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ORIGINAL ARTICLE

Secretome analysis of the basidiomycete *Phanerochaete chrysosporium* grown on ammonia-treated lignocellulosic biomass from birch wood

Kiyoshi Sakuragi[1,3] · Chiaki Hori[1,4] · Kiyohiko Igarashi[1,2] · Masahiro Samejima[1]

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Abstract

Ammonia pretreatment is a promising technique for enhancing enzymatic saccharification of lignocellulosic biomass. However, an enzymatic cocktail suitable for the breakdown of pretreated biomass samples is still being developed. The basidiomycete *Phanerochaete chrysosporium* is a well-studied fungus with regard to bioconversion of lignocellulosic biomass. In the present work, we analyzed proteins secreted by *P. chrysosporium* grown on untreated and ammonia-treated birch wood meal. Fungal growth, xylanase activity, and extracellular protease activity increased in the media containing the ammonia-treated biomass; however, cellulase production decreased compared to that observed in the untreated biomass. Secreted extracellular proteins were separated by two-dimensional electrophoresis and identified by liquid chromatography ion–trap mass spectrometry. Fifty-five spots corresponding to secreted proteins were chosen for further analysis. In the culture with ammonia-treated biomass, the relative concentration of a xylanase belonging to glycoside hydrolase (GH) family 11 increased, while acetyl xylan esterases belonging to carbohydrate esterase family 1 decreased. Moreover, GH family 10 xylanases were promoted proteolysis in the culture of ammonia-treated biomass, leading to the loss of family 1 carbohydrate-binding modules. These results indicated that *P. chrysosporium* produced enzymes related to the recognition of structural changes on xylan with de-acetylation and introduction of nitrogen by ammonia pretreatment of birch wood meal.

Keywords *Phanerochaete chrysosporium* · Secretome analysis · Lignocellulosic biomass · Ammonia pretreatment · Birch wood

Introduction

Lignocellulosic biomass, the most abundant renewable organic carbon source on earth, consists mainly of cellulose, hemicellulose, and lignin [1]. Appropriate conversion of biomass offers enormous potential for the production of biofuels and biochemicals.

Various pretreatment methods are used to enhance the enzymatic saccharification of lignocellulosic biomass [2]. Among them, one of the most promising approaches for lignocellulosic biomass from grass plants is pretreatment with liquefied ammonia, and this methodology has been extensively investigated [3–5]. The ammonia cleaves ester bonds in lignocellulosic biomass and generates amides [6, 7]. It has been demonstrated that ammonia pretreatment also alters the polymorphic form of crystalline cellulose with a low water content, transforming the natural crystalline form (cellulose I) to cellulose III_I [8], which is far more susceptible to enzymatic degradation [9]. Moreover, we have recently reported

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✉ Masahiro Samejima
amsam@mail.ecc.u-tokyo.ac.jp

[1] Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

[2] VTT Technical Research Centre of Finland, P.O.Box 1000, Tietotie 2, 02044 VTT Espoo, Finland

[3] Present Address: Energy Engineering Research Laboratory, Central Research Institute of Electric Power Industry, 2-6-1 Nagasaka, Yokosuka, Kanagawa 240-0196, Japan

[4] Present Address: Graduate School and School of Engineering, Hokkaido University, North 13, West 8, Sapporo, Hokkaido 060-8628, Japan

that ammonia pretreatment is effective for improving enzymatic saccharification of not only grass biomasses but also hardwood biomasses with high xylan and low lignin contents such as birch wood and willow wood [10]. Nevertheless, searching for effective enzymes for the conversion of ammonia-treated hardwood biomass is still under development.

Wood-decay fungi are omnipotent degraders of lignocellulosic biomass and the basidiomycete *Phanerochaete chrysosporium* has been one of the best-studied examples [11–13]. *P. chrysosporium* produces a variety of extracellular enzymes including glycoside hydrolases (GHs), carbohydrate esterases (CEs) and oxidative enzymes with auxiliary activities (AAs) to degrade lignocellulosic biomass [14–18]. The sequenced genome of *P. chrysosporium* has revealed many genes that encode extracellular enzymes [19], and the results involving GHs, CEs, and AAs have been deposited into the Carbohydrate-Active enZymes (CAZymes) database [20]. Moreover, extensive proteomic analysis of extracellular proteins, or the secretome, has been performed to map the lignocellulolytic system of the fungus [14, 16, 18, 21, 22].

In the present study, we aimed to examine the effect of ammonia treatment on the extracellular proteins of *P. chrysosporium* to degrade lignocellulosic biomass by performing a comparative secretome analysis of *P. chrysosporium* grown on untreated or ammonia-treated lignocellulosic biomass from birch wood.

Materials and methods

Preparation and chemical analysis of biomass

The preparation and chemical analysis of untreated or ammonia-treated birch wood meal were previously reported [10]. In brief, a birch wood block was milled, and then sieved to obtain 40-mesh-passed meal. The wood meal was Soxhlet extracted with benzene–ethanol (2:1 v/v) and used for experiments as untreated biomass (UB). UB was treated with liquefied ammonia at 140 °C for 1 h and used for experiments as ammonia-treated biomass (AB).

Cultivation of *Phanerochaete chrysosporium* and enzyme assays

The cultivation of *Phanerochaete chrysosporium* and enzyme assays were performed according to the method in our previous paper [23] with slight modifications. In brief, *P. chrysosporium* strain K-3 [24] was cultivated in Kremer and Wood medium [25] for 3 days with 2.0% (w/v) UB or AB from birch wood as the sole carbon source. The media were sterilized by autoclaving at 121 °C for 20 min before inoculation. The fungal growth and protein concentration were determined daily. To evaluate fungal growth, 1 mL cultures were collected in a measuring tube and left to stand for 30 min; the volume of sediment was used as a representative metric for the growth of fungal mycelia. The protein concentration of the culture was determined by Bradford assay (Bio-Rad Laboratories, Inc., California, US). Avicel (Funakoshi Co., Ltd., Tokyo, Japan), xylan from birch (Sigma-Aldrich Co., Missouri, US), and azoalbumin (Sigma-Aldrich Co.) were used as a substrate for assaying cellulase, xylanase and protease activities of culture. The amount of reducing sugar released by enzymatic reactions was measured using the *p*-hydroxybenzoic acid hydrazide (PHBAH; Wako Pure Chemical Industries, Ltd.) method [26, 27]. One unit of cellulase and xylanase activities were defined as the amount of enzyme required to release 1 μmol reducing sugar per min under the assay conditions using a predetermined standard curve obtained with glucose and xylose, respectively. One unit of protease activity was defined as the amount of enzyme required for release of 0.001 Abs at 335 nm per min under the assay conditions. Each conversion data point represents the mean of three measurements with the standard deviation. Statistically significant differences between the two series were assessed by Student's *t* test or Aspin–Welch's *t* test following an *F* test assessment of variance.

Separation and identification of proteins from *Phanerochaete chrysosporium*

The separation and identification of proteins from *P. chrysosporium* have been described in detail [23]. In brief, the proteins from culture were separated by two-dimensional electrophoresis (2DE). The stained 2DE gels were scanned and individual protein spots on different gels were matched and quantified. Fifty-five spots of protein were digested by trypsin and analyzed by ultra-high-performance liquid chromatography–mass spectrometry. The peptides were identified by an in-house-licensed mascot search engine (Matrix Science K. K., London, UK) with 10,048 annotated gene models from the *P. chrysosporium* version 2.0 genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html) [23]. The obtained amino acid sequences were analyzed by a BLASTP search against the NCBI non-redundant database with default settings to confirm gene function [23].

Results

Fungal growth, protein concentration, and enzymatic activity

P. chrysosporium was cultivated in synthetic media containing the biomass either with AB and UB. As shown in Fig. 1a, the mycelial volume constituted 0.1 mL of the

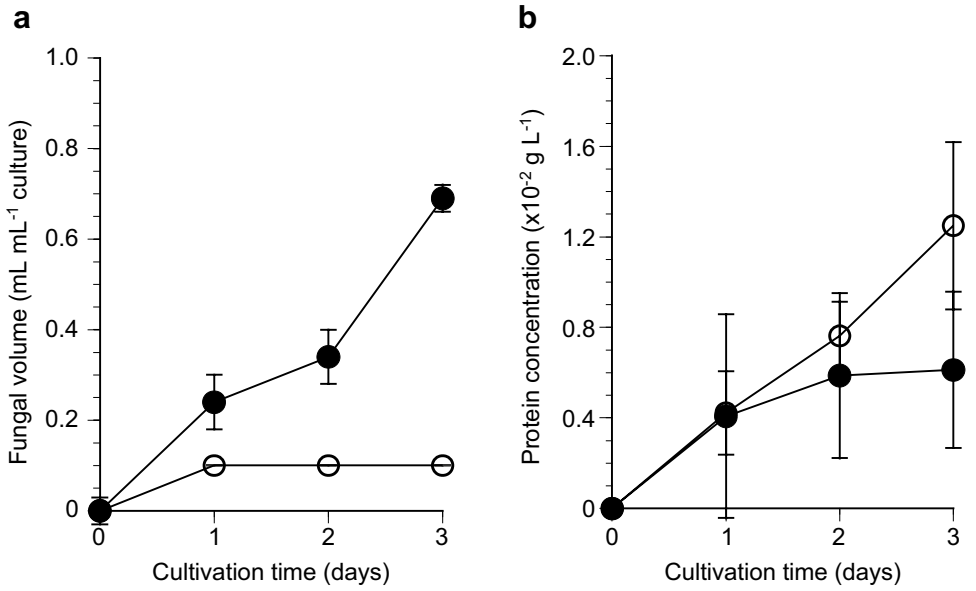

Fig. 1 Fungal growth and extracellular protein concentration of *P. chrysosporium*. **a** Fungal growth and **b** extracellular protein concentration of *P. chrysosporium* cultivated for 3 days in synthetic medium containing 2.0% (w/v) UB (white circle) and 2.0% (w/v) AB (black circle) as carbon sources. The mycelium volume per 1 mL of culture filtrate was measured as described in Materials and Methods. Protein concentration of the culture filtrate was estimated by the Bradford method. *UB* untreated birch wood meal, *AB* ammonia-treated birch wood meal. Each data point represents the mean ± standard deviation (SD) of three replicates. Where the error bars are not visible, they fit within the symbols

1 mL UB culture on day 1, and it remained at this level until day 3. Conversely, the mycelial volume increased remarkably in the 1 mL AB culture, reaching 0.7 mL after 3 days ($p < 0.01$). In the UB culture, 1.2×10^{-2} g L^{-1} of extracellular protein was secreted after 3 days of cultivation, compared to 0.6×10^{-2} g L^{-1} in the AB culture (Fig. 1b). Although the changes in protein concentration of the cultures were not significantly different after 3 days of cultivation ($p =$ n. s.), ammonia pretreatment greatly enhanced the growth of *P. chrysosporium*.

Cellulase, xylanase, and protease activity in the 3-day culture filtrates were measured and compared (Fig. 2). In the UB culture, 1.6 U L^{-1} of cellulase activity was measured after 3 days of cultivation, compared to 0.95 U L^{-1} in the AB culture (Fig. 2a). The specific activity of cellulase (activity/mg of total proteins) between AB and UB cultures was not significantly different ($p =$ n. s.). This may be because the major proteins secreted by *P. chrysosporium* are cellulases. On the other hand, the specific activity of xylanase and protease were markedly increased in the AB culture compared to the UB control culture. Xylanase and protease activity increased 7.8- and 2.2-fold in the AB culture compared to the UB control culture ($p < 0.01$).

Secretome analysis of proteins

Extracellular proteins in the culture filtrates were separated by 2DE (Fig. 3). For both UB and AB filtrates, most of the protein spots focused at a p*I* of 4.5–5.5, although their molecular masses ranged from 37 to 100 kDa. Of these spots, 55 spots were subjected to LC–MS/MS analysis (Table 1). These proteins were selected on the basis of the amount of protein and the sharpness of the spot. Most of the proteins were classified as CAZymes such as GHs (34 spots), CEs (10 spots), and AAs (3 spots). In addition, four thaumatin-like (*thn*) proteins and one aldose 1-epimerase (*a1e1*) were detected. Protein functional analysis showed that many of the proteins were cellobiohydrolases and endoglucanases participate in cellulose degradation, as well as xylanases and accessory enzymes participate in xylan degradation [14, 16, 18, 21–23, 28]. Spot numbers 5, 7, 9, 10, 11, 12, 19, 22, 24, 30, 35, 48, and 52 were identified in both the UB and AB cultures. The major spots corresponded to cellobiohydrolases (Cel6A: spot 5; Cel7C: spots 11, 12, and 13; and Cel7D: spots 7 and 9), endoglucanases (Cel5A: spot 4; and Cel5B: spot 2), and xyloglucanase (Xgh74B: spot 31).

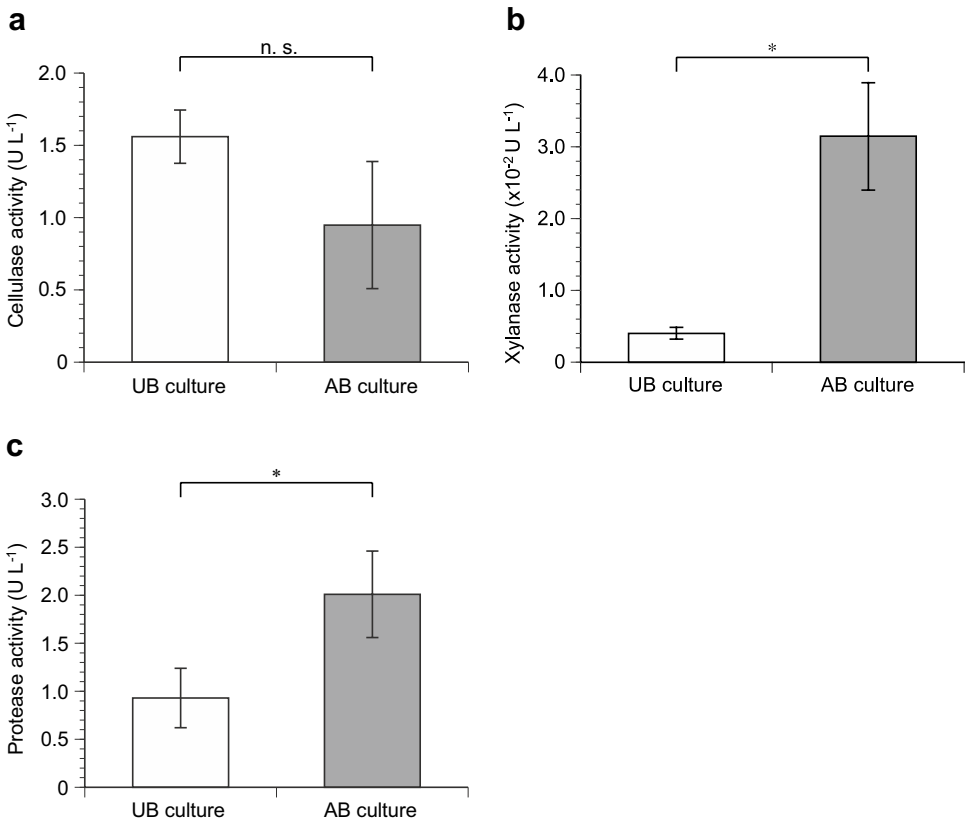

Fig. 2 Comparison of cellulase, xylanase, and protease activities in UB and AB cultures after 3 days. Enzyme activity was measured in sodium acetate (pH 5.0) at 30 °C. **a** Avicel and **b** xylan from birch wood were used as substrates to measure enzyme activity in the UB and AB cultures. The newly formed reducing ends were estimated using the PHBAH method [26, 27] with some modifications. **c** Azoalbumin was used as a substrate. A_{335nm} of the reaction was measured. *UB* untreated birch wood meal, *AB* ammonia-treated birch wood meal. Each data point represents the mean ± standard deviation (SD) of three replicates. Statistical significance was determined using Student's or Welch's *t* test (*$p < 0.01$; *n. s.* not significant)

To investigate the effects of ammonia pretreatment on protein expression ratios, the normalized average volume of each protein spot in the AB culture was compared to that of the corresponding spot in the UB culture. For measured total protein in 2DE, 49% (UB culture) and 51% (AB culture) protein were identified. Cellobiohydrolases of GH family 7 were the most common extracellular proteins in both UB and AB cultures, accounting for 40% of total protein. In the AB culture, 10 protein spots exhibited more than double the normalized average volume compared to the corresponding spots in the UB culture (Table 2). Among them, both spot 22 assigned to the GH family 11 xylanase (Xyn11B) and spot 23 of the proteolytic product derived from Xyn11B were remarkably enhanced in the AB culture compared to that in the UB culture. In contrast, six other protein spots in the AB culture showed less than 50% of the volume of the corresponding spots in the UB culture (Table 3). Spot 20 assigned to the GH family 10 xylanase (Xyn10C) was decreased in the AB culture, while spot 19 of the proteolytic product derived from Xyn10C was increased in the AB culture. In addition, it was notable that both Xyn10C (spot 20) and Xyn11B (spot 22) have a type I carbohydrate-binding module (CBM I) connected to the catalytic domain by a linker region, while any fragment assigned to CBM I was not detected from their proteolytic products corresponding to spot 19 and spot 23 with a lower molecular weight. A part of Xyn10C (spot 20) and Xyn11B (spot 22), therefore, may have been proteolytically degraded in the AB culture (Table S1, S2 and Fig. S1, S2).

Discussion

Ammonia pretreatment is particularly effective for improving the enzymatic saccharification of lignocellulosic biomass from grass plants [3–5]. In addition, we have recently reported that ammonia pretreatment is also effective for improving the enzymatic saccharification of hardwood biomasses such as birch wood with high xylan and low lignin contents [10]. The biomass recovery after ammonia pretreatment of birch wood meal was 99% and this pretreatment did not change the neutral sugar and lignin contents, while the content of nitrogen increased and the content of acetyl group decreased after ammonia pretreatment, suggesting that the acetyl substitution of xylan was eliminated by the cleavage of ester linkages during ammonia pretreatment [10].

In the present study, we found that whereas protein secretion by *P. chrysosporium* was depressed in the AB, fungal growth was higher in the AB culture than in the UB culture. Although we must consider adsorption of enzymes to biomass residue, the above-mentioned finding indicated improved fungal digestion of the ammonia-treated biomass

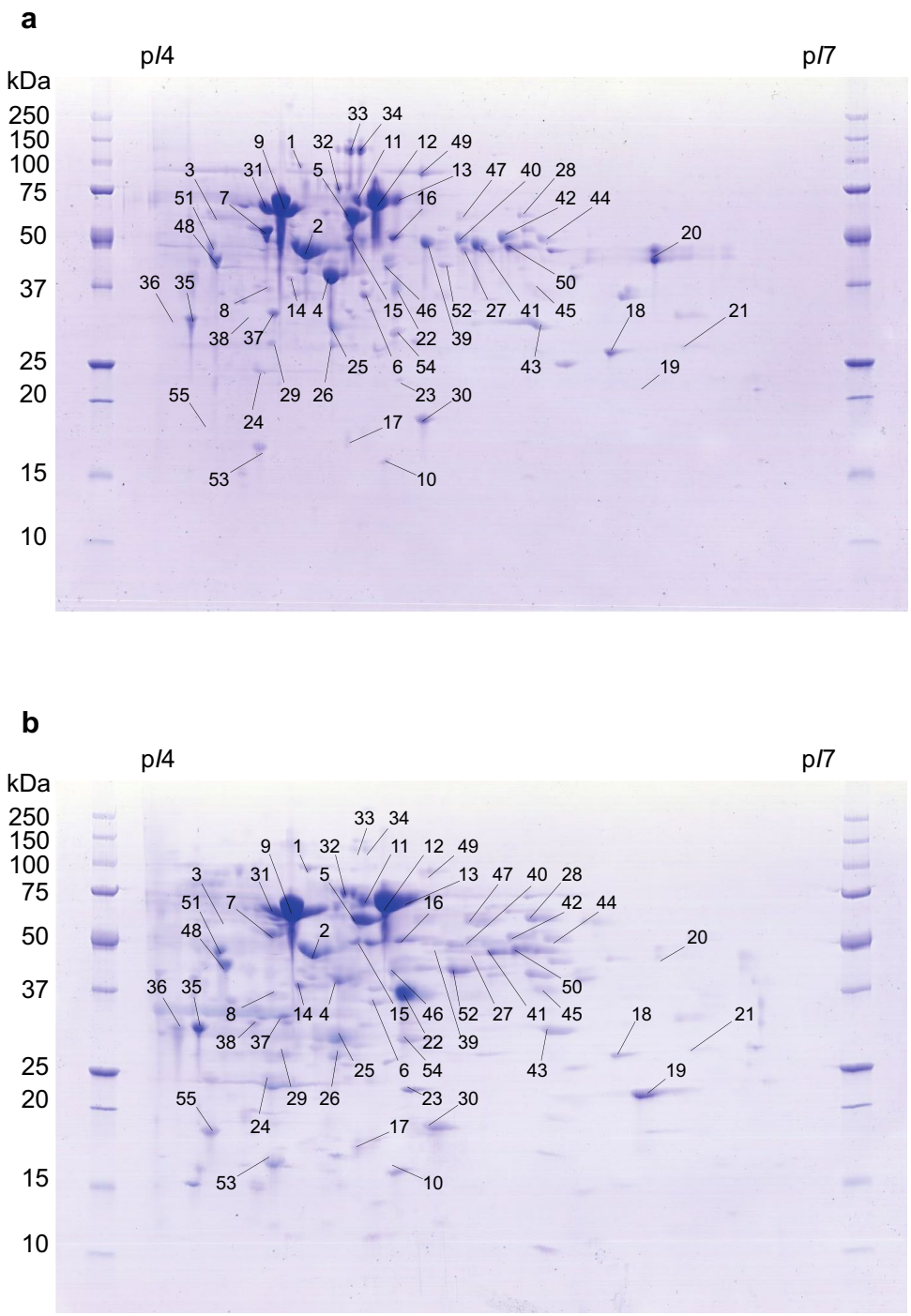

Fig. 3 Two-dimensional electrophoresis (2DE) of the UB and AB cultures stained with Coomassie brilliant blue. **a** UB; **b** AB. *P. chrysosporium* was cultivated in synthetic medium containing 2.0% (w/v) UB or 2.0% (w/v) AB for 3 days. An aliquot (100 µg) of extracellular protein from each sample was separated as described in "Materials and methods". The horizontal axis of the gel spans pH 4–7, and the vertical axis spans 250–10 kDa. Spot numbers correspond to the proteins in Table 1

and catabolite repression of overall protein secretion during biomass degradation. Thus, this ammonia pretreatment process effectively enhances the hydrolysis of lignocellulosic biomass from birch wood.

There has been great interest in finding an optimal enzyme that hold a key role in biomass degradation can not only enable the supplementation of commercially available enzyme mixtures, but also the design of tailor-made enzyme cocktails that can be optimized for each substrate [30, 31]. In the present study, we used comparative secretome analysis to examine the effect of ammonia pretreatment on the levels of proteins secreted by *P. chrysosporium* grown on birch wood meal.

It is known that the concentration of nitrogen is the critical parameter for the production of lignin decomposition enzymes in *P. chrysosporium* [32]. However, since we cultivated *P. chrysosporium* in Kremer and Wood medium [25] containing a high level of nitrogen source, detection

Table 1 Proteins secreted by *P. chrysosporium* after cultivation with untreated or ammonia-treated birch wood meal

Spot number	Score[a]	Protein ID[b]	Function (gene or domain)	Protein family	CBM[c]	Th Mw[d]	Th p*I*[d]	Sequence coverage (%)
1	830	129849	Glucan β-glycosidase (*gly3B*)	GH 3	–	87.142	4.72	36
2	336	4361	Endo-β-1,4-glucanase (*cel5B*)	GH 5	N	49.730	6.21	17
3	308	5115	β-Mannanase *(man5C*)	GH 5	N	41.058	4.27	10
4	845	6458	Endo-β-1,4-glucanase (*cel5A*)	GH 5	N	39.856	5.04	49
5	989	133052	Cellobiohydrolase II (*cel6A*)	GH 6	N	48.967	5.04	41
6	343	133052	Cellobiohydrolase II (*cel6A*)	GH 6	N	48.967	5.04	30
7	299	137372	Cellobiohydrolase 58 (*cel7D*)	GH 7	C	59.497	4.96	25
8	416	137372	Cellobiohydrolase 58 *(cel7D*)	GH 7	C	59.497	4.96	34
9	2046	137372	Cellobiohydrolase 58 (*cel7D*)	GH 7	C	59.497	4.96	52
10	224	137372	Cellobiohydrolase 58 (*cel7D*)	GH 7	C	59.497	4.96	19
11	1326	127029	Cellobiohydrolase 62 (*cel7C*)	GH 7	C	56.128	5.03	54
12	2010	127029	Cellobiohydrolase 62 (*cel7C*)	GH 7	C	56.128	5.03	55
13	1448	127029	Cellobiohydrolase 62 (*cel7C*)	GH 7	C	56.128	5.03	50
14	683	127029	Cellobiohydrolase 62 (*cel7C*)	GH 7	C	56.128	5.03	45
15	1173	138345	Endo-β-1,4-xylanase (*xyn10A*)	GH 10	N	44.065	5.21	55
16	409	138345	Endo-β-1,4-xylanase (*xyn10A*)	GH 10	N	44.065	5.21	55
17	498	7045	Endo-β-1,4-xylanase (*xyn10C*)	GH 10	N[e]	31.213	6.89	42
18	962	7045	Endo-β-1,4-xylanase (*xyn10C*)	GH 10	N[e]	31.213	6.89	76
19	572	7045	Endo-β-1,4-xylanase (*xyn10C*)	GH 10	N[e]	31.213	6.89	62
20	1033	7045	Endo-β-1,4-xylanase (*xyn10C*)	GH 10	N[e]	31.213	6.89	76
21	537	7045	Endo-β-1,4-xylanase (*xyn10C*)	GH 10	N[e]	31.213	6.89	75
22	428	133788	Endo-β-1,4-xylanase (*xyn11B*)	GH 11	C	30.811	5.72	21
23	114	133788	Endo-β-1,4-xylanase (*xyn11B*)	GH 11	C	30.811	5.72	17
24	184	7048	Putative endo-xyloglucanase (*cel12B*)	GH 12	–	27.084	4.74	10
25	179	8466	Endo-β-1,4-glucanase (*cel12A*)	GH 12	–	26.461	4.79	19
26	176	8466	Endo-β-1,4-glucanase (*cel12A*)	GH 12	–	26.461	4.79	8
27	277	29397	Putative rhamnogalacturonase (*rgh*28C)	GH 28	–	44.801	5.22	51
28	674	29397	Putative rhamnogalacturonase (*rgh28C*)	GH 28	–	44.801	5.22	50
29	91	4822	Endo-1,5-α-L-arabinanase	GH 43	–	33.101	4.82	9
30[f]			Endo-β-1,4-glucanase (*cel45*)	GH 45	–	18.169	5.04	
31	595	28013	Glycoside hydrolase family 74 (*xgh74B*)	GH 74	–	66.641	4.56	44
32	604	138226	Putative xyloglucanase (*gly74A*)	GH 74	–	77.792	4.72	36
33	973	134556	Xyloglucanase (*xgh74B*)	GH 74	C	89.860	5.05	43
34	1014	134556	Xyloglucanase (*xgh74B*)	GH 74	C	89.860	5.05	41
35	123	3280	Thaumatin-like protein (*thn2*)		–	27.768	4.74	14
36	97	3280	Thaumatin-like protein (*thn2*)		–	27.768	4.74	14
37	240	5297	Thaumatin-like protein (*thn1*)		–	29.288	4.99	14
38	143	5297	Thaumatin-like protein (*thn1*)		–	29.288	4.99	14
39	697	126075	Putative acetyl xylan esterase (*axe1*)	CE 1	–	35.970	5.91	29
40	511	126075	Putative acetyl xylan esterase (*axe1*)	CE 1	–	35.970	5.91	29
41	772	126075	Putative acetyl xylan esterase (*axe1*)	CE 1	–	35.970	5.91	29
42	391	126075	Putative acetyl xylan esterase (*axe1*)	CE 1	–	35.970	5.91	29
43	776	126075	Putative acetyl xylan esterase (*axe1*)	CE 1	–	35.970	5.91	29
44	275	129015	Carbohydrate esterase family 1 protein (*axe1*)	CE 1	N[e]	30.753	6.20	21
45	289	129015	Carbohydrate esterase family 1 protein (*axe1*)	CE 1	N[e]	30.753	6.20	13
46	439	132137	Pectin methylesterase	CE 8	–	36.287	5.84	36
47	554	130517	Putative glucuronoyl esterase	CE 15	N	49.741	5.55	28
48	245	3097	Fungal cellulose binding domain-containing protein	CE 16	N	43.216	5.08	15

Table 1 (continued)

Spot number	Score[a]	Protein ID[b]	Function (gene or domain)	Protein family	CBM[c]	Th Mw[d]	Th p*I*[d]	Sequence coverage (%)
49	821	11098	Iron reductase domain/cellobiose dehydrogenase (*cdh*)	AA8-AA3_1	–	82.189	5.19	31
50	313	41563	Lytic polysaccharide monooxygenase	AA9	C	33.203	5.39	29
51	301	121193	Lytic polysaccharide monooxygenase	AA9	C	32.562	4.31	25
52	207	138479	Aldose 1-epimerase (*a1e1*)		–	38.454	4.98	27
53	316	8221	Hypothetical protein (IPR010829 Cerato-platanin)		–	14.970	4.69	33
54	453	3085	Hypothetical protein		–	40.214	5.02	35
55	117	2416	Hypothetical protein		–	17.519	8.44	27

[a]In-house MASCOT score

[b]Protein model number from the *P. chrysosporium* genome database version 2.0

[c]Deduced amino acid sequence with and without a carbohydrate binding module (CBM); *N* N-terminal, *C* C-terminal

[d]Theoretical molecular mass (Th Mw, kDa) and p*I*

[e]Manual annotation revealed existence of a signal peptide and/or a CBM sequences in the gene

[f]MS/MS dataset of spot number 30 searched against genome version 2.0 in Scaffold 6:1798039–1798139 [29]

Table 2 Increased 2DE spot volumes between ammonia-treated and untreated birch wood meal cultures

Spot number	Protein family	Function (gene or domain)	Normalized average volume		Relative degree
			UB × 10^7	AB × 10^7	
36		Thaumatin-like protein	0.13	0.64	5.0
22	GH 11	Endo-β-1,4-xylanase (*xyn11B*)	0.60	2.9	4.9
19	GH 10	Putative endo-β-1,4-xylanase (*xyn10C*)	0.25	1.2	4.7
55		Hypothetical protein	0.07	0.28	4.2
23	GH 11	Endo-β-1,4-xylanase (*xyn11B*)	0.12	0.52	4.2
52	A1e1	Aldose 1-epimerase (*a1e1*)	0.24	0.83	3.4
38		Thaumatin-like protein	0.09	0.27	3.0
14	GH 7	Cellobiohydrolase 62 (*cel7C*) (Fragment)	0.30	0.70	2.4
24	GH 12	Putative endoxyloglucanase (*cel12B*)	0.43	1.0	2.4
13	GH 7	Cellobiohydrolase 62 (*cel7C*) (Fragment)	1.6	3.6	2.2

Spots showing a normalized average volume increase by twofold or more on 2DE gels in AB vs. UB cultures. Spot numbers correspond to those in Fig. 3 and Table 1, and the intensity was analyzed by Prodigy 2D ver. 1.0. The relative degree calculation equals the normalized average volume of AB divided by the normalized average volume of UB

UB untreated birch wood meal, *AB* ammonia-treated birch wood meal

of lignin-decomposing enzymes in the culture was precluded. In addition, protease activity was increased 2.2-fold in the AB culture compared to the UB control culture and the 2DE profiles of the AB culture revealed many low molecular weight proteins, which may be the result of protease-mediated cleavage during cultivation. For example, one of the spots corresponding to a GH family 10 xylanase (Xyn10C) was repressed in the AB culture, while other spots of Xyn10C were enhanced compared to the UB culture. This appeared to be mainly due to proteolytic cleavage of Xyn10C. Therefore, we considered that the total amounts of Xyn10C were essentially the same in the AB and UB cultures.

On the other hand, increased concentration of a GH family 11 xylanase was noted when AB was used as the carbon source. Notably, GH family 11 xylanase is expressed by various white-rot fungi, including *P. chrysosporium* [33], which is known to secrete three endo-1,4-β-xylanases. Two *xyn* genes (*xynA* and *xynC*) encode GH family 10 glycoside hydrolases, whereas the third (*xynB*) encodes a protein in GH family 11 [34, 35]. XynA and XynC, corresponding to Xyn10A and Xyn10C in the present study, also showed activity toward *p*-nitrophenyl-β-D-cellobioside and *p*-nitrophenyl-β-D-xylopyranoside, whereas XynB, corresponding to Xyn11B in the present study, hydrolysed only xylan [34]. In addition, XynA and XynC mostly produced

Table 3 Decreased 2DE gel spot volumes in ammonia-treated vs. untreated birch wood meal cultures

Spot number	Protein family	Function (gene or domain)	Normalized average volume		Relative degree
			UB × 10^7	AB × 10^7	
33	GH 74	Xyloglucanase (*xgh74B*)	0.45	0.04	0.087
20	GH 10	Putative endo-β-1,4-xylanase (*xyn10C*)	0.92	0.12	0.14
34	GH 74	Xyloglucanase (*xgh74B*)	0.81	0.17	0.21
27	GH 28	Putative rhamnogalacturonase (*rgh28C*)	0.14	0.04	0.30
49	AA8-AA3_1	Iron reductase domain/cellobiose dehydrogenase (*cdh*)	0.56	0.20	0.36
39	CE 1	Putative acetyl xylan esterase (*axe1*)	0.71	0.32	0.45

Spots showing a normalized average volume decreased by 0.5-fold or greater on 2DE gels in AB vs. UB cultures. Spot numbers correspond to those in Fig. 3 and Table 1, and the intensity was analyzed by Prodigy 2D version 1.0. The relative degree was calculated as the normalized average volume of AB divided by the normalized average volume of UB

UB untreated birch wood meal, *AB* ammonia-treated birch wood meal

products resembling xylobiose and xylotetraose, whereas XynB released products resembling xylobiose and xylopentaose [34]. We previously observed a strong induction of Xyn10C in soluble xylan from oat spelt-containing cultures, whereas the production levels of Xyn11B was unaltered [23]. Xylan in oat spelt has α-L-arabinose side chains, whereas that in birch wood has additional side chains of 4-*O*-methyl-β-D-glucuronic acid. Although the lack of response to oat spelt appears to conflict with the results of our present study, a plausible explanation may be that *P. chrysosporium* recognizes changes in the structure of xylan on modification and solubility during ammonia treatment, leading to the enhancement of Xyn11B production. Notably, supplementation with the commercially available GH family 11 xylanase from *Neocallimastix patriciarum* significantly increased the yields of both glucose and xylose from AB of birch wood [10]. The previous results of enzymatic saccharification show a good correlation with the present results.

Furthermore, ammonia treatment of the biomass reduced the concentration of GH family 74 xyloglucanase (Xgh74B), GH family 28 polygalacturonase (Rgh28C), AA8–AA3 cellobiose dehydrogenase (CDH), and CE family 1 acetyl xylan esterase (Axe1). Notably, in a previous study, we found that the production of these enzymes was enhanced, rather than reduced, when soluble xylan from oat spelt was added to a cellulolytic culture [23]. These results support the idea that *P. chrysosporium* employs complex, so-far-unidentified mechanisms for recognition of its environment.

In conclusion, we used the comparative secretome analysis to examine the expression levels of proteins secreted by *P. chrysosporium* grown in UB and AB cultures. In the AB culture, relative production of Xyn11B, as a key enzyme for enzymatic saccharification of xylan, was increased. While relative production of Xgh74B, Rgh28C, CDH, and Axe1 decreased. The ammonia pretreatment promoted proteolysis of Xyn10C, leading to a loss of family 1 carbohydrate-binding modules. These results indicated that *P. chrysosporium* produced enzymes related to recognition of structural changes on xylan with de-acetylation and introduction of additional nitrogen by ammonia pretreatment of birch wood meal. However, the complex mechanism that *P. chrysosporium* recognizes and regulates its enzymes is still unknown. Further studies on the enzymatic saccharification of the ammonia pretreated biomass from birch wood using a defined enzyme cocktail from *P. chrysosporium* might provide clues leading to a better understanding of the complex mechanisms of biomass degradation by *P. chrysosporium*, as well as being helpful for optimizing enzyme cocktails for efficient saccharification towards the production of biofuels and biochemicals from lignocellulosic biomass.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest associated with this manuscript.

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