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Comparative secretomic analysis of lignocellulose degradation by *Lentinula edodes* grown on microcrystalline cellulose, lignosulfonate and glucose

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ABSTRACT

Lentinula edodes has the potential to degrade woody and nonwoody lignocellulosic biomass. However, the mechanism of lignocellulose degradation by L. *edodes* is unclear. The aim of this work is to explore the profiling of soluble secreted proteins involved in lignocellulose degradation in L. *edodes*. For that, we compared the secretomes of L. *edodes* grown on microcrystalline cellulose, cellulose with lignosulfonate and glucose. Based on nanoliquid chromatography coupled with tandem mass spectrometry of whole-protein hydrolysate, 230 proteins were identified. Label-free proteomic analysis showed that the most abundant carbohydrate-active enzymes involved in polysaccharide hydrolysis were *endo*-β-1,4-glucanase, α-galactosidase, polygalacturonase and glucoamylase in both cellulosic secretomes. In contrast, enzymes involved in lignin degradation were most abundant in glucose culture, with laccase 1 being the predominant protein (13.13%). When the cellulose and cellulose with lignosulfonate secretomes were compared, the abundance of cellulases and hemicellulases was higher in cellulose with lignosulfonate cultures, which was confirmed by enzyme activity assays. In addition, qRT-PCR analysis demonstrated that the expression levels of genes encoding cellulases and hemicellulases were significantly increased (by 32.2- to 1166.7-fold) when L. *edodes* was grown in cellulose with lignosulfonate medium.
Biological significance: In this article, the secretomes of L. *edodes* grown on three different carbon sources were compared. The presented results revealed the profiling of extracellular enzymes involved in lignocellulose degradation, which is helpful to further explore the mechanism of biomass bioconversion by L. *edodes*.

1. Introduction

Lentinula edodes (order Agaricales), commonly known as Xianggu or shiitake, is the second most commercially produced mushroom species, surpassed only by the white button mushroom (*Agaricus bisporus* [Lange] Imbach) [1]. In addition to its commercial value, *L. edodes* shows potential in biotransformation and fiber bleaching as well as bioremediation [2–4]. In nature, *L. edodes* is a saprobic, wood-colonizing white-rot species that degrades lignocellulose in wood. Therefore, its cultivation materials have been dead trees or wood chips in China for the last eight centuries [5]. Currently, sawdust-based cultivation of L. *edodes* consumes forest resources, preventing ecological conservation. Replacing wood or wood chips with other lignocellulose biomass such as agricultural straws is necessary for cultivation of this species. Moreover, the biological efficiency of L. *edodes* could improve with growth on new cultivated materials. Understanding the mechanism of lignocellulose degradation by L. *edodes* is critical.

The wood decay mechanism of white-rot fungi has been investigated largely in Polyporales species because of the availability of genomic information, often combined with transcriptomic and secretomic analyses. Lignin degradation relies on a wide array of extracellular oxidoreductases produced by white-rot fungi. Of these enzymes, fungal class-II lignin modifying peroxidases (CAZy auxiliary activity family 2, AA2) including lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs) play major roles in lignin modification [6,7]. Class-II peroxidases require hydrogen peroxide as an oxidant, which may be generated by copper radical oxidases (CROs, AA5) including glyoxal oxidase (GLOX), glucose–methanol–choline oxidoreductases (GMCs, AA3) such as aryl-alcohol oxidase, methanol oxidase, and pyranose oxidase [8,9]. In addition to peroxidases, laccases have been implicated in lignin degradation [10]. Laccases are phenol-oxidizing multicopper oxidases (MCOs, AA1) that may act on lignin substructures with the aid of aromatic mediator compounds [6].

Crystalline cellulose is depolymerized by white-rot fungi with the aid of lytic polysaccharide monooxygenase (LPMO, CAZy family AA9) enzymes [11,12]. Depolymerized cellulose is completely digested by cellobiohydrolases (CAZy glycoside hydrolase family GH6 and GH7) and endoglucanases (GH5, GH9, GH12, GH44 and GH45), in conjunction with β-glucosidases (GH1 and GH3) [6,13]. In addition, a wide array of

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other carbohydrate-active enzymes such as carbohydrate esterases and polysaccharide lyases (PLs) degrade plant cell wall components including hemicelluloses and pectins [13]. Interestingly, the lignin degradation process may involve modulation of the hydrolytic enzymes commonly associated with cellulose and hemicellulose [11]. The genes expressed and proteins produced during growth on plant material reflect the specific lifestyle of each fungal species and its strategy for lignocellulose conversion [6].

While Polyporales species are generally grown on woody substrates, members of the Agaricales order such as *Pleurotus* naturally grow on wood, leaf litter, and other lignocellulosic agricultural wastes, suggesting diverse strategies for lignocellulose conversion. The diversity of strategies could be reflected in the differences among the secretomes. For example, *Pleurotus ostreatus* secretomes shared only 39% of proteins when grown on polar wood or wheat straw [11,14]. A number of studies have revealed that the composition of the secretome changes in response to different carbon sources, as reviewed by McCotter et al. [15]. In general, the availability, quality, and complexity of the carbon source influence the secretome composition [15]. This suggests that various carbon sources from lignocellulosic biomass can be used as inducers to investigate the mechanism of lignocellulose degradation of L. *edodes* using secretome analysis.

Several (hemi)cellulolytic enzymes including xylanase, endoglucanases (GH12) and cellobiohydrolases (GH6 and GH7) and enzymes involved in lignin degradation (laccases and MnP) in L. *edodes* have been biochemically characterized [16–22]. Recently, the genome of strain W1–26, a monokaryon from a commercial dikaryon of L. *edodes*, was sequenced in our lab (*L. edodes* genome database website: http://LEgdb.chenlianfu.com; [23]), and the annotated information is available in the NCBI database (GenBank accession number LDAT00000000). According to the genome sequence, selective ligninolysis by L. *edodes* [24] is probably attributable to the presence of an arsenal of lignin-degrading enzymes, including 14 MCOs, 2 MnPs, 1 VP and a set of MCOs and GMCs [23]. Moreover, secretomic analysis combined with genomic information will offer insight into the enzymatic mechanism involved in the decay of not only lignins but also other lignocellulose constituents by saprotrophic fungi (reviewed in reference [25]).

The present work aims to characterize potentially important enzymes involved in degradation of lignocellulose and to provide insight into extracellular enzyme gene expression patterns in L. *edodes*. This will reveal additional reagents to increase the efficiency of biomass conversion to useful chemical feedstocks and will lead to improvements in mushroom crop yields. Here, we use glucose, microcrystalline cellulose, and sodium lignosulfonate (a natural chemically modified product of lignin and a byproduct from pulping spent liquor, usually used as anionic polymeric surfactant) as carbon sources to investigate the unique enzyme system of L. *edodes* and to evaluate its capacity for bioconversion of agricultural materials.

2. Materials and methods

2.1. Fungal strain and culture conditions

The L. *edodes* commercial heterokaryon strain WX1 (ACCC 50926) has been deposited in the Agricultural Culture Collection of China (Wuhan). Stock cultures were activated on complete yeast extract medium (CYM) plates with 2% glucose, 0.2% yeast extracts, 0.2% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.046% KH_2PO_4, and 2% agar.

For preparation of extracellular enzymes, 9-mm-diameter agar plugs from the leading mycelia edge of the stock cultures were transferred to 25 mL liquid modified CYM containing 2% (wt/vol) glucose (glucose), 2% microcrystalline cellulose (cellulose) or 1.9% microcrystalline cellulose plus 0.1% sodium lignosulfonate (cellulose-SLS) as the major carbon source. The cultures (triplicate for each condition) were incubated at 25 °C under stationary conditions for 20 days.

For the three media conditions, culture supernatants were collected, centrifuged twice at 10,000 ×*g* for 15 min to remove the mycelia and then clarified by filtration through a 0.45 μm filter. 10 mL of filtrate from each condition was extracted for enzyme activity assays, and the rest used for proteomic analysis.

2.2. Secreted protein preparation

Triplicate filtrates for each condition were combined equally [11]. Next, 5 mL of saturated Tris-phenol was added to each 25 mL filtrate sample in a 50 mL centrifuge tube; the samples were mixed by inversion and then held at room temperature for 10 min. Following centrifugation at 10,000 ×*g* for 10 min at 4 °C, 1 mL of phenol solution containing the dissolved proteins (at the bottom of the centrifuge tube) was collected and placed in a new centrifuge tube, in which proteins were precipitated via the addition of five volumes of cold 0.1 M sodium acetate in pure methanol. The sample was centrifuged, and after decanting the supernatant, the pellet was washed several times with cold 80% acetone. Pelleted proteins were re-solubilized and denatured in 40 μL of a 9 M urea solution (9 M urea, 2% CHAPS, 1 mM PMSF, 50 mM DTT). The Bradford method was employed to determine the protein concentration using bovine serum albumin (BSA) to generate a standard curve. Protein solutions were diluted to the same concentration, and 200 μL (100 μg) of each sample was pipetted and purified using a readyprep 2-D Cleanup kit (BIO-RAD, USA) according to the manufacturer's instructions. Purified proteins were re-solubilized in 25 mM NH_4HCO_3 at pH 8.2 for tryptic digestion (100 ng/μL Trypsin Gold from PROMEGA Corp. in 25 mM NH_4HCO_3).

2.3. Secretomic analysis

Mass spectrometric analysis of soluble extracellular proteins was performed as described for *P. ostreatus* [11]. Digestion was conducted overnight at 30 °C. Peptides were extracted at 37 °C in 100% ACN followed by 0.5% trifluoracetic acid, dried, cleaned using ZipTip with a 0.6 μL C18 resin (Millipore), and reconstituted in 5 μL of 0.1% formic acid in 2% ACN. Peptide samples (100-fold diluted, 2 μL volume) were injected and analyzed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled with nanoEasy high-performance liquid chromatography equipment (Proxeon). Three technical replicates were run for the LC-MS/MS analysis. Peptides were first trapped onto a 2 cm C18-A1 ASY-Column precolumn (Thermo Scientific) and then eluted onto a Biosphere C18 column (75 μm inner diameter, 15 cm length, 3 μm particle size) (NanoSeparations) via a 130 min gradient elution from 0 to 45% buffer B (buffer B: 0.1% formic acid in pure ACN) at a flow rate of 250 nL/min. The LTQ-Orbitrap was set to acquire MS/MS spectra in data-dependent mode as follows: MS survey scans from *m/z* 300 to 1600 were collected with a target value of 1,000,000 at a resolution of 30,000 (at *m/z* 400), and MS2 spectra were acquired in the linear ion trap with a target value of 10,000 and normalized collision energy of 35%. Precursor ion charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of one and an exclusion duration of 30 s.

Raw MS/MS data were searched against the genome of the L. *edodes* database [23], using MaxQuant (version 3.2.2.0) as the search engine. The MS/MS search was performed with the following settings: (i) trypsin was used to digest the proteins, allowing two missed cleavages, (ii) carbamidomethylation of cysteine residues was selected as a fixed modification, and (iii) error tolerances on the precursor and fragment ions were set to 4.5 ppm and 0.5 Da, respectively. For protein identification, the desired false discovery rate (FDR) at peptide spectrum match (PSM) level was set to 1%. PSMs were further filtered with the cutoff of Andromeda score (>40) and delta score (>6). Protein identifications were accepted if they contained at least 2 identified peptides and one unique peptide. The protein FDR was set as 1%. For abundance

calculation (label-free quantification), mass spectrometric signal intensities (MaxQuant) of peptide precursor ions belonging to each protein were divided by the total abundance of all detected proteins in each culture condition. Protein abundance value was calculated from the normalized values of the three technical replicates. The presence or absence of a signal peptide was predicted with SignalP 4.1 [26].

2.4. Enzyme activity assays

Enzymatic hydrolyses of the polysaccharides were performed in a sodium acetate buffer solution (SABF, 0.2 M, pH 4.8). The filter paper activity (FPA) and endoglucanase (CMCase), xylanase, cellobiohydrolase (*p*NPCase), and β-glucosidase (*p*NPGase) activities of the culture supernatants were assayed according to the methods reported by Li et al. [27], with a few differences. The control to account for the presence of any reducing sugars in the crude enzyme samples was not boiled, but rather digested with proteinase K (PROMEGA). The SABF used for the FPA assay included 1% β-glucosidase (Sigma # C6105). Independent triplicate cultures were sampled and analyzed.

2.5. Real time quantitative PCR analysis

L. edodes mycelia grown in liquid CYM for 20 days were washed twice with sterilized water and transferred to glucose, cellulose or cellulose-SLS medium. Cultures were inoculated at 25 °C and sampled at 1, 3 and 5 d, respectively. Mycelia that were not transferred served as controls. All samples were centrifuged twice at 10,000 × g to remove supernatants, washed and stored at −80 °C for total RNA preparation.

For total RNA isolation, approximately 100 mg of L. *edodes* mycelia was ground in liquid nitrogen and extracted using RNAiso plus (TaKaRa, Japan) according to the manufacturer's instructions. RNA integrity and quantity were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the RNA6000 Nano Assay, respectively. First-strand cDNA was synthesized with 2 μg of RNA and 0.5 μg of oligo(dT) in 20-μL reactions using TranScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China) according to the manufacturer's instructions. The primer sequences of the tested genes and the reference gene are listed in Table S3. Quantitative RT-PCR was performed using a CFX Connect real-time PCR system (BIO-RAD). Each reaction consisted of 1 μL each of the forward and reverse primers (10 mM), 30 ng of sample cDNA, 10 μL of AceQ qPCR SYBR Master Mix (Vazyme, China) and water to a final volume of 20 μL. The cycling parameters were 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 20 s. A dissociation curve was generated to verify that a single product was amplified. Transcript levels were normalized to the transcript levels of the actin gene (*L. edodes* genome Gene ID: LE01Gene04556) and quantified according to the formula $2^{-\Delta\Delta CT}$ [28]. Control reactions included a sample from mycelia that were not transferred. Three biological and three technical replicates were analyzed.

3. Results

3.1. Secretome profiles of L. edodes cultured in glucose, cellulose and cellulose-SLS media

Proteomic analysis using nanoLC-MS/MS was performed to investigate the lignocellulolytic enzyme profiles in the secretome of L. *edodes* grown on glucose, cellulose or cellulose-SLS. A total of 230 proteins were identified in the three different culture media (listed in Table S1, and total matched peptides were listed in Table S2). As summarized in Fig. 1, 175, 193 and 212 extracellular proteins were found in the glucose, cellulose and cellulose-SLS media, respectively. In total, 64.3% of the identified proteins were produced under all three conditions; 8.3% were exclusive to cellulose-SLS cultures; 15.2% were shared by cellulose-SLS and cellulose cultures; and 0.9% and 4.3% were exclusive to cellulose and glucose cultures, respectively. Proteins unique to the

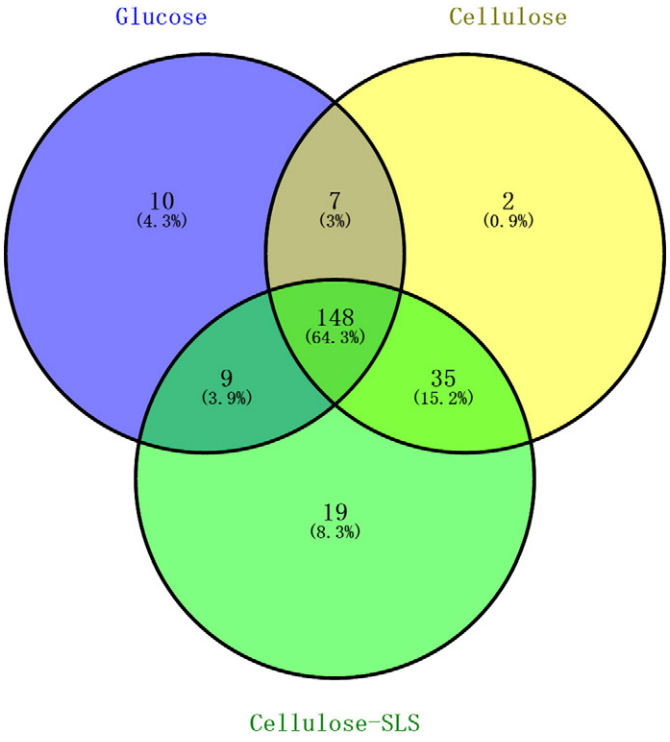

Fig. 1. Venn diagram of total protein numbers for the L. *edodes* secretomes in glucose, cellulose and cellulose-SLS media. (See Table S1 for the complete protein lists for each of the secretomes.)

cellulose-SLS conditions include several enzymes involved in lignocellulose degradation, such as lytic polysaccharide monooxygenase (AA9), cellobiohydrolase (GH7), *endo*-β-xylanase (GH10), α-galactosidase (GH27, CBM1), pyranose dehydrogenase and cellobiose dehydrogenase (AA3) (Table 1). The molecular weight of the identified proteins ranged from 8 to 220 kDa (Table S1).

The secreted proteins were classified by function into the following types: CAZyme, oxidoreductase, lipase, phosphatase, protease and peptidase, hypothetical protein, and other protein. The CAZyme family consisted of cellulase, hemicellulase, lignin-degrading protein, pectinase, amylase, other polysaccharide-degrading protein, cell wall remodeling protein and chitinase. The three secretomes had a similar composition in terms of the protein types present. *L. edodes* released the most CAZymes (103 proteins) into the cellulose-SLS medium, with 97 and 91 proteins secreted into the cellulose and glucose media, respectively (Fig. 2). Moreover, 7.3% of the 110 CAZymes were found exclusively in the cellulose-SLS secretome, with 0.9% and 2.7% exclusively present in the cellulose and glucose secretomes, respectively (Fig. S1). The number of proteins involved in degrading cellulose, hemicellulose, lignin, pectin and other polysaccharides present in cellulose and cellulose-SLS media was greater than that in glucose media (Fig. 2).

3.2. Abundance of the proteins involved in plant cell wall degradation in the three L. edodes secretomes

There were moderate changes in the number of proteins involved in plant cell wall degradation produced by L. *edodes* in the three media. However, noteworthy differences were observed in the abundance of the secreted proteins, especially those involved in cellulose, hemicellulose, pectin and lignin degradation (Table 1).

Efficient and complete degradation of cellulose requires the synergistic action of three types of cellulases: cellobiohydrolases (CBHs), endoglucanases (EGLs) and β-glucosidases (BGLs). The relative abundance of cellulases was lower in the glucose medium (0.36%) than in the cellulose-SLS medium (0.81%), however, the relative abundance of

Table 1
Relative abundance of the individual proteins involved in cellulose, hemicellulose, pectin and lignin degradation.

Protein ID[a]	CAZy Family[b]	Protein description[a]	Protein abundance (% of total MS intensity)[c]			SP[d]
			Glucose	Cellulose	Cellulose–SLS	
Cellulose–degrading proteins						
04541	GH12	endo–β–1,4–glucanase	– –[e]	0.09	0.21	Y
04829	GH7–CBM1	1,4–β–D–glucan cellobiohydrolase	0.03	0.02	0.18	Y
10050	GH6–CBM1	1,4–β–D–glucan cellobiohydrolase	0.01	0.03	0.11	Y
08136	GH5	endo–β–1,4–glucanase	0.00	0.02	0.10	Y
12864	GH7–CBM1	1,4–β–D–glucan cellobiohydrolase	0.00	0.01	0.07	Y
10512	GH3	β–glucosidase	0.29	0.00	0.06	Y
07961	GH7–CBM1	1,4–β–D–glucan cellobiohydrolase	0.01	0.00	0.02	Y
10266	AA9	lytic polysaccharide monooxygenase	– –	– –	0.02	Y
04089	GH7–CBM1	1,4–β–D–glucan cellobiohydrolase	– –	– –	0.02	Y
10634	GH3–CBM1	β–glucosidase	– –	0.00	0.01	Y
07574	GH3	β–glucosidase	0.02	0.00	0.00	Y
Hemicellulose–degrading proteins						
06823	GH27–CBM35	α–galactosidase	0.46	0.98	0.74	Y
05323	GH27	α–galactosidase	0.00	0.10	0.69	Y
06046	GH51	α–L–arabinofuranosidase	0.11	0.27	0.66	Y
00068	GH43–CBM35	galactan 1,3–beta–galactosidase	0.00	0.11	0.33	Y
03223	GH10–CBM1	endo–1,4–β–xylanase	0.10	0.10	0.30	Y
05156	GH3	exo–1,4–β–xylosidase	0.01	0.05	0.19	Y
10175	GH43	galactan1,3–β–galactosidase	– –	0.04	0.14	N
09625	GH27	α–galactosidase	0.13	0.12	0.14	N
12913	GH2	β–mannosidase	0.20	0.14	0.14	Y
05961	GH35	β–galactosidase	0.03	0.07	0.12	Y
09692	GH51	α–L–arabinofuranosidase	0.07	0.08	0.12	Y
11487	CE15–CBM1	glucuronoyl methylesterase	0.13	0.07	0.09	Y
06248	CE3	acetyl xylan esterase	0.02	0.01	0.08	Y
04279	GH95	α–fucosidase	0.04	0.06	0.04	N
05655	GH115	xylan α–glucuronidase	0.00	0.02	0.04	Y
02639	GH10	endo–1,4–β–xylanase	– –	0.00	0.03	Y
01882	GH27–CBM1	α–galactosidase	– –	– –	0.01	Y
07273	GH74–CBM1	xyloglucanase	– –	– –	0.01	Y
07975	GH10–CBM1	endo–1,4–β–xylanase	– –	– –	0.01	Y
00726	GH5	mannan endo–1,4–β–mannosidase	0.04	0.01	0.00	Y
13906	GH37	trehalase	0.01	– –	– –	N
05191	GH95	α–L–fucosidase	0.00	0.00	0.00	Y
Lignin–degrading proteins						
08330	AA1	laccase 1	13.13	7.92	1.81	Y
13044	AA1	laccase 6	0.05	0.00	0.91	Y
13975	AA7	glucooligosaccharide oxidase	0.64	0.06	0.73	Y
00046	AA7	glucooligosaccharide oxidase	0.57	0.35	0.53	Y
01372	AA1	laccase 5	0.00	0.04	0.09	Y
02763	AA7	glucooligosaccharide oxidase	0.11	0.05	0.08	Y
08997	AA2	manganese peroxidase 2	0.01	0.00	0.06	Y
03487	AA3	aryl–alcohol oxidase	0.03	0.06	0.02	Y
04633	AA5	glyoxal oxidase	0.02	0.01	0.01	Y
02931	AA7	glucooligosaccharide oxidase	0.02	0.01	0.01	Y
04793	AA5	copper radical oxidase	0.05	0.01	0.00	Y
09425	AA3	glucose oxidase	0.01	0.00	0.00	Y
02092	AA5	glyoxal oxidase	– –	0.01	0.00	N
07621	AA3	glucose oxidase	– –	0.00	0.00	Y
04660	AA1	laccase 8	0.00	– –	0.00	Y
13226	AA3	pyranose dehydrogenase	– –	– –	0.00	Y
04737	AA	laccase 13	– –	0.00	– –	Y
12439	AA3	glucose oxidase	0.00	0.01	– –	Y
04836	AA3	aryl–alcoholoxidase	0.02	– –	– –	N

(continued on next page)

Table 1 (*continued*)

Pectin–degrading proteins						
06016	GH28	polygalacturonase	0.61	1.78	2.03	N
14061	CE8	pectinesterase	0.06	0.25	0.17	N
03020	GH28	polygalacturonase	0.08	0.06	0.13	Y
08349	GH28	polygalacturonase	– –	0.06	0.02	Y
06973	GH28	polygalacturonase	0.01	0.00	0.02	Y
07596	GH78	α–L–rhamnosidase	0.00	0.00	0.01	Y
02734	GH28	rhamnogalacturonase	– –	0.01	0.01	Y
05074	GH53	endo–β–1,4–galactanase	0.00	0.01	0.00	Y
12252	GH106	α–L–rhamnosidase	– –	0.00	0.00	Y
01030	GH105	rhamnogalacturonyl hydrolase	0.00	0.00	– –	Y

[a]Protein ID and protein description were obtained from our *Lentiula edodes* genome database (website: http://LEgdb.chenlianfu.com). ID number is short for LE01Gene00000.
[b]Family information was obtained from the carbohydrate-active enzyme database (CAZy).
[c]Abundance values are given for proteins identified by peptide LC-MS/MS.
[d]means proteins were not detected from the corresponding condition.
[e]Prediction of signal peptides was based on SignalP analysis.

cellulases was lowest in the cellulose medium (0.16%; Fig. 3). As shown in Tables 1, 5 CBHs (GH6 and GH7) were significantly abundant (0.40%) in the cellulose-SLS medium, while they were less abundant and not represented in the glucose (0.04%) and cellulose (0.06%) cultures, respectively. Two EGLs (GH5 and GH12) exhibited the highest abundance in the cellulose-SLS medium (0.31%), followed by the cellulose medium (0.11%), whereas no EGL was detected in the glucose cultures. In contrast, three BGLs (GH3s) were most abundant in the glucose medium (0.31%) and were hardly expressed in the cellulose or cellulose-SLS condition (less than 0.1%). Lytic polysaccharide monooxygenase (LPMO) is an auxiliary enzyme currently used to supplement cellulase preparations. An LPMO classified as AA9 was detected only in the cellulose-SLS culture at a low abundance of 0.02%.

Hemicellulose degradation requires the concerted action of both main chain and side-group hemicellulases. The relative abundance of hemicellulases was lower in the glucose (1.47%) than in the cellulose media (2.44%) and was highest in the cellulose-SLS media (3.94%), as shown in Fig. 3. The α-galactosidase (GH27-CBM35) was the most abundant of this type due to its high abundance in the glucose, cellulose and cellulose-SLS cultures (0.46%, 0.98%, and 0.74%, respectively, Table 1). Most hemicellulases, including a β-xylosidase (GH3), two α-galactosidases (GH27), a β-galactosidase (GH35), two galactan-β-galactosidase (GH43), an α-L-arabinofuranosidase (GH51) and a xylan α-glucuronidase (GH115), were upregulated in both cellulose and cellulose-SLS cultures compared to glucose cultures, while an endo-1,4-β-xylanase (GH10) and an acetyl xylan esterase (CE3) were upregulated only in cellulose-SLS cultures. A β-mannosidase (GH5) and a trehalase (GH37) were identified only when L. *edodes* was grown on glucose, although at low abundance (0.04% and 0.01%, respectively).

Pectin is a highly branched structural heteropolysaccharide, so a broad range of enzymes are required for its degradation. The relative abundance of pectinase significantly increased when L. *edodes* was grown on cellulose (2.17%) and cellulose-SLS (2.39%) media, compared to glucose media (0.76%, Fig. 3). The most abundant enzyme in the pectinase category was a polygalacturonase (GH28) with an abundance of 0.61%, 1.79% and 2.03% in the glucose, cellulose and cellulose-SLS cultures, respectively (Table 1). The expression pattern of pectinases included four polygalacturonases (GH28), a pectinesterase (CE8) and an *endo*-β-1,4-galactanase (GH53) that were upregulated either under both cellulose and cellulose-SLS conditions or under only one of those conditions.

In contrast to the polysaccharide degradation enzymes mentioned above, the relative abundance of lignin-degrading enzymes was highest in the glucose cultures (14.72%), followed by the celluloses (8.54%), and the cellulose-SLS cultures (4.27%; Fig. 3). Laccase 1 (AA1) was extremely abundant in the glucose secretome (13.13%), while its abundance was lower in the cellulose and cellulose-SLS secretomes (7.92% and 1.81%, respectively; Table 1). Additionally, some proteins with relatively low abundance, including three glucooligosaccharide oxidases (AA7), a glyoxal oxidase (AA5), a copper radical oxidase (AA5) and an aryl-alcohol oxidase (AA3), were slightly more abundant in the glucose secretome than in the cellulose and cellulose-SLS secretomes. In contrast, laccase 5 (AA1) and a manganese peroxidase (AA2) were upregulated in the cellulose and cellulose-SLS cultures, while laccase 6 (AA1),

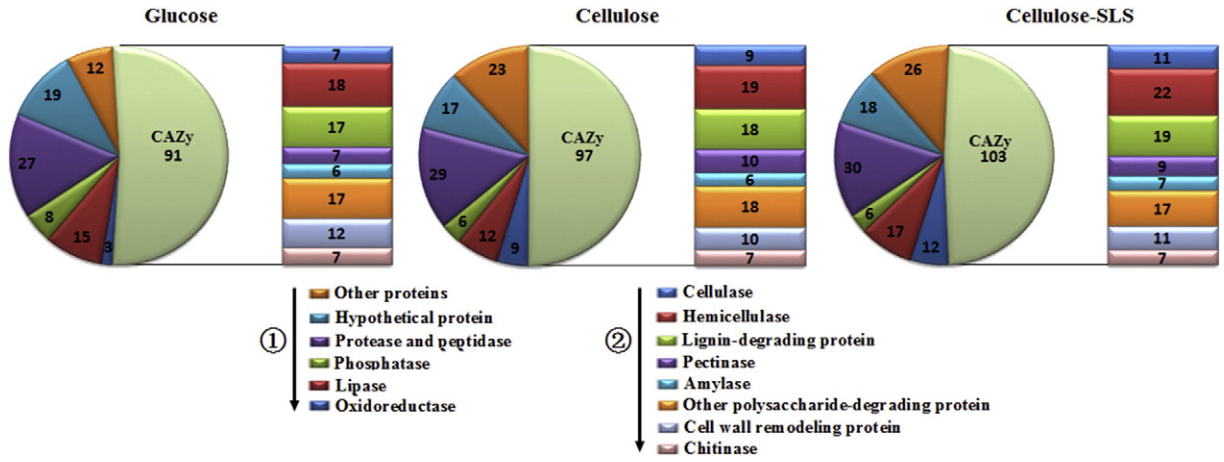

Fig. 2. Functional classification of the proteins released by L. *edodes* grown on glucose, cellulose, and cellulose-SLS. ①: Legends of circular area (left); ②: Legends of square area (right); Arrow: the order of corresponding parts in the chart (circular area or square area).

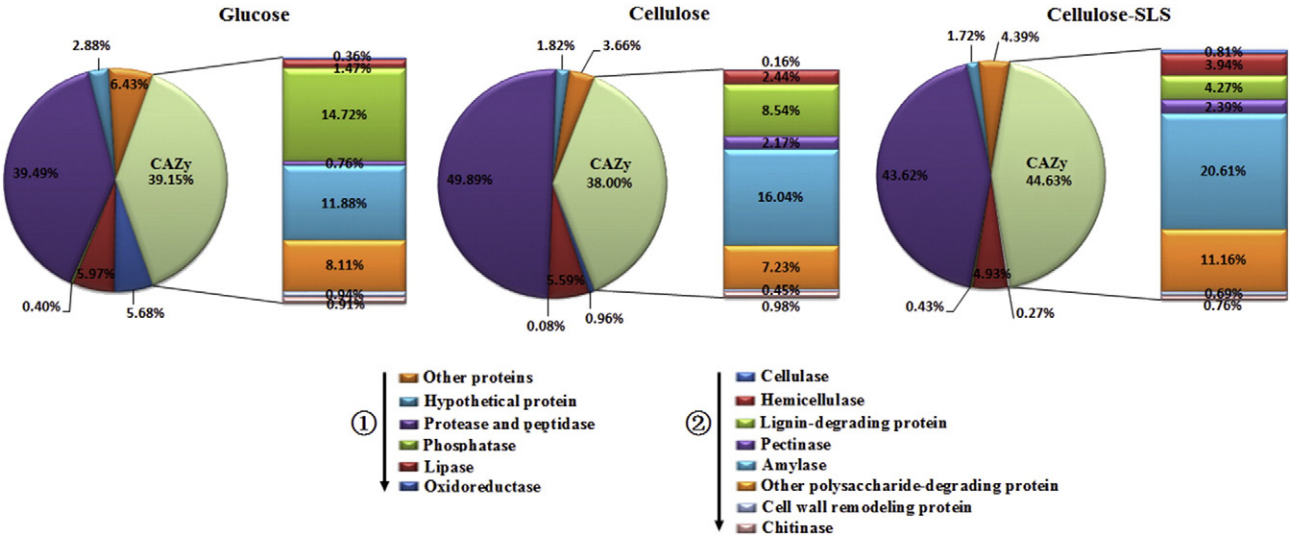

Fig. 3. Relative abundance of proteins in the L. *edodes* secretomes in glucose, cellulose, and cellulose-SLS media. ①: Legends of circular area (left); ②: Legends of square area (right); Arrow: the order of corresponding parts in the chart (circular area or square area).

an aryl-alcohol oxidase (AA3) and a glucose oxidase (AA3) were upregulated either in the cellulose or cellulose-SLS cultures.

3.3. Determination of the activities of several extracellular enzymes

The activities of xylanase, endoglucanase (CMCase), cellobiohydrolase (*p*NPCase) and β-glucosidase (*p*NPGase) and FPA were evaluated. As shown in Fig. 4A, L. *edodes* produced higher levels of FPA, CMCase activity and xylanase activity in cellulose and cellulose-SLS media than in the glucose medium. *p*NPCase and *p*NPGase activities showed no significant differences between cellulose and glucose media (Fig. 4B) but greatly increased activities in the cellulose-SLS media. In general, a similar pattern of concordance between extracellular enzyme activities and secretomic profiles of enzymes with cellulolytic and hemicellulolytic functions was observed, although a few differences were observed.

3.4. qRT-PCR analysis of cellulose- and hemicellulase-encoding gene expression.

To validate the expression profiles obtained by secretomic analysis, the transcript levels of 9 cellulase- and hemicellulase-encoding genes identified in the secretomes were tested by qRT-PCR. This process was performed after transformation of L. *edodes* mycelia to glucose, cellulose or cellulose-SLS culture conditions for 1, 3 or 5 d. The transcript levels of these 9 enzyme-encoding genes showed no significant differences from the controls at 1 d, but their expression was gradually increased at 3 d and 5 d in all three conditions. Notably, the increased expression of these genes was even greater in the cellulose condition than in the glucose condition, and the highest expression occurred in the cellulose-SLS condition at 5 d, with a range of 32.2- to 1166.7-fold increases (Fig. 5). The expression trends of the selected genes obtained by qRT-PCR confirmed those observed from the secretome data. Furthermore, the qRT-PCR analysis results suggested that SLS enhanced the expression of cellulase- and hemicellulase-encoding genes at the transcript level.

4. Discussion

This work described the expression profiles of CAZy proteins in the secretome of a white-rot edible mushroom, *L. edodes*, cultured on different carbon sources. With a recently sequenced and annotated genome [23], it is accessible to analyze the proteome of biomass-degrading enzymes. Moreover, we investigated the relative changes in the abundance of L. *edodes* extracellular CAZy proteins expressed on cellulose and cellulose-SLS media compared to glucose medium. Our results suggested that the lignocellulose-degrading enzyme repertoire of L. *edodes* was composed of a variety of CAZy families, including a selection of hydrolases, esterases, lyases, auxiliary oxidoreductases, a lignin-modifying peroxidase, and H_2O_2-producing enzymes, all of which are needed for the complete degradation of polymeric lignocellulose components.

4.1. CAZy proteins involved in lignin degradation in the L. edodes secretomes

The L. *edodes* genome includes fourteen laccase (multicopper oxidase, MCO) genes, together with three class-II peroxidase genes [23].

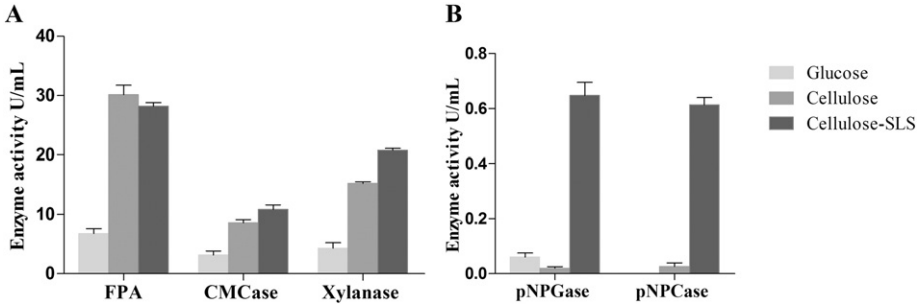

Fig. 4. Activities of several enzymes identified in the secretomes of L. *edodes* grown in glucose, cellulose, and cellulose-SLS media. The activities of total cellulase (FPA), endoglucanase (CMCase) and endoxylanase (Xylanase) are shown in A. The activities of cellobiohydrolase (*p*NPCase) and β-glucosidase (*p*NPGase) are shown in B. The values shown are the means of three replicates, and the *error bars* indicate standard deviations from the mean values.

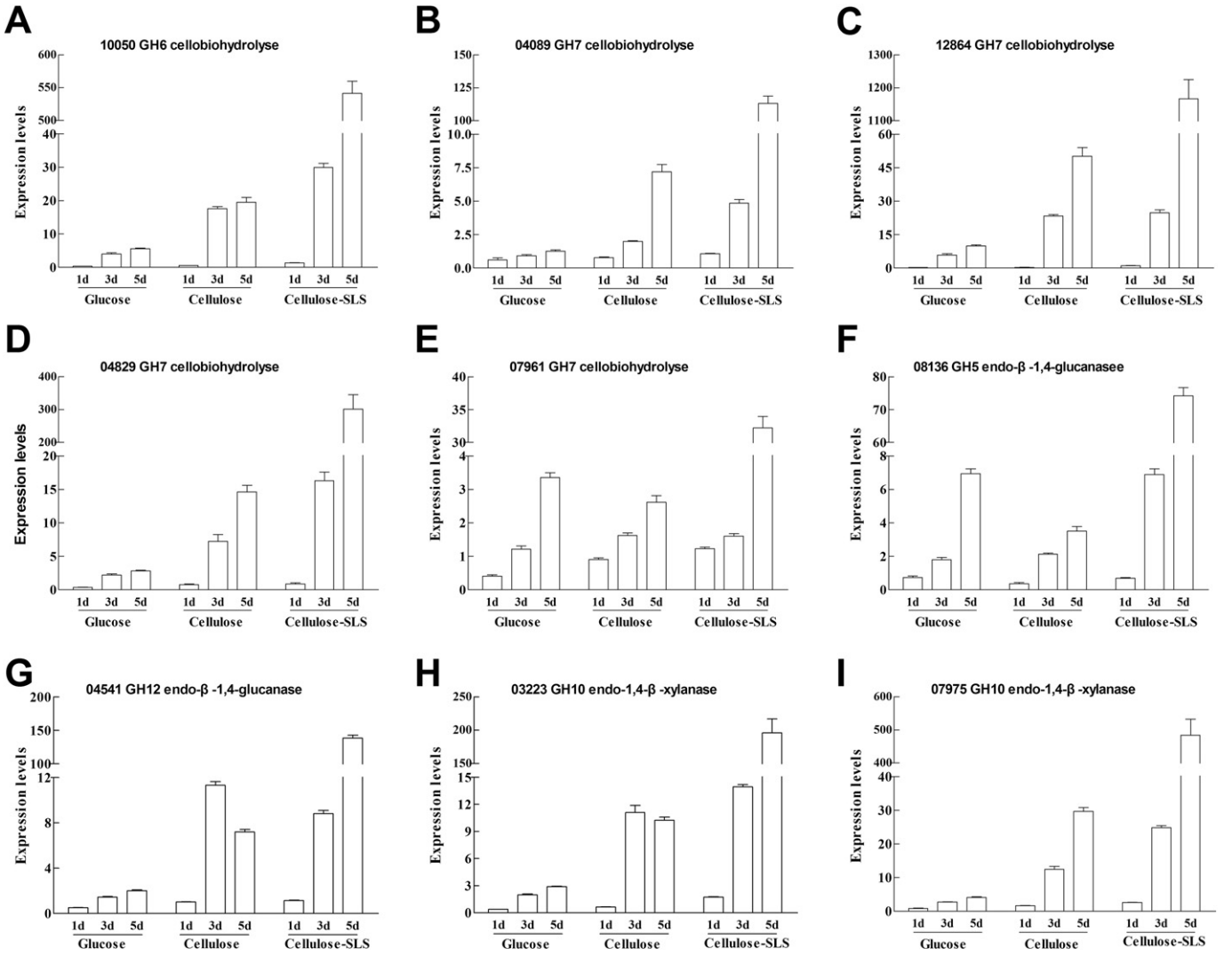

Fig. 5. Quantitative RT-PCR analysis of 9 selected cellulase and hemicellulase-encoding genes. Total RNA was isolated from mycelia of L. *edodes* following transfer to glucose, cellulose or cellulose-SLS for 1, 3, or 5 days. The relative expression levels were normalized to the expression level of the actin gene (ID: LE01Gene04556). The values shown are the means of three replicates, and the *error bars* indicate standard deviations from the mean values.

Two class-II peroxidase genes have been isolated and characterized as MnPs [16,18,29], and one was predicted to be a VP [23]. A MnP was identified in present study, with relatively higher protein abundance in cellulose-SLS cultures. This finding was consistent with a previous study reporting that L. *edodes* produces manganese peroxidase as its main lignolytic enzyme [16].

Laccases can strongly degrade non-phenolic lignin in lignocellulosic materials in the presence of redox mediators [30–32]. Three laccases, LACC1 [19,33], LACC4 (previously designated LACC2; [22]), and LACC6 [34] have been purified from L. *edodes*. And the corresponding genes of LACC1 and LACC4 have been cloned [35,36]. Additional laccase-encoding genes (lacc7–11) were reported in L. *edodes* strain L45A, and LACC1, LACC4, LACC5, and LACC7 were found to be expressed in *Pichia pastorisfor* [21]. Three novel laccases (lacc12-lacc14) were found in L. *edodes* through genomic sequencing [23,37]. At least five laccases were secreted by L. *edodes* when grown on cellulose or cellulose-SLS, as shown in the present secretomic study. LACC1 is the most highly expressed protein of the lignin-degrading protein category in the cellulose-SLS secretome (1.81%); however, its expression was much higher in the glucose secretome (13.13%). This indicates that LACC1 may have a protective role rather than a direct role in lignocellulose deconstruction. A rich available glucose in the medium leads to oxidative stress cellular. It has been observed that oxidative stress can stimulate the extracellular laccase activity of some white rot basidiomycetes such as *Fomes fomentarius*, *Tyromyces pubescens*, *Trametes versicolor*, and *Abortiporus biennis* [38,39]. The expression of the laccase gene from white rot fungus *Trametes* sp. 5930 in *Pichia pastoris* can enhance the resistance of yeast to H_2O_2-mediated oxidative stress by stimulating the glutathione-based antioxidative system to protect the cell from oxidative damage [40]. LACC1 might increase fungal resistance against oxidative stress. Additionally, the *lacc1* RNAi mutants in L. *edodes* caused morphological phenotypes including not forming a thick aerial mycelium mat on agar medium, with many short branches with low mycelial density, a thin cell wall, and few fibrous layers [20]. LACC6 and LACC5 were upregulated in the cellulose-SLS culture, emphasizing their roles in the L. *edodes* secretome regarding processing of the growth substrate and carbon source. LACC8 and LACC13 were also produced by L. *edodes* in cellulose or cellulose-SLS cultures, although at low protein abundance. The above results support the findings of a previous transcriptional analysis of multicopper oxidases in the same fungus, except for LACC8, whose encoding gene was not identified in the genome [37].

Laccases and MnPs have been reported in the secretomes of several white-rotting basidiomycetes, including the Agaricales species *Agaricus bisporus*, *P. ostreatus*, *P. eryngii*, and *Trametes trogii* [11,41–43], the Polyporales species *Ceriporiopsis subvermispora*, *Phlebia radiata*, and *Ganoderma lucidum* [44–47], and the *Schizophyllum commune* [48].

Interestingly, in another Agaricales species, *P. ostreatus*, laccases (LACC10, LACC2, LACC9 and LACC6) are also the main proteins in the polar wood and wheat straw secretomes [11]. However, the model ligninolytic basidiomycetes *P. chrysosporium* completely lacks laccase-encoding genes in its genome [49]. The presence of at least six different *P. chrysosporium* LiPs and MnPs has been reported in carbon- or nitrogen-limited glucose and lignocellulose-containing cultures [50–52], although some studies failed to detect any LiPs in lignocellulose-containing media [53,54]. In this respect, different white-rot fungi demonstrate distinct non-LiP-dependent or non-laccase-dependent strategies for lignin degradation.

GMCs from the CAZy family AA3, together with CROs/GLOXs from the CAZy family AA5, may supply extracellular hydrogen peroxide for fungal lignin modification and class-II peroxidases, and AA3 oxidoreductases may also couple activities with aryl-alcohol dehydrogenases [43]. Both AA3 and AA5 proteins were detected in L. *edodes* secretomes and were relatively less abundant in cellulose-SLS cultures than in glucose cultures. These proteins may be as helpful as LACC1 for increasing fungal resistance to oxidative stress.

4.2. CAZy proteins involved in polysaccharide hydrolysis in the L. edodes secretomes

In addition to lignin attack, cellulose degradation by white-rot fungi occurs through the activity of a combination of several divergent protein families: cellulases of the GH5, GH6, GH7, GH9, GH11, GH12, GH44 and GH45 families [6,55,56], LPMOs and CDH [11,57]. The cellulases identified in the present study were upregulated in cellulose media, especially in cellulose-SLS media, compared to control media, as validated by qRT-PCR analysis and enzyme activity assays. The components and expression patterns of GH family cellulases identified in the secretome of L. *edodes* are similar to those in *P. chrysosporium* cultured in cellulosic medium [54]. The GH12 protein was the most abundantly and specifically expressed in the cellulosic conditions, which illustrated a dominant '*endo*-' enzyme activity. Three GH12 family enzymes in L. *edodes* were characterized and found to be upregulated in cellulose media [58]. The L. *edodes* genome contains one GH6- and four GH7-encoding genes [23], all of which were identified in the secretome of L. *edodes* grown on cellulose-SLS. The GH6-encoding gene and one of the four GH7-encoding genes have been isolated and characterized as cel6B and cel7A, respectively, and the transcript levels of both were significantly upregulated when L. *edodes* was cultured in the presence of crystalline cellulose [59]. In addition, the transcript abundance of both genes and one GH5 encoding gene, which was also identified in the present secretomic analysis, greatly increased when the formation of brown film by L. *edodes* was induced by light during solid state fermentation [60]. This suggests that these cellulases participate in polysaccharide degradation and are involved in life cycles. LPMOs from CAZy family AA9 play important roles in cellulose and hemicellulose degradation, with cellobiose dehydrogenase (CDH) acting as a source of electrons for LPMOs [12]. An LPMO enzyme (LE01Gene10266) and a CDH were identified only in the cellulose-SLS conditions, in line with the report of Harris et al. [61] that a protein belonging to the AA9 family enhances the activity of cellulose hydrolysis in the presence of lignocellulose but not pure cellulose. Although cellulases were upregulated in the cellulose-SLS conditions, the overall abundance of cellulases was much lower than that of lignin-degrading proteins, which could be related to the selective degradation of lignin by L. *edodes* [24].

Hemicellulose in hardwood mainly contains xylan, and its degradation requires xylanase, β-xylosidase, glucuronidase, α-L-arabinofuranosidase and acetyl xylan esterase [62], all of which were identified in the L. *edodes* secretome except for glucuronidase. In this work, hemicellulose main chain-cleaving enzymes, including a GH10 xylanase and a CE3 acetyl xylan esterase, showed significant upregulation in cellulose-SLS medium. In addition, two GH2 and GH5 β-mannosidases in low abundance and the absence of mannanase (hydrolyzing the backbone of mannan) encoding genes in the genome [23] suggested a low softwood-hydrolyzing potential of L. *edodes*, since mannan is the main hemicellulose in softwood fiber. Moreover, it is well known that the production of mannanase can be induced by cellulose in many fungi, including *P. chrysosporium* [54]. The L. *edodes* hemicellulose degradation system has been less extensively studied, with only a purified xylanase reported [17]. The pectin-degrading enzymes of L. *edodes* have rarely been documented, but this study indicated the expression and regulation of polygalacturonase (the most abundant pectinase in the three secretomes), pectin esterase, rhamnogalacturonase, and *endo*-β-1,4-galactanase in cellulose and cellulose-SLS media. Now that the L. *edodes* genome has been sequenced, transcript and protein expression profiling studies of lignocellulose-degrading genes should be performed to clarify the role of each protein in this fungus.

In this work, we found that SLS greatly enhanced the expression of cellulase- and hemicellulase-encoding genes in L. *edodes* at both the transcriptional and secretory levels. It has been reported that SLS enhanced the biodegradation of polychlorinated biphenyls by *Pseudomonas* sp. 7509 [63]. SLS and lignin-based polyoxyethylene ether enhance enzymatic hydrolysis of lignocellulose by dispersing cellulase aggregates, thereby significantly reducing nonproductive adsorption of cellulase on lignin [64–68]. However, the mechanism by which SLS enhances the expression of cellulase- and hemicellulase-encoding genes at the transcript level is still unclear. Future studies will clarify this mechanism as well as the mechanism for the transcriptional regulation of cellulase- and hemicellulase-encoding genes in L. *edodes*.

In addition to extracellular enzymes, different culture substrates affected the taste component content and taste quality of L. *edodes* [69]. Accordingly, quality characters including organoleptic and medical characteristics of fruiting bodies should be considered when L. *edodes* is cultivated on woody and nonwoody lignocellulosic substrates.

5. Conclusions

Our integrative analysis of the data set obtained from L. *edodes* grown on glucose, cellulose or cellulose-SLS demonstrated the polysaccharide and lignin degradation capacities of L. *edodes*. Under lignocellulose-free conditions (glucose medium), *L. edodes* produces only a small amount of essential lignocellulose-degrading enzymes (GH5, GH6, and GH7 for cellulose degradation; GH10 for hemicellulose degradation; GH28 for pectin degradation; and AA2 for lignin degradation). During the degradation of cellulose or cellulose-SLS, *L. edodes* secretes large amounts of lignocellulose-degrading enzymes, especially under cellulose-SLS conditions. Additionally, the transcript expression levels of genes encoding cellulases and hemicellulases were significantly increased by SLS.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.04.023.

Conflict of interest

The authors have declared that no conflict of interest exists.

Abbreviations

AA	auxiliary activities
ACN	acetonitrile
BSA	bovine serum albumin
CAZy	carbohydrate-active enzyme
CBM	carbohydrate-binding module
CDH	cellobiose dehydroge- nase
CE	carbohydrate esterase
CMC	carboxymethyl cellulose
CRO	copper radical oxidases
FPA	filter paper activity

GH	glycoside hydrolase
GLOX	glyoxal oxidase
GMC	glucose-methanol-choline oxidases/dehydrogenases
LACC	laccase
LiP	lignin peroxidase
LPMO	lytic polysaccharide monooxygenase
MnP	manganese peroxidase
*p*NPC	*p*-nitrophenol-D-cellobioside
*p*NPG	*p*-Nitrophenyl β-D-glucopyranoside
SLS	sodium lignosulfonate
	qRT-PCR Real time quantitative PCR
SABF	sodium acetate buffer solution
VP	versatile peroxidase

Transparency document

The Transparency document associated with this article can be found, in the online version.

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