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Secretome analysis of the thermophilic xylanase hyper-producer *Thermomyces lanuginosus* SSBP cultivated on corn cobs

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Abstract *Thermomyces lanuginosus* is a thermophilic fungus known for its ability to produce industrially important enzymes including large amounts of xylanase, the key enzyme in hemicellulose hydrolysis. The secretome of *T. lanuginosus* SSBP was profiled by shotgun proteomics to elucidate important enzymes involved in hemicellulose saccharification and to characterise the presence of other industrially interesting enzymes. This study reproducibly identified a total of 74 proteins in the supernatant following growth on corn cobs. An analysis of proteins revealed nine glycoside hydrolase (GH) enzymes including xylanase GH11, β-xylosidase GH43, β-glucosidase GH3, α-galactosidase GH36 and trehalose hydrolase GH65. Two commercially produced *Thermomyces* enzymes, lipase and amylase, were also identified. In addition, other industrially relevant enzymes not currently explored in *Thermomyces* were identified including glutaminase, fructose-bisphosphate aldolase and cyanate hydratase. Overall, these data provide insight into the novel ability of a cellulase-free fungus to utilise lignocellulosic material, ultimately producing a number of enzymes important to various industrial processes.

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Introduction

With the growing worldwide demand for clean and sustainable alternative fuel sources, much work is focussed on developing technologies that can utilise agri-waste biomass as feedstocks for biofuel production, thus reducing the use of food crops for energy. Enzymatic degradation of lignocellulose requires the combined action of ligninases, cellulases and hemicellulases, to release hexose (primarily glucose) and pentose (primarily xylose) sugars for subsequent fermentation. Current commercially available enzyme cocktails designed for saccharification of lignocellulosic material contain enzymes mostly from the mesophilic fungus *Trichoderma reesei*. While these cocktails are able to degrade cellulosic biomass, their efficiency is less than optimal for large-scale commercial production.

Thermomyces lanuginosus is a thermophilic filamentous fungus commonly isolated from soil and compost heaps [10]. Its ability to grow on lignocellulosic biomass is due to its hyper-production of a thermostable, pH tolerant GH11 xylanase [18]. Interestingly, this fungus does not appear to produce a cellulase under any growth conditions currently reported [10, 38]. In addition to the production of xylanase, *T. lanuginosus* produces a number of enzymes currently utilised in a variety of industries, including lipase [7] and amylase [11].

Genome analysis of *T. lanuginosus* SSBP revealed the presence of 224 genes that fell into the Carbohydrate Active EnZymes (CAZy) family of proteins [24]. Of these genes, 94 are glycoside hydrolases, enzymes involved in

Table 1 Activities of key lignocellulosic degrading enzymes from *T. lanuginosus*

Enzyme	Activity (U/ml)	Optimal pH	Optimal Temp (°C)	Enzyme Stability		References
				pH range[a]	Temp (°C)[b]	
β-Xylanase	3,575	6.5	70	3–12	50–80	37, 36, 35
β-Xylosidase	0.4	6.5	50	5–9	50	37
α-Galactosidase	90	5	65	3–7.5	60	30, 28
α-Arabinosidase	0.1	6.5	50	5–8	40	37
β-Glucosidase	4.2	6	65	5–12	50	16
β-Mannosidase	0.1	5	80	5–6	60	37

Maximal activities, pH, temperature and stabilities of lignocellulosic enzymes reported from various strains of *T. lanuginosus*

[a] Maintains at least 80 % activity over 30 min at the optimum temperature

[b] Maintains at least 80 % activity over 30 min at the optimum pH

the hydrolysis of glycosidic bonds in complex sugars. Previously reported activities of a number of enzymes involved in hydrolysis of lignocellulosic biomass from various *T. lanuginosus* strains are outlined in Table 1 [37]. The primary lignocellulosic enzyme activity present in the supernatant of all strains grown on any tested agri-waste biomass is the β-xylanase, with the highest reported activity of any known xylanase to date of 3,575 U/mL [38]. Other hemicellulases present and studied in *T. lanuginosus* strains include β-xylosidase, α-galactosidase, α-arabinosidase and β-mannosidase. Although it is reported that *T. lanuginosus* lacks endo- and exo-glucanase activities, the presence of β-glucosidase activity (4.2 U/mL) has been reported in the supernatant following growth on corn cobs [17].

Recently, there have been a number of investigations into the secretomes of various fungi to elucidate mechanisms important for the successful saccharification of lignocellulose with the aim of improving efficiency within the biofuel industry. Such secretomes include those from *Trichoderma* [13], *Fusarium* [29], *Aspergillus* [20] and *Thermoascus* [23]. These studies include analysis following growth on a number of different carbon sources including simple substrates such as xylose, lactose and maltose, as well as complex lignocellulosic substrates including wheat straw, sugarcane bagasse and corn stover. All of the fungal secretomes analysed to date have had a particular focus on characterising the cellulase components secreted by these fungi as these are considered the most critical, efficiency-limiting enzymes in lignocellulose degradation. Characterisation of hemicellulases, however, is also essential to develop commercial enzymatic cocktails required for complete saccharification of lignocellulosic biomass.

In this study, we present the first secretome profile of the thermophilic fungus *T. lanuginosus*. This particular fungus is unique in that it is cellulase-free, compensating by producing large quantities of a GH11 xylanase. Thus, the data presented reveal important hemicellulases and accessory enzymes necessary for the hydrolysis of plant biomass. This information could provide insight into essential ancillary enzymes required to produce optimised enzymatic cocktails that could be used by the bioethanol industry. In addition, the presence of several other industrially important enzymes is revealed, many for the first time in *T. lanuginosus*, making this both a novel, and industrially relevant study.

Methods

Reagents and raw material

All reagents used were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Corn cobs were obtained from local markets. Kernels were removed and the cobs were cut into pieces 1–2 cm thick. The pieces were dried overnight at 60 °C, milled, sieved to collect particles of 2–7 mm and autoclaved at 121 °C for 15 min.

Fungal strain and growth conditions

Thermomyces lanuginosus strain SSBP, previously identified in this lab, was maintained on PDA plates and subcultured every 7 days. For growth on lignocellulosic material, *T. lanuginosus* was grown in a medium modified from Schlacher et al. [35], containing prepared corn cobs 30 g/L; KH_2PO_4 5 g/L; yeast extract 15 g/L; pH 6. Growth was carried out at 50 °C with 180 rpm shaking in 250 mL flasks containing 100 mL medium, inoculated with a 9-mm mycelial disk from the peripheral region of an actively growing PDA plate culture. After 5 days, the culture medium was centrifuged at 3,500×g at 4 °C for 20 min and concentrated using an AmiconUltracel with a 10 kDa cut off. Samples for plate analysis were stored at −20 °C. Samples for MS analysis were lyophilised and stored at room temperature until use. The pellets were removed and placed at 80 °C until a constant dry weight was achieved (24–48 h) for fungal biomass determinations.

Protein determination, SDS-PAGE and zymography

Protein determination was performed using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and with BSA as the standard.

For SDS-PAGE, proteins (10 μg) were separated on a 4–16 % Mini-PROTEAN®TGX gel (Bio-Rad) under denaturing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. The protein marker used was the PageRuler prestained molecular marker (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Zymogram analysis of the xylanase enzyme was performed based on the overlay method by Royer and Nakas [33]. Separation of proteins was done under native conditions on a 4–16 % Mini-PROTEAN® TGX gel (Bio-Rad). Following protein separation, an overlay gel (2 % agarose, 0.5 % xylan in 0.2 M citrate buffer, pH 6.5) was placed over the native gel and the sandwich incubated at 50 °C for 30 min. The overlay gel was then developed in 95 % ethanol until the background turned white. Activity was visualised by hydrolysis zones on the gel.

Plate assays

Plate assays were performed with modifications according to the method of Manavalan et al. [22]. All plates contained 1.8 % agar in 0.2 M citrate buffer, pH 6.5 plus the respective substrate. Crude supernatant (10, 20, 30, 40 or 50 μL) was loaded into wells on each assay plate. A control well contained 50 μL of uninoculated media. Xylanase activity was detected by addition of 0.2 % RBB-xylan as the substrate and the plate incubated overnight at 50 °C. Activity was visualised by zones of hydrolysis. β-xylosidase activity was detected by addition of 2 mM 4-methylumbelliferyl-β-D-xylopyranoside(MUX) as the substrate and the plate incubated overnight at 50 °C. Activity was visualised by UV fluorescence in a BioRad GelDock XR (Bio-Rad Laboratories, Hercules, CA, USA). β-glucosidase activity was detected similarly to the assay β-xylosidase, except that 2 mM 4-methylumbelliferyl-β-D-glucopyranoside(MUG) was used instead. Cellulase activity was determined using 0.5 % CMC as the substrate and the plate incubated at 50 °C overnight. Plates were then stained with 1 % Congo Red for 20 min, destained with 1 M NaCl and fixed with 0.5 % acetic acid.

Sample preparation for analysis by LC–MS/MS

The protein content of the concentrated culture medium samples was estimated using the Bio-Rad Protein Assay and a total of 500 μg protein from each of the three replicates was aliquoted and lyophilized. The protein pellets were resuspended in 8 M urea, 50 mM Tris-HCl (pH 7.5) and 10 mM DTT. After sample re-suspension, iodoacetamide was added to a final concentration of 10 mM and the samples incubated for 30 min at room temperature. The samples were diluted to 1 M urea using 50 mM Tris-HCl (pH 7.5) and 20 μg trypsin was added to each. Samples were incubated overnight at 37 °C, desalted and peptides concentrated using C18 Macro Spin Columns (Harvard Apparatus) and finally concentrated in a CentriVap Cold Trap (Labconco). Samples (peptides) were resuspended in a solution of 2 % acetonitrile, 0.1 % formic acid prior to analysis by mass spectrometry.

Identification of proteins by LC–MS/MS

The digested secretome samples were analysed on an Agilent 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies) coupled to an Agilent 1290 LC system (Agilent). The desalted peptide samples (400 μg) were loaded onto a Ascentis Peptides ES-C18 column (2.1 mm × 100 mm, 2.7 μm particle size; Sigma-Aldrich) via an Infinity Autosampler (Agilent Technologies) with buffer A (2 % acetonitrile, 0.1 % formic acid) flowing at 0.400 ml/min. The column compartment was set at 60 °C. Peptides were eluted into the mass spectrometer via a gradient with initial starting conditions of 5 % buffer B (98 % acetonitrile, 0.1 % formic acid) increasing to 30 % buffer B over 30 min, then to 50 % buffer B in 5 min. Subsequently, buffer B concentration was increased to 90 % over 1 min and held for 7 min at a flow rate of 0.6 ml/min followed by a ramp back down to 5 % buffer B over one minute, where it was held for 6 min to re-equilibrate the column. Peptides were introduced to the mass spectrometer from the LC via a Dual Agilent Jet Stream ESI source operating in positive-ion mode. A second nebulizer was utilised for the introduction of reference masses for optimal mass accuracy. Source parameters employed Gas Temp (250 °C), Drying Gas (14 L/min), Nebulizer (35 psig), Sheath Gas Temp (250 °C), Sheath Gas Flow (11 L/min), VCap (3,500 V), Fragmentor (180 V), OCT 1 RF Vpp (750 V). The data were acquired with the Agilent MassHunter Workstation Software, LC/MS Data Acquisition B.05.00 (Build 5.0.5042.2) operating in Auto MS/MS mode. A maximum of 20 precursors per cycle were selected for MS/MS analysis, limited by charge states 2, 3 and >3, within a 300–1,400 m/z mass range and above a threshold of 1,500 counts. The acquisition rate was set to 8 spectra/s. MS/MS spectra were collected with an Isolation Width at Medium (~ 4 m/z) resolution and collision energy dependent on the m/z to optimize fragmentation ($3.6 \times (m/z)/100 - 4.8$). MS/MS spectra were scanned from 70 to 1,500 m/z and were acquired until 40,000 total counts were collected or for a maximum accumulation time of 333 ms. Former parent ions were excluded for 0.1 min following selection for MS/MS acquisition.

MS/MS data analysis

The acquired data were exported as .mgf files using the Export as MGF function of theMassHunter Workstation Software, Qualitative Analysis (Version B.05.00 Build 5.0.519.13 Service Pack 1, Agilent Technologies) using the following settings: Peak Filters (MS/MS) the Absolute height (≥20 counts), Relative height (≥0.100 % of largest peak), Maximum number of peaks (300) by height; for Charge State (MS/MS) the Peak spacing tolerance (0.0025 m/z plus 7.0 ppm), Isotope model (peptides), Charge state Limit assigned to (5) maximum. Resultant data files were interrogated with the Mascot search engine version 2.3.02 (Matrix Science) with a peptide tolerance of ±50 ppm and MS/MS tolerance of ±0.1 Da; variable modifications Acetyl (N-term), Carbamidomethyl (C), Deamidated (NQ), Oxidation (M); up to one missed cleavage for trypsin; Peptide charge 2+, 3+ and 4+; and the instrument type was set to ESI-QUAD-TOF.

Searches were performed against an internally compiled protein dataset derived from the genome sequence of *Thermomyces lanuginosus* SSBP [24]. The database comprised 5,220 sequences (2,793,099 residues) and included proteins comprising the common Repository of Adventitious Proteins (cRAP v2012.01.01 from The Global Proteome Machine). The ions score or expected cut-off for peptides in each sample was the following, Tlang-Sec-1 (27), Tlang-Sec-2 (26) and Tlang-Sec-3 (26). Peptides with ions scores below these values are included in Table S1; ions scores ≥ the cut-off value indicate identity or extensive homology ($p < 0.05$). Protein and peptide matches identified after interrogation of MS/MS data by Mascot were filtered and validated using Scaffold (version 4.1.1, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0 % probability by the Peptide Prophet algorithm [14] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0 % probability and contained at least 1 identified peptide (at 95 % and greater). Protein probabilities were assigned by the Protein Prophet algorithm [25]. This resulted in false discovery rates of 0.1 % for protein and 0.8 % for peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Functional annotation

Proteins identified by mass spectrometry were functionally annotated using the BLAST algorithm at National Center for Biotechnology Information [3]. The CAZy family annotations were obtained using the CAZymes Analysis Toolkit (http://mothra.ornl.gov/cgi-bin/cat/cat.cgi) [26].

Signal sequence predictions were undertaken using SignalP 4.1 with default D-cut-off values (http://www.cbs.dtu.dk/services/SignalP/) [27] and SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/) [5]. BLAST matches to the Fungal Secretome Database (FSD) [6] were undertaken against the Characterized Sequences (1,027 Predicted Secretome) dataset using default parameters (Limit Expect Value to 10^{-5}).

Results and discussion

Growth and qualitative enzyme analysis

Total biomass produced by the fungus following 5 days growth was approximately 29.2 g/L ± 2.8, revealing a clear ability for the fungus to utilise the lignocellulosic biomass as a carbon source. Visual analysis of the media revealed remaining corn cob material was softened, with a majority being degraded, thus most of the dry weight measured can be attributed to fungal mycelia and only a small portion to remaining corn cob material (data not shown).

Protein profiling by SDS-PAGE revealed the presence of a number of faint protein bands, with an intense band at 24 kDa (Fig. 1a). Previous studies have shown that this fungus produces extremely high quantities of a GH11 xylanase, with a molecular mass of 24.3 kDa [18, 35]. Native zymogram analysis confirmed that this predominant band was the xylanase (Fig. 1b), and that the enzyme apparently undergoes various post-translational modifications, evidenced by multiple activity bands.

Plate assays of key lignocellulosic enzymes were performed to provide a qualitative assessment of the secretome (Fig. 2a–d). Activities of xylanase, β-xylosidase and β-glucosidase were present, while no cellulase activity was detected. These results are consistent with previous studies [37, 38], supporting samples appropriate for secretome analysis.

Proteomic profile of the *T. lanuginosus* secretome

The growth media from three independent 5-day old cultures of *T. lanuginosus* grown on 3 % corn cob material were collected and proteins precipitated prior to digestion with trypsin and analysis by LC–MS/MS. Collectively, 142 unique proteins (non-redundant) were identified from the three independent biological samples when stringent protein and peptide-based filtering was applied on the initial search results (Table S1). After data filtering the Tlang-Sec-1 sample yielded a total of 131 proteins, while only 66 and 58 proteins were identified from the Tlang-Sec-2 and Tlang-Sec-3 samples, respectively. To obtain a reproducible set of identified proteins from the *T. lanuginosus*
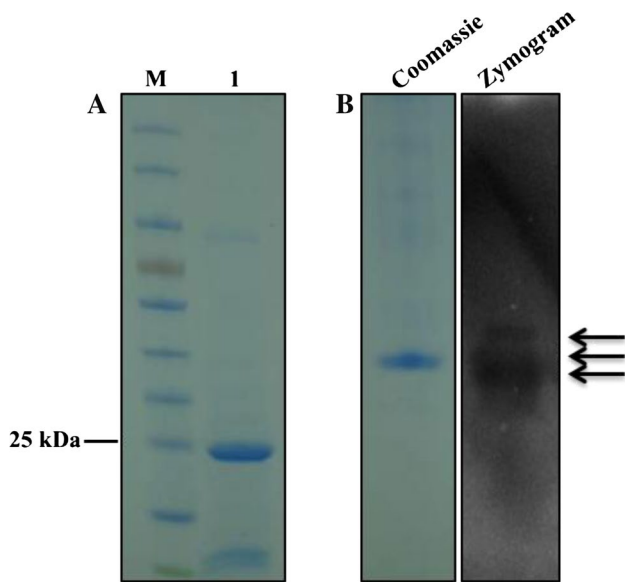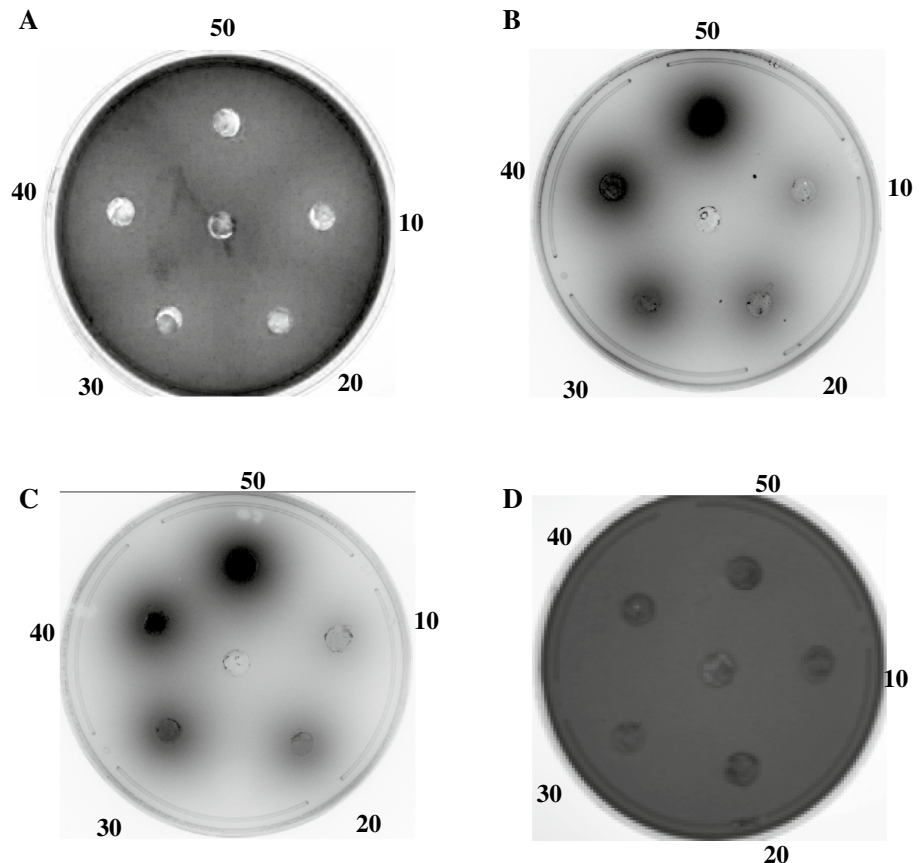
Fig. 1 Protein profile of the *T. lanuginosus* secretome grown on 3 % corn cobs. **a** SDS-PAGE protein profile of 10 µg of supernatant proteins. *M* marker, *1* separated supernatant proteins. **b** Native-PAGE coomassie and zymogram analysis of the xylanase enzyme. *Arrows* indicate activity bands
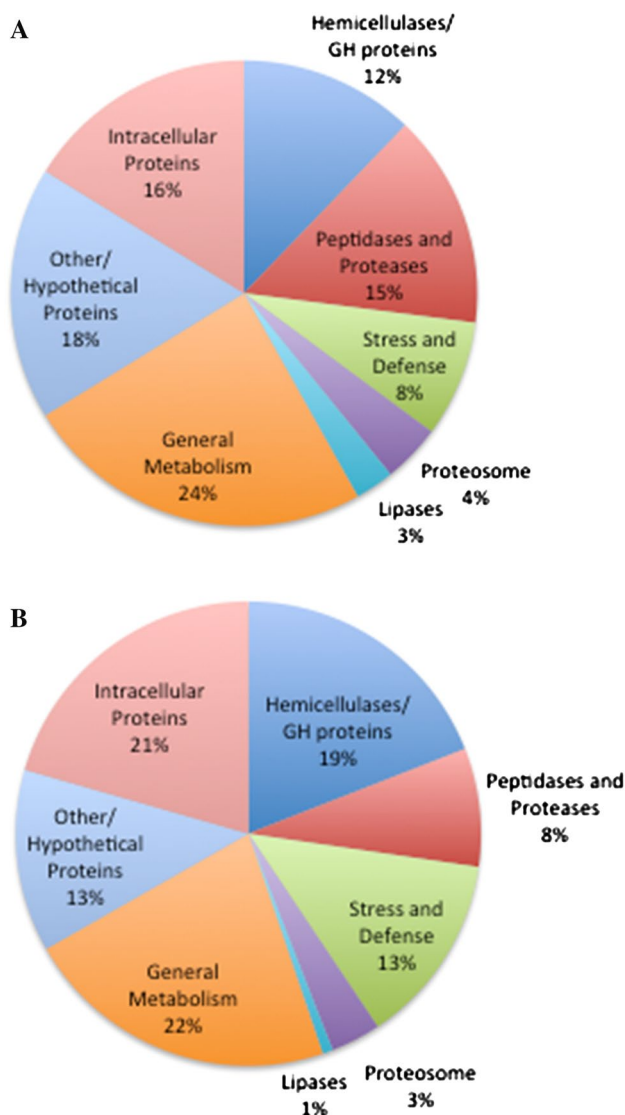
secretome, only proteins identified in at least two of the independent samples were considered representative. This resulted in a collection of 74 proteins that were consistently identified in *T. lanuginosus* secretome samples by LC–MS/MS (Figure S1).

Functional analysis of the *T. lanuginosus* secretome

Initial functional assignments for the 74 identified proteins were obtained using BLAST [18] and CAZy annotations [26] with the final functional breakdown manually curated (Table S2). A broad functional analysis of these proteins revealed 9 (12 %) hemicellulose/GH proteins, 11 (15 %) peptidases/proteases, 6 (8 %) stress and defence proteins, 3 (4 %) proteasome proteins, 2 (3 %) lipases, 18 (24 %) proteins involved in general metabolism, 13 (18 %) other/hypothetical proteins and 12 (16 %) putative intracellular contaminating proteins (Fig. 3a). This functional breakdown was somewhat similar if protein abundance was used (average Normalized Spectral Abundance Factor) to estimate the abundance of proteins in each category (Fig. 3b). Based on this weighting, only a major

Fig. 2 Plate assays of various lignocellulosic enzymes from the supernatant of *T. lanuginosus* after growth on 3 % corn cobs. Crude supernatant (10 to 50 µl as indicated) was loaded into wells and incubated at 50 °C for the appropriate time. At the centre of each plate is the control of 50 µl uninoculated production media. **a** xylanase, **b** β-xylosidase, **c** β-glucosidase, **d** cellulase

A

B

Fig. 3 Functional classification of the 74 proteins identified in the *T. lanuginosus* secretome grown on 3 % corn cobs. **a** classification based on enzymatic activity. **b** classification based on protein weighting in the total sample

decrease in the peptidases/proteases (8 %) functional category was observed with generally only minor increases to other groups. Signal sequence predictions were performed using both SignalP for traditional signal peptides, and SecretomeP for non-traditional signal peptides. In total, 54 proteins identified in the secretome contain a signal sequence. Seventeen proteins were predicted to contain a signal peptide by SignalP. All 54 proteins were predicted by SecretomeP, of which 39 are considered true non-classical signal peptides (i.e. not identified by SignalP; Table S2). Of the 54 signal peptide-containing proteins, nine are involved in hemicellulose degradation or belong to the GH families, seven are peptidases/proteases, two are components of the proteasome, two are lipases, three are involved in

stress and defence, 11 are involved in general metabolism, 11 with other or unknown functions and nine are typically intracellular proteins (mitochondrial located). The signal analysis suggests the majority of the identified enzymes (73 %) are likely to be secreted and validates the presence of the enzymes found in the *T. lanuginosus* secretome. The remaining 20 proteins are considered to be contaminants from burst mycelium, either during sample preparation or from naturally senescing fungi. However, it is possible that some of these 20 proteins are truly extracellular but lack a known signal peptide or they have been secreted via an alternative, unrecognised pathway.

To further assess our *T. lanuginosus* secretome, we compared the identified proteins with characterized secretome proteins at the Fungal Secretome Database (FSD) [6]. A total of 27 proteins had significant BLAST matches to previously characterized secreted fungal proteins found at FSD (Table S2). Most of the hemicellulases, GH proteins and protease families in our dataset have previously been characterized in other fungal secretomes, except for the alpha-galactosidase and alpha-trehalose glucohydrolase. Proteins identified in other functional categories were less represented in FSD, reflecting either species specific components of the *T. lanuginosus* secretome or cellular contaminants. However, the characterized secretome dataset at FSD only contains 1,024 proteins (from 281 secretomes). Currently no data are available from the genus *Thermomyces*, further highlighting the additional utility of our secretome to community.

As this fungus has the ability to grow well on lignocellulosic material, analysis on the secretome was performed using the CAZymes Analysis Toolkit [26]. A total of 14 proteins identified in the secretome belong to the CAZy family of enzymes (Table 2). Determination of the number of putative gene families for each of the CAZy enzymes was performed by a BLAST search against the *T. lanuginosus* SSBP database. It is interesting to note that the primary hemicellulase enzymes xylanase, β-xylosidase and α-galactosidase are all single gene families within the *T. lanuginosus* genome. β-glucosidase comprises 6 gene families, although only one member was identified in the secretome. Other single family CAZy enzymes in the secretome are the α-amylase, the α-trehalose glucohydrolase, a putative aminopeptidase and the IgE-binding protein. All other CAZy enzymes have at least one other homologue in the genome, suggesting gene redundancy, differential expression or similar yet different functions. Expression analysis could assist in determining which of the above applies.

Enzymes involved in lignocellulosic degradation

Thermomyces lanuginosus is known for its ability to hyper-express xylanase, while having no cellulase activity

Table 2 CAZy enzymes identified from the supernatant of *T. lanuginosus* after growth on corn cobs

Tlang ID	Protein name	Accession number	Molecular mass (kDa)	pI	CAZy family	Signal peptide	Gene families
Hemicellulases							
NOKS4_002370-RA	β1,4-Endo-xylanase	gi|335371365	24.3	4.77	GH11	YES	1
NOKS4_000883-RA	β-Glucosidase	gi|304651073	92.7	4.9	GH3	YES	6
NOKS4_002883-RA	β-1,4-Xylosidase	gi|321150563	38.1	5.16	GH43	YES	1
NOKS4_000066-RA	α-Galactosidase C precursor (Melibiase)	gi|67902302	82.1	5.32	GH36	YES	1
NOKS4_004192-RA	Glucooligosaccharide oxidase, putative	gi|242796746	52.2	5.24	AA7	YES	7
Other enzymes							
NOKS4_002970-RA	GPI-Anchored cell wall beta-1,3-endoglucanase EglC	gi|70985687	45.9	4.58	GH17	YES	3
NOKS4_001715-RA	α-Amylase A type-1/2	gi|317144680	58.8	4.67	CBM20 GH15	YES	1
NOKS4_000016-RA	β-1,3-1,4-Glucanase	gi|301070474	33.3	4.48	GH16	YES	4
NOKS4_001248-RA	α, α-Trehalose glucohydrolase TreA/Ath1	gi|242814874	118.4	5.15	GH65	YES	1
NOKS4_003269-RA	Glutaminase GtaA	gi|242824809	77.5	5.02	CBM13	YES	2
NOKS4_004762-RA	IgE-binding protein	gi|557729830	20.7	4.68	CBM52	YES	1
NOKS4_000642-RA	Mitochondrial-processing peptidase subunit β	gi|212531699	53.1	5.96	GH12	YES	3
Proteases/Peptidases							
NOKS4_004381-RA	Serine protease	gi|290749783	48.4	5.43	CBM13	YES	2
NOKS4_002176-RA	Aminopeptidase, putative	gi|242794073	42.3	4.78	CBM12	YES	1

CAZy family assignments were conducted using the CAZymes Analysis Toolkit. Presence of a signal peptide by either SignalP or SecretomeP is indicated. Putative number of gene families was determined by BLAST analysis of the *T. lanuginosus* SSBP genome

[10]. With respect to hemicellulose degradation, the characterisation of the secretome revealed the presence of a β-1,4-endo-xylanase (NOKS4_002370-RA) responsible for cleaving 1,4-linkages in xylan and a β-xylosidase (NOKS4_002883-RA) which removes xylose residues from the non-reducing end of xylan. The presence of these two xylan degrading enzymes was further examined based on their abundance in the secretome using average spectral counts (NSAF: normalized spectral abundance factor). The β-1,4-endo-xylanase was ten-fold more abundant with an average NSAF of 0.147 compared to the β-xylosidase with an average NSAF of 0.015 (Table S2). In fact, the β-1,4-endo-xylanase dominated the secretome and was many times more abundant than the vast majority of identified proteins in the secretome samples. This observation is supported by both the banding pattern of secretome samples using SDS-PAGE indicating a prominent ca. 24 kDa protein and the corresponding zymogram indicating the bands xylanase activity.

In addition to the xylan degrading enzymes, an α-galactosidase (NOKS4_000066-RA) was also identified and is likely responsible for the hydrolysis of melibiose to galactose and glucose. The analysis also revealed the presence of a GH3 β-glucosidase (NOKS4_000883-RA) and a glucooligosaccharide oxidase (NOKS4_004192-RA), which are typically reported to be involved in cellulose degradation. However, glucooligosaccharide oxidase has a strong role in the oxidation of a number of oligosaccharides that are present in hemicellulose and lignocellulosic biomass generally [41]. Therefore, we propose that this enzyme is more likely involved in supporting hemicellulose degradation rather than the degradation of cellulose in *Thermomyces*. Analysis of other recent fungal secretomes such as *Penicillium echinulatum* [31] and *Trichoderma* [1] revealed the overwhelming presence of cellulolytic enzymes. While there are fungal secretomes, such as *Aspergillus nidulans* [34] and *Fusarium verticilloides* [29], which show a greater relative percentage of hemicellulases, these secretomes also contain a number of cellulases (7 and 5 % cellulases, respectively). Uniquely, the secretome data presented here support the notion that the filamentous fungus *T. lanuginosus*, is capable of hydrolysing lignocellulosic biomass by exclusively employing hemicellulases.

Interestingly, no typical lignin degrading enzymes were found in the secretome. It has been reported that the lignin component of corn cobs is typically 6.7 to 13.9 % [8] and is easily disrupted by mechanical shearing (blender), heat treatment (autoclaving) and aqueous swelling [21]. It is

Table 3 Commercially interesting enzymes expressed in the secretome of *T. lanuginosus*

Major commercially available enzymes found in the secretome of *T. lanuginosus* grown on 3 % corn cobs are listed in alphabetical order. Current commercially available preparations of enzymes from *T. lanuginosus* are indicated

[a] Includes pulp and paper industry, textile industry, chemical industry, animal feed industry, bioremediation

[b] Available commercially

Enzyme	Industry				*T. lanuginosus* enzyme[b]
	Food	Pharma	Biofuel	Other[a]	
α-Amylase	X	X			**Yes**
α-Galactosidase			X		No
β-1,4-Endo-xylanase	X		X	X	**Yes**
β-1,4-Xylosidase	X		X		No
β-Glucosidase			X		No
Cyanate hydratase				X	No
Endo-1,3(4)- β-glucanase	X				No
Fructose-bisphosphate aldolase		X			No
Glucan endo-1,3-β-glucosidase	X				No
Glutaminase	X	X			No
Lipase	X		X	X	**Yes**
Various aminopeptidases	X				No

possible that following the preparation of the corn cobs in this study, the lignin was no longer tightly bound to the hemicellulose component, thus allowing enzymatic access and degradation of xylan. In addition, some of the enzymes identified in the secretome have activities that produce H_2O_2 (e.g. glucooligosaccharide oxidase and various oxidoreductases), a chemical proposed to be able to soften and remove lignin [9, 12]. It is thus possible that even in the absence of a chemical pretreatment, *T. lanuginosus* is able to remove lignin to enable enzymatic access to hemicellulose/cellulose, likely by way of H_2O_2 production from the action of extracellular enzymes.

Other industrially important enzymes

Thermostable enzymes are highly sought after in a variety of industries, both to replace current use of toxic chemicals, as well as to generate "natural" products, increasingly desired by consumers. Currently, most of the commercial enzymes produced are derived from mesophilic *Aspergillus* or *Trichoderma* species, although a few thermostable enzymes produced by *T. lanuginosus* are available. These include lipase, for use in food and biodiesel industries [7], amylase for use in food and pharmaceutical industries [15] and xylanase for use in biofuel, pulp and paper and food industries [38]. Therefore, a search of the secretome for putative commercially useful enzymes was compiled (Table 3).

Interestingly, besides the xylanase, both a lipase and amylase were also identified in the secretome of *T. lanuginosus* grown on corn cobs, a media specifically optimised for cellulosic enzyme production. In addition to the current commercially produced enzymes of *T. lanuginosus*, a number of other industrially interesting enzymes were found to be expressed by the fungus (Table 3). Glutaminase

is considered an antileukemic agent [39], an antiretroviral agent [32] and is used as a flavour and aroma enhancer in the food industry [19]. Fructose-bisphosphate aldolase is used as a biocatalyst in the synthesis of important pharmaceutical compounds, although stability of the current industrially used enzyme, particularly at high temperatures, remains a problem [14]. In addition, various aminopeptidases were identified in the *T. lanuginosus* secretome which are involved in maturation and activation of proteins and many have applications in the food industry [40]. A critical enzyme found in the secretome, cyanate hydratase, is responsible for detoxification of cyanide. This enzyme holds great potential for bioremediation of contaminated industrial sites [4].

A number of enzymes with proposed putative industrial functions were also identified in the secretome. These include the GDSL lipase for the food and biodiesel industries [2] and glucooligosaccharide oxidase for the biofuel industry [41]. These data, coupled with the inherent thermostability and broad pH activity of the enzymes produced by this fungus, render *T. lanuginosus* an exciting repository of industrially and commercially relevant enzymes.

Conclusion

Gaining an insight into the secretome of this thermophilic fungus has revealed the most prominent enzymes involved in hemicellulose degradation and a number of as yet unexploited enzymes. Optimising growth parameters for the induction of these enzymes is currently underway. Combining current commercialised enzymes, with the identification of novel industrial enzymes presented in this paper, we propose *T. lanuginosus* as a potential source of unique commercial enzymes. In addition, with relatively

few proteins present in the supernatant, combined with the strong expression capacity of the xylanase gene, *T. lanuginosus* is also of interest as an expression host. We are currently developing *T. lanuginosus* in this capacity, utilising native regulatory elements, which will provide a novel fungal expression system for the high yield production of thermostable enzymes, including the production of *T. lanuginosus* enzymes identified in this study.

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