquid chromatography (HPLC, Aminex HPX-87H column, 300 × 7.8 mm, at 65 °C with RI detector) with 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 mL/min.

Statistical analysis

All experiments were conducted in triplicate. Data are presented as the mean ± standard errors. The differences between ECI and ECII were confirmed by Student's *t* test. Statistical significance was considered when *p* < 0.05.

Results and discussion

Enzyme production induced by corncob and cellulose

Figure 1 showed the production of the main lignocellulolytic enzymes by *T. reesei* Rut C30 using corncob or cellulose as the sole carbon source. Higher activities of FPase, CMCase, β-glucosidase and xylanase in ECI (induced by corncob) were obtained compared to those in ECII (induced by cellulose). After 120 h induction, the activities of FPase, CMCase, β-glucosidase and xylanase in ECI were 86.5, 46.9, 120.9 and 291.2% higher than those in ECII. The relative expression quantities of three cellulase genes (*cbh1*, *bgl1* and *egl1*), a xylanase gene *xyn1* and three transcription factor (TF) genes (*xyl1*, *ace1* and *ace2*) were provided in Fig. 2. After 18 h induction, the transcript abundance of *cbh1*, *bgl1* and *egl1* induced by corncob was 3.41-, 3.66- and 4.08-fold of those by cellulose. The expression of *xyn1*, encoding the main endoxylanase, induced by corncob at 9, 18 and 36 h was 2.37-, 7.44-, and 8.11-fold of those by cellulose, respectively. Meanwhile,

the expression quantity of *xyl1* (encoding the main positive regulator TF XYR1) induced by corncob was always higher than that by cellulose, and the expression level of *xyl1* was reported always in a positive correlation with the gene expressions of cellulase and xylanase in *T. reesei* [9]. Gene expression confirmed the increase of enzyme activity induced by corncob from transcription level. In addition, the concentration of secreted protein induced by corncob was about 2.2-fold of that by cellulose after 120 h induction.

The specific activities as well as values of FPase:β-glucosidase and FPase:xylanase of these two crude multi-enzymes after 120 h induction were shown in Table 1. The specific activities of FPase and CMCase in ECI were relatively lower than those in ECII due to the high protein production in ECI. Moreover, the specific activities of xylanase and β-glucosidase in ECI increased compared to those in ECII, especially, that of xylanase nearly doubled. Besides, values of FPase:β-glucosidase and FPase:xylanase in ECI were both lower than those in ECII, which indicated

that the composition proportion of the crude multi-enzyme was significantly different between ECI and ECII, and this would lead to the different performances in the hydrolysis of lignocellulose biomass.

These results were consistent with the previous reports that agricultural wastes were superior to pure cellulose at inducing enzymes for biomass degradation (Alriksson et al. 2009). It is well known that cellulose and lignocellulose themselves cannot directly trigger the induction of lignocellulolytic enzymes because it is insoluble. And their hydrolysates, soluble saccharides, such as cello-oligosaccharide, cellobiose, galactose and xylooligosaccharide, actually induce the synthesis of lignocellulolytic enzymes in *T. reesei* (Karaffa et al. 2006). Corncob as a complex inducer could improve not only the xylanase activity but also the activities of other enzymes such as FPase, CMCase and β-glucosidase. However, an interesting thing was reported that adding xylan to cellulose medium significantly decreased the FPase activity but had little effect on xylanase activity in *T. reesei* Rut C30 (Liao et al. 2014). Of

Fig. 1 Activities of the main lignocellulolytic enzymes in the culture supernatants of *T. reesei* Rut C30 on corncob and cellulose. The culture supernatants from *T. reesei* Rut C30 were obtained at different

times during the induction. **a** FPase; **b** CMCase; **c** β-glucosidase; **d** xylanase. Error bars indicate the standard deviation of three replicates

Fig. 2 Relative expressions of the main lignocellulolytic enzymes and transcription factor genes to steady-state *act* reference gene in *T. reesei* Rut C30. The strain was pregrown on glucose and then

transferred into induction medium with corncob (gray column) and cellulose (black column) for 0 (a), 9 (b), 18 (c) and 36 h (d) induction. Error bars indicate standard deviations

course, the composition of corncob is not limited to cellulose and xylan, but the induction mechanism is much too complicated so that it is far from being understood. Multiple synergies might occur during this induction system.

Secreted protein profiles induced by corncob and cellulose

The secreted protein profiles by SDS-PAGE were shown in Fig. 3. Under the same loading amount of proteins, the numbers of protein bands and the intensity of protein bands were different between ECI and ECII. According to the CAZy database summarized by Pribowo et al. (Pribowo et al. 2012), the molecular weight range of cellulase and hemicellulase was from 20 to 120 kDa. Because the secreted protein of *T. reesei* is consisted of 20–36% endoglucanase, 60–80% exoglucanase and only 1% β -glucosidase (Herpoël-Gimbert et al. 2008), the most intense band at 50–60 kDa in each lane in Fig. 3 mainly corresponded to exoglucanase (CBH I and CBH II). The different band numbers of the corresponding lanes

Fig. 3 Protein profile by SDS-PAGE of the secretome by *T. reesei* Rut C30 on corncob and cellulose. The secreted protein samples were obtained after 120 h induction. (Lane 1) ECI induced by corncob; (lane 2) ECII induced by cellulose; (lane M) protein standard marker. The protein bands in the red box labeled were selected as the interested differential ones for further LTQ-MS/MS analysis

indicated that the species of the total secreted protein induced by corncob and cellulose were different.

To analyze the difference in the secreted protein profiles between ECI and ECII, matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (LTQ-FT MS/MS) was used to identify the selected protein bands

Table 2 Identification of the selected different bands on the SDS-PAGE of the secreted proteins by *T. reesei* Rut C-30 grown on corncob and cellulose by LTQ-MS/MS

Accession number	Predicted protein function	Cazy family	MW (kDa)	pI	Corncob		Cellulose	
					Score	Area	Score	Area
Cellulases								
50402144	Exoglucanase I	GH7	54.0	4.81	–	–	594.52	5.579E7
572280773	Endoglucanase VII	GH61	26.8	7.72	–	–	442.81	1.419E8
170549	Endoglucanase EG-II	GH5	44.2	5.22	–	–	143.31	1.117E7
299892806	Endoglucanase IV	GH61	35.5	53.23	–	–	5.90	6.002E6
Hemicellulases								
7328936	Beta-xylanase	GH10	38.1	7.44	388.80	6.857E7	6405.21	6.618E8
358384163	Beta-xylanase	GH10	38.5	8.13	–	–	4453.78	5.280E8
31747160	Xyloglucanase	GH74	87.1	5.78	892.01	3.103E8	4213.43	1.937E8
380293100	Beta-xylosidase	GH3	87.2	5.72	327.82	6.450E7	–	–
2791278	Beta-xylosidase	GH3	87.1	5.78	–	–	3035.53	2.313E8
1054936	Arabinofuranosidase/B-xylosidase	ArabFuran-catal	51.1	6.34	40.17	2.659E5	–	–
Other glycoside hydrolases								
358381827	Glycoside hydrolase family 2	GH2	104.8	5.55	–	–	54.02	3.585E5
358393086	Glycoside hydrolase family 3	GH3	86.7	5.81	242.90	4.877E7	1460.07	1.940E8
340519849	Glycoside hydrolase family 3	GH3	84.5	5.68	69.52	5.356E5	–	–
340521228	Glycoside hydrolase family 5 (fragment)	GH5	40.1	5.38	47.64	1.227E5	255.89	4.962E6
340515048	Glycoside hydrolase family 16	GH16	30.9	5.49	30.85	2.188E5	–	–
340517806	Beta-1,3-endoglucanase (fragment)	GH17	42.2	4.67	0	4.087E5	129.11	3.464E6
340514746	Glycoside hydrolase family GH30	GH30	48.5	7.40	–	–	45.09	1.388E6
340514293	Glycoside hydrolase family 31	GH31	98.5	6.76	50.44	2.157E6	0	6.121E5
340518458	Glycoside hydrolase family 55	GH55	83	6.25	35.92	1.451E5	66.76	3.102E5
358382817	Glycoside hydrolase family 62	GH62	34.3	7.99	169.30	2.279E7	–	–
358383338	Glycoside hydrolase family 64	GH64	39.8	5.38	39.80	5.966E5	–	–
340515404	Glycoside hydrolase family 65	GH65	116.6	6.90	–	–	71.71	2.667E7
358380926	Glycoside hydrolase family 74	GH74	87.9	5.92	–	–	1094.00	1.065E8
340516490	Glycoside hydrolase family 92	GH92	87.5	5.48	118.33	2.209E6	459.08	5.934E6
358386597	Glycoside hydrolase family 92	GH92	89.1	6.07	91.16	3.685E5	–	–
399936313	Glucanase (fragment)	GH7	52.3	4.64	250.63	3.227E7	–	–
202072836	Glucanase	GH6	49.6	5.21	166.81	1.915E7	–	–
303297110	Glucanase	GH7	48.1	4.94	72.13	3.494E6	212.55	9.215E6
52547947	Glucanase	GH6	49.5	5.10	–	–	868.66	8.025E7
572275914	Endo-beta-1,4-glucanase (fragment)	GH12	22.3	7.94	–	–	87.36	3.750E6
Accessory proteins								
358383120	Expansin-like protein	nd	51.4	5.60	237.06	9.432E6	–	–
31747158	Cip1	nd	32.9	5.19	172.39	1.915E7	111.38	8.272E6
8052455	Swollenin	nd	51.5	5.02	–	–	287.08	5.090E6
358396788	Expansin module family protein	nd	51.7	5.35	–	–	19.17	4.217E6
Other proteins								
340520819	Aminopeptidase	nd	98.8	5.52	163.81	7.718E5	–	–
340517301	NADH-cytochrome b5 reductase	nd	36.0	9.38	3.558E5	35.73	–	–
340515114	Malate dehydrogenate	nd	35.1	8.98	2.155E5	32.16	–	–
340517515	Proteasome subunit beta type	nd	29.0	6.16	54.68	2.906E5	–	–
133242	Ribonuclease Trv	nd	25.9	4.45	74.30	36.14	–	–
354551318	Trypsin-like protease	nd	25.8	5.47	2.120E6	1.094E7	–	–
340522816	Carbohydrate esterase family 5	CE5	21.9	6.64	885.86	5.75 E8	939.98	5.90E8

Table 2 continued

Accession number	Predicted protein function	Cazy family	MW (kDa)	pI	Corncob		Cellulose	
					Score	Area	Score	Area
340519961	Carbohydrate esterase family 9	CE9	102.6	6.06	282.60	2.211E6	38.72	1.009E5
340515357	Cell wall protein	nd	41.2	5.41	89.95	7.62E5	67.61	6.78E5
340522672	Predicted protein	nd	20.9	5.35	350.25	1.205E8	71.67	2.209E7
340519026	Predicted protein	nd	24.7	4.97	150.00	1.082E7	321.22	1.420E7
340515525	Predicted protein	nd	93.3	5.36	192.16	1.65E6	500.67	7.02E6
340516817	Predicted protein	nd	38.3	5.94	28.80	6.212E5	110.68	3.659E6
572278710	Uncharacterized protein	nd	38.4	8.21	36.97	1.489E6	29.52	9.690E7
572280540	Uncharacterized protein	nd	30.6	4.45	27.08	2.853E5	–	–
340521732	Predicted protein	nd	44.2	5.83	190.02	8.632E6	–	–
340515421	Predicted protein	nd	29.8	5.95	31.08	4.317E5	–	–
340517951	Predicted protein	nd	26.9	5.06	35.98	1.084E7	–	–
340522571	Trypsin-like serine protease	nd	26.4	6.28	–	–	52.01	1.146E7
340517798	Predicted protein	nd	28.2	4.55	–	–	215.19	527.08
572283977	Uncharacterized protein	nd	28.1	5.19	–	–	1.330E6	3.174E6

GH glycoside hydrolase, *nd* not detected, *CE* carbohydrate esterase

Table 3 Volume dosage and content analysis for each crude multi-enzyme under the same hydrolysis conditions

Crude enzyme ^a	Enzyme loading (FPU/g) ^b	β-glucosidase activity (IU) ^c	Volume (mL) ^c	Protein content (mg) ^c	Substrate concentration (g/L)
ECI	8	1.83	6.96	3.69	20
ECII		1.51	12.97	3.11	
C1.5L		16.03	0.90	59.43	

^a ECI and ECII were induced by corn cob and cellulose for 120 h, respectively. C1.5L is a commercial *T. reesei* cellulase product Celluclast 1.5L (sigma C2730)

^b Enzyme loading was 8 FPU per g substrate (IU/g) for these three different enzymes

^c Needed volume of each enzyme under the same enzyme loading (8 FPU/g) and their corresponding content of β-glucosidase and protein

which were shown in Fig. 3. The main differential proteins identified in content and species were shown in Table 2. Most proteins identified were functionally grouped into cellulase, hemicellulase, other glycoside hydrolase, esterase, glucanase, and accessory protein in the degradation of lignocellulosic biomass, predicted protein and other proteins. This result was consistent with the results in previous studies (Liao et al. 2014; He et al. 2014). One exoglucanase and three endoglucanases were found specifically in ECII rather than in ECI. Among the differential hemicellulases, two β-xylosidases were found specifically in ECI rather than in ECII. Additionally, some other glycoside hydrolases (such as family 16, 62 and 64) with low molecular weight were found specifically in ECI, while glycoside hydrolases (such as family 2, 65 and 74) with high molecular weight were found specifically in ECII. Meanwhile, some predicted proteins with unknown function and accessory proteins such as Cip1 and expansin-like protein were also found at a higher content in ECI than in ECII. These proteins and the diverse glycoside hydrolases with

low molecular weight might play a role in the induction of lignocellulolytic enzyme by corn cob. In addition, it was reported that xylose reductase, galactokinase and methyltransferase were essential for the cellulase induction on lactose in *T. reesei* (Karaffa et al. 2006; Pribowo et al. 2012; Herpoël-Gimbert et al. 2008). These hemicellulases might be contained in the ECI secretome. However, the induction mechanism was too complicated because so many proteins and hydrolysates of insoluble lignocellulose participated in this process, and further researches on the functions of these differential proteins are needed to reveal the induction mechanism of lignocellulolytic enzymes in *T. reesei* by lignocellulosic biomass.

Enhancing saccharification performance of the crude multi-enzyme

Saccharification performances of ECI, ECII and commercial Celluclast 1.5L (C1.5L) on microcrystalline cellulose and different pretreated lignocellulosic biomasses were

Fig. 4 Saccharification performance of the crude multi-enzymes (ECI, ECII and C1.5L) in the hydrolysis of different substrates. **A** total reducing sugar yield; **B** individual sugar concentrations. The capital letters in the two figures refer to different substrates: **a** alkali-

pretreated corn straw; **b** steam explosion corn straw; **c** acid-pretreated corncob; **d** microcrystalline cellulose. Error bars indicate the standard deviation of three replicates

compared. From Table 3, it could be seen that at the same amount of enzyme loading and substrate concentration, the volume of ECI required was only half of that of ECII, and the corresponding content of β -glucosidase (expressed in IU) in ECI was 21% higher than that in ECII.

The hydrolysis results of different substrates were shown in Fig. 4. After 48 h hydrolysis, the reducing sugar yields of different substrates hydrolyzed by ECI were all higher than that by ECII except that of cellulose. The reducing sugar yields of acid-pretreated corncob (substrate C) and alkali-pretreated corn straw (substrate A) hydrolyzed by enzyme I increased 12.5 and 7.6% compared to those by enzyme II ($p < 0.05$), while there was no obvious difference in the reducing sugar yield of steam explosion corn straw (substrate B) hydrolyzed by enzyme I and enzyme II ($p > 0.05$). In addition, the yields of glucose and xylose of the pretreated lignocellulosic biomass (A–C) hydrolyzed by ECI were all higher than those by ECII, but higher glucose yield of cellulose (substrate D) was obtained by ECII. The yields of glucose and xylose of acid-pretreated corncob (substrate C) hydrolyzed by enzyme I increased up to 33.4 and 21.1% compared to those by enzyme II ($p < 0.05$). These results indicated that the enzymes induced by the substrate itself could efficiently hydrolyze the corresponding substrate. Compared to commercial enzyme C1.5L, ECI had higher glucose yields for substrates A–D, and higher xylose yields for substrates A, B and C although the protein content in the hydrolysis system by C1.5L was 16 times of that in the system by ECI. This might be caused by the low hemicellulase activity in C1.5L. These data indicated that enzyme proportion and composition as well as the pretreatment method significantly influenced the yields of the reducing sugar in the enzymatic hydrolysis of lignocellulosic biomass. On the

whole, the composition proportion with lower FPase: β -glucosidase and FPase:xylanase in ECI was more efficient for the hydrolysis of lignocellulosic biomass considering the yield of total reducing sugar.

Ng et al. (2011) reported that β -glucosidase could not only prevent the inhibition by cellobiose in *T. reesei*, but also enhance the activities of endo- and exo-cellulases in cellulosic bioconversion. Therefore, the increase in the hydrolysis efficiency of ECI compared with that of ECII might be due to the optimization of the enzyme composites and the synergetic effect among the cellulase and hemicellulase. It was speculated that the production increase of each enzyme component induced by corncob in Fig. 1 might be also related to the synergistic induction between cellulose and hemicellulose components in corncob. Another advantage of ECI induced by corncob was to reduce the enzyme dosage (46.5% decrease in volume at the same FPU loading) in lignocellulosic biomass hydrolysis, which was greatly significant to reduce the biorefinery cost of lignocellulosic biomass.

Conclusion

In this study, an efficient crude multi-enzyme induced by corncob with optimized composition improved the saccharification yield of the lignocellulose biomass and reduced the enzyme dosage. The enzyme proportion of this efficient multi-enzyme induced by corncob could guide the genetic modification of *T. reesei*. Corncob or other lignocellulosic biomass as a rather cheap raw material could be a potential candidate for the low cost production of lignocellulolytic multi-enzymes for efficient lignocellulose degradation.

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Compliance with ethical standards

Ethical approval This study was focused on analyzing the efficient crude multi-enzyme produced by *Trichoderma reesei* using corncob for the hydrolysis of lignocellulose. Every parts of the research did not involve human participants and other animals. Our manuscript complies with the Ethical Rules applicable for 3 Biotech.

Conflict of interest The authors have declared that no conflicts of interest exist.

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