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Integrated Functional-Omics Analysis of *Thermomyces lanuginosus* Reveals its Potential for Simultaneous Production of Xylanase and Substituted Xylooligosaccharides

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Abstract

Thermophiles have several beneficial properties for the conversion of biomass at high temperatures. *Thermomyces lanuginosus* is a thermophilic filamentous fungus that was shown to secrete 40 glycoside hydrolases and 25 proteases when grown on different carbon sources. Among the 13 identified glycoside hydrolases with high expression levels, 9 were reduced sugar glycosidases (RSGs) belonging to seven GH families, and 7 of the 10 identified proteases were exopeptidases belonging to six different protease families. High expression of RSGs and exopeptidases may allow the fungus to efficiently degrade oligosaccharides and oligopeptides in saprophytic habitats. There were no xylan side chain-degrading enzymes predicted in the genome of *T. lanuginosus*, and only one thermophilic GH11 xylanase (g4601.t1) and one GH43 xylosidase (g3706.t1) were detected by liquid chromatography-mass spectrometry/mass spectrometry when *T. lanuginosus* grown on xylan, which led to the accumulation of substituted xylooligosaccharides (SXOS) during corncob xylan degradation where SXOS output made up more than 8% of the total xylan. The SXOS are beneficial prebiotics and important inducers for enzymes secretion of microorganisms. Thus, *T. lanuginosus* exhibits distinct advantages in utilizing cheap raw materials producing one thermostable xylanase and the high value-added SXOS as well as microbial inoculants to compost by batch fermentation.

Keywords *Thermomyces lanuginosus* · Integrated functional-omics · Secretome · Industrial applications · Substituted xylooligosaccharides (SXOS)

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Introduction

Thermophiles are a large category of microorganisms found ubiquitously in nature that have optimum growth temperatures of 50 °C or higher. [1, 2] Thermophiles participate in degrading lignocellulose at high temperatures and play a primary role in both the carbon cycle and biomass deconstruction. [2] Because high temperatures accelerate lignocellulose deconstruction and reduce the risk of contamination, [2–5] the fermentation conditions of thermophiles are ideal for many industrial applications. [2, 6–8]

Thermomyces lanuginosus is a thermophilic filamentous fungus and cellulase-free xylanase superproducer that was first isolated from potatoes by Tsiklinskaya in 1899. [1, 9, 10] *T. lanuginosus* is widely distributed in compost and soil environments and has an optimum growth temperature of 50 °C. [10, 11] A recent research demonstrated that *T. lanuginosus* dominates (> 90%) self-heating maize straw compost fungal communities when fermentation temperatures reach above 60 °C. [12] The extracellular enzymes secreted by *T. lanuginosus* have exceptional properties, such as high thermostability and a wide range of optimal pH values that allow for extensive industrial application of the enzymes. [11, 13–18] A large number of these enzymes have been cloned and engineered in previous studies. [19–23] However, the cost could be decreased when cheap biomass materials are used as substrates to product these enzymes. Because the composition unit and content of biomass materials are different, and the effects of different composition units on enzyme secreted by *T. lanuginosus* have not been systematically studied, their industrial application is limited. Proteomics can provide comprehensive information associated with extracellular enzymes, for example, species, concentration, modification, and subcellular location. [24] Therefore, functional-omics studies integrating genomics and proteomics are needed to further explore the potential industrial applications of this widespread fungus.

The whole genome of *T. lanuginosus* was sequenced and its genome size was found to be 19.16 Mb, which is one third less than the genomes of several other lignocellulosic fungi, such as *Aspergillus niger*, *Trichoderma reesei*, and *Penicillium oxalicum* in Table S1. [25] Genes annotated as lignocellulolytic enzymes included mainly hemicellulose-degrading enzymes (Fig. S1, Table S1). [25, 26] In addition to carbon sources, nitrogen sources are also indispensable for the rapid growth of saprobiotic microorganisms, and the secreted proteases could degrade the proteins in the environment into small peptides and amino acids, which could be directly assimilated and utilized as nitrogen sources, so as to maintain the continuous secretion of more extracellular proteins by the microorganisms. It is necessary for *T. lanuginosus* SD01 to efficiently obtain carbon and nitrogen sources from saprophytic environment to support its rapid growth; thus, the secreted GH enzymes and peptidases, which could degrade glycan and proteins into absorbable carbon and nitrogen sources, respectively, by *T. lanuginosus* SD01 under different substrates, were analyzed at the same time. Whole-genome sequencing of *T. lanuginosus* and the rapid development of proteomics technologies has made it possible to systematically study the physiological and biochemical characteristics of this fungus and explore the potential of further industrial applications.

In this study, the species and functions of the proteome expressed by *T. lanuginosus* SD01 (CGMCC 3.15828), a filamentous fungus isolated from self-heating maize straw compost, were comprehensively assessed. The main goal of this research was to study the growth specificity and the potential industrial applications of *T. lanuginosus* SD01 based on integrated functional-omics approaches.

Material and Methods

Material

Glycerol (Gly), glucose (Glc), maltose (Mal), starch (Sta), arabinose (Ara), xylose (Xyl), xylooligosaccharide (XOS), xylan (XY), mannose, galactose (Gala), galactomannan (Mannan), cellobiose (Cello), microcrystalline cellulose (MCC), and $NaCNBH_3$ were purchased from Sangon Biotech Co. (Ltd. Shanghai, China). 7-amino-1, 3-naphthalenedisulfonic acid monopotassium salt monohydrate, trichloroacetic acid, dithiothreitol (DTT), iodoacetamide, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Strain Growth Conditions and Protein Extraction

The filamentous fungi *T. lanuginosus* SD01 (CGMCC 3.15828) was previously isolated from maize straw compost by our laboratory and had been deposited in China General Microbiological Culture Collection Center (CGMCC). Conidia (7.0×10^7/mL) were stored in 80% (*v*/*v*) glycerol at − 20 °C. To compare colony diameter, the strain was grown on potato dextrose agar (PDA) plates at 30, 40, 50, and 60 °C for 5 days. Gly, Glc, Mal, Sta, Ara, Xyl, XOS, XY, mannose, Gala, Mannan, Cello, and MCC at 1% (*w*/*v*) were supplemented into minimal medium (MM) prepared as described previously. [27] All media were sterilized at 115 °C for 30 min. Suspensions of 100 μL of fresh *T. lanuginosus* SD01 conidia were inoculated into 100 mL medium and incubated at 50 °C with shaking at 200×*g* for 5 days. Samples were harvested every day. Substrates and fungal biomass were removed using eight layers of gauze and centrifugation at 10,000×*g* at 4 °C for 10 min. The supernatant was further filtered through a 0.22-μm membrane (Dingguo, Beijing, China). The pH of the supernatant was measured using a pH meter (Sartorius, Beijing, China). The supernatant was stored at 4 °C and used as the crude enzyme extract in further experiments. The dry weights of the fungal biomass and substrates were obtained by determining the constant weight at 105 °C for 48 h. The fungal biomass was obtained by subtracting the dry weights of the substrate at day 0. Three independent replicates were conducted for each carbon source.

Extracellular Reducing Sugar and Enzyme Activity Assays

The Bradford method [28] was used to determine protein concentrations in the crude enzyme extract. Briefly, 100 μL of enzyme was mixed with 1 mL of Coomassie Brilliant Blue G-250 dye, after being reacted at room temperature for 10 min; then, a microplate spectrophotometer (Tecan, Morrisville, NC, USA) was used to measure all mixtures at 595 nm. Protein content was measured in triplicate by subtracting protein content at day 0. Bovine serum albumin (0.1 mg/mL) was used to obtain the standard curve.

The dinitrosalicylic acid method was applied to quantitatively measure the concentrations of extracellular reducing sugars and xylanase activity. [29] XY (1%, *w*/*v*) dissolved in sodium hydrogen phosphate/citric acid buffer (pH 5.5) was used as a substrate to measure xylanase activity. Four hundred microliters of enzyme and 600 μL of substrate were reacted at 70 °C for 30 min; then, 800 μL of DNS was immediately added to each sample to terminate the reaction and boiled for 10 min. Water (8.2 mL) was added to the system and mixtures were determined by ultraviolet spectrophotometer (Puyuan Instruments, Ltd., Shanghai, China) at 550 nm.

Xylanase activity was defined as the international unit, and one unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute. Standard curves were prepared using 0.1 mg/mL xylose or glucose.

Fluorescence-Assisted Carbohydrate Electrophoresis

The Fluorescence-assisted carbohydrate electrophoresis (FACE) method described by Zhang et al. [30] was used to identify and quantify polysaccharide hydrolysate species and concentrations. Electrophoresis was performed on a miniaturized vertical gel system (Bio-Rad, Hercules, CA, USA). Each enzyme sample or maker (5 μL) was mixed with 5 μL of 0.2 M 7-amino-1, 3-naphthalenedisulfonic acid monopotassium salt monohydrate dissolved in 15% acetic acid. The reaction was kept in the dark for 1 h to label the samples. Then, 5 μL of 1 M $NaCNBH_3$ was added and the mixture was incubated at 42 °C overnight. For each plate, 7 μL of each labeled sample was loaded per well and electrophoresis was run at 7 mA. Gels were scanned using a ChemiDoc™MP System (Bio-Rad). The gray values of bands were measured using the Quantity One software (Bio-Rad). Cellulose was used to prepare cellodextrins for using as a glucose marker. [31] XOS mixed xylose was used as xylose marker.

Analysis of the Product Profiles of Arabinoxylan Arabinofuranohydrolase Degrading the Hydrolysates of XY

The GH 62 family arabinoxylan arabinofuranohydrolase (AXH, EC:3.2.1.55, GAQ35594.1) of *A. niger* An76 was heterologously expressed in *Escherichia coli*. The plasmids connecting the AXH gene were transformed into *E. coli* BL21. About 10 single colonies were picked into 400 mL lysogeny broth (LB) media containing ampicillin, and shock cultured at 37 °C until the OD600 attained 0.6–0.8. IPTG with a final concentration of 1 mmol/mL was added into media and cultured at 20 °C for 20 h. Microorganism were collected by centrifuging at 8000×*g* for 10 min, and then resuspended by NaH_2PO_4/NaCl buffer (pH 8.0), and were ultrasonicated for 30 min. After being centrifuged at 11000×*g* for 30 min, the supernatant was collected and filtered through a 0.22-μm membrane (Dingguo) as the crude enzyme. Transferring the crude enzyme into a Ni2+ affinity column and eluting the column using imidazole buffer, the eluant was collected in Eppendorf (EP) tubes. Pure proteins were collected and concentrated by 10-kDa membrane (Dingguo) and the activity of AXH was found to be 1.113 ± 0.001 IU/mg.

AXH was diluted 50-, 20-, 10-, and 5-fold in Tris-HCl buffer (pH 3.0) and used to hydrolyze the hydrolysates of XY. Then, 5 μL AXH was mixed with 100 μL of each hydrolysate of XY and the reaction was maintain at 45 °C for 5 min. The reaction was then terminated by boiling for 10 min. XOS bands were detected by FACE. The gray values of bands were measured using the Quantity One software (Bio-Rad).

Analysis of Protein Profiles by SDS-PAGE and Native Zymograms

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze changes in protein profiles. [32] The concentration of the separation gel was 12% (*w*/*v*) and the loading volume of the sample was 20 μL/well. The gel was stained with Coomassie brilliant blue R250 for 30 min and then destained with destaining solution containing 100 mL glacial acetic acid, 100 mL absolute ethanol, and 800 mL distilled water per liter. The gels were scanned using a BenQ scanner 7550R (BenQ, Jiangsu, China).

The native zymogram method modified by Zhang et al. was used to detect differences in xylanase activity between different carbon sources. [33] XY solution (2%, *w*/*v*) in sodium hydrogen phosphate/citric acid buffer at pH 5.5 was used as the substrate. The concentration of the separation gel was 10% (*w*/*v*) and the volume of the sample was 15 μL/well. The gels were soaked in xylan solution at 70 °C for 30 min after electrophoresis, then, dyed with 0.5% (*w*/*v*) Congo Red dissolved in 10% (*v*/*v*) alcohol for 15 min, and decolored using 1 M NaCl. Subsequently, the gels were scanned using a BenQ scanner 7550R (BenQ).

The gelatinase zymogram method modified by Pan et al. was used to determine protease activity. [34] A substrate of 0.1% gelatin was added to a 13.6% (*w*/*v*) separation gel. The loading volume of the samples was 7 μL/well. After electrophoresis, the gels were washed twice with 2.5% (*v*/*v*) Triton X-100 dissolved in Tris-HCl buffer at pH 8.0 for 15 min to remove the SDS, and then washed twice with Tris-HCl buffer at pH 8.0 for 20 min to remove Triton X-100. The gels were incubated at Tris-HCl buffer (pH 8.0) at 50 °C for 2 h to recover the protease activity. The gels were stained and destained according to SDS zymogram and they were scanned using a BenQ scanner 7550R (BenQ).

T. lanuginosus SD01 Secretome Analysis by LC-MS/MS

LC-MS/MS was used to analyze the secretomes of *T. lanuginosus* SD01 on different substrates as previously described. [12] Extracellular crude enzymes secreted by *T. lanuginosus* SD01 for 4 days were ultrafiltered using a 3-kDa cutoff membrane (Dingguo) and mycelia were further filtered for extraction of total intracellular protein. Concentrated enzymes were precipitated using 10% (*w*/*v*) trichloroacetic acid and then dissolved in high-performance liquid chromatography (HPLC) grade water. [35] After determining protein concentrations using the Bradford method, 50 μL of degeneration buffer (0.5 M Tris-HCl, 2.75 mM EDTA, 6 M guanidine-HCl, and 30 μL 1 M DTT) and 30 μL of 1 M DTT were added to 50 μg of protein and the mixtures were incubated at 37 °C for 2 h. Samples were alkylated using 50 μL of 1 M iodoacetamide and the reaction was left in the dark for 1 h. After the MicroconYM-10 membrane (3-kDa cutoff, Dingguo) was washed with HPLC grade water, 360 μL of 25 mM NH_4HCO_3 was added and the proteins were washed four times by centrifugation at 14,000×*g*. The proteins were then digested with trypsin at a ratio of 1:25 (*w*/*w*, trypsin/protein) at 37 °C overnight. After desalination through a C18 Ziptip (Dingguo), the peptide samples were dissolved in 0.1% (*v*/*v*) trifluoroacetic acid. Eluted peptides were analyzed using a Prominence nano LC system (Shimadzu, Tokyo, Japan) coupled with an LTQ-Orbitrap Velos Pro ETD mass spectrometer (Thermo Scientific, MA, Germany). An LC system equipped with a custom silica column (75 μm × 15 cm) containing Reprosil-Pur 120 C18-AQ (particle size 3 μm; Dr. Maish, Germany) was used to separate peptides, which were eluted with a stepping gradient of solvent A (2.0% (*v*/*v*) ACN in water with 0.1% (*v*/*v*) formic acid) and solvent B (98% (*v*/*v*) ACN in water with 0.1% (*v*/*v*) formic acid). A nanospray ion source (2000 V electrospray voltage and 275 °C transfer capillary temperature) was then used to spray the separated peptides into the mass spectrometer. The LTQ-Orbitrap Velos Pro ETD was run in the data-dependent acquisition mode using the Xcalibur 2.2.0 software (Thermo Scientific) for tandem MS experiments. Full-scan MS spectra (from 400 to 1800 m/z) were detected in the orbitrap with a resolution of 60,000 at 400 m/z. The ten most intense precursor ions greater than the threshold of 5000 counts in the linear ion trap with a normalized collision energy of 35% were selected for MS/MS fragmentation analysis. In order to avoid selecting repeated peptides, dynamic exclusion was set to within 60 s. A total of three replicates were performed for each sample.

Analysis of *T. lanuginosus* SD01 Intracellular Proteins by LC-MS/MS

The trichloracetic acid-acetone method was used to extract total intracellular proteins as described previously with slight modifications. [36] Simply, 5 g of mycelia powder ground with liquid nitrogen was added to 50 mL of acetone containing 13.3% (*w*/*v*) TCA and 0.093% (*v*/*v*) β-mercaptoethanol and left overnight at − 20 °C. The mixture was centrifuged at 14,000×*g* at 4 °C for 20 min, the supernatant was discarded, and the pellet was washed twice with precooled acetone containing 0.07% (*v*/*v*) β-mercaptoethanol. The pellet was resuspended in 30 mL lysis buffer (7 M urea, 2 M thiourea, 4% (*w*/*v*) CHAPS, 0.8% ampholytes, 20 mM Tris, and 20 mM DTT) and ultrasonicated for 30 min to disrupt cells. The mixture was centrifuged at 20,000×*g* at 16 °C for 30 min and the supernatant was collected for intracellular protein analysis. Protein precipitation and LC-MS/MS analysis were conducted for analysis of the secretome.

Database Searches

Database searches were conducted using the Proteome Discoverer software 1.4 (Thermo Scientific) and the SEQUEST search engine. The reference database used for *T. lanuginosus* SD01 was the detailed annotated genome of *T. lanuginosus* SSBP. Parameters for protein searches were set as follows: (1) trypsin was selected to digest proteins, (2) a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da were set for mass tolerance, (3) oxidation of methionine was chosen as the dynamic modification, and (4) carbamidomethyl of cysteine residues was selected as the fixed modification. The results contained only peptides with at least six amino acid residues with 95% certainty ($q \leq 0.05$). At least two peptides ($q < 0.05$) were required to identify proteins and the false discovery rate was set at 1%. The relative abundance of proteins was determined based on the relative number of peptide spectrum matches (PSMs). Previous studies have demonstrated a linear correlation between PSMs and protein abundance. [37] The SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the secretion signals of proteins. ANOVA analysis was used to detect significant differences with a cutoff value of $p < 0.05$.

Results

Dynamic Changes in Extracellular Reducing Sugar and Protein Concentrations and Xylanase Activity

T. lanuginosus SD01 was isolated from thermophilic maize straw composts. The diameter of the colonies was greatest when they were incubated on PDA solid medium at 50 °C for 5 days, demonstrating the thermophilic nature of the fungus (Fig. 1a). [11] During the liquid fermentation process, the extracellular pH increased from 5.1 ± 0.3 to 8.6 ± 0.3 (Fig. 1b, Table S2), which is consistent with previous reports. [11, 18, 38] *T. lanuginosus* SD01 grew on soluble sugars (Glc, Mal, Sta, Ara, Xyl, XOS, mannose, Gala, and Cello) rapidly, which corresponded with a significant decrease in extracellular reducing sugar concentrations after 2–3 days and a majority of sugars (> 90%) was absorbed after the fourth day (Fig. 1c). When *T. lanuginosus* SD01 grew on polysaccharides (MCC, Mannan, XY) and non-reducing sugars (Gly) for 5 days, the concentrations of reducing sugars in crude enzyme were all lower than 1 mg/ml,

which made up little proportion in the column chart (Fig. 1c). Thus, the reducing sugar amount of Gly, XY, Mannan, and MCC were not visible in Fig. 1c. The biomass of *T. lanuginosus* SD01 was higher when the fungus was grown on soluble sugars compared with insoluble substrates (XY, Mannan, and MCC; Fig. 1d). This result is consistent with a previous study that optimal *T. lanuginosus* SSBP growth occurred on readily available carbon sources. [39] Thus, soluble sugars could be used as substrates to achieve rapid strain rejuvenation in industrial production. However, XY and MCC induced protein secretion by *T. lanuginosus* SD01 more efficiently than soluble sugars (Fig. 1e), suggesting a key role for polysaccharides in protein production. In filamentous fungi, soluble sugars with high concentration in the initial cultivation period always resulted in carbon catabolite repression, [40] when the soluble sugars were nearly exhausted; a litte amount of enzymes could be induced because of carbon starvation, whereas, when filamentous fungi exposed to polysaccharides such as XY and MCC, only a little amount of available sugars were released from polysaccharides; the carbon starvation allows low-level expression of a set of enzymes to degrade polysaccharides into soluble sugars; because of the low release-rate and high absorption-rate high of soluble sugars, little amount of extracellular sugar remains, which was beneficial for the continuous secretion and accumulation of enzymes. In addition, the induction mechanism of insoluble polysaccharides recognized by carbohydrate-binding modules (CBM) [41] may be another key reason for the higher amount of protein secretion in XY and MCC, and they needed further research.

Activity of xylanase by *T. lanuginosus* SD01 was efficiently and specifically induced by Xyl, XOS, and XY starting on day 2, especially when XOS and XY were used as the substrates (Fig. 1f).

Analysis of the Dynamics of Degradation and Uptake of Oligosaccharides

FACE has previously been used to identify and quantify oligosaccharide species and concentrations. [42] As shown in Fig. 2, the gray values of reducing sugars decreased significantly on day 3 and were undetectable on day 4, which corresponds to the reducing sugar concentration measurements (Fig. 1c). When *T. lanuginosus* SD01 was grown on Mal, the reducing sugar concentration on day 2 did not change significantly compared with day 0 (Fig. 1b), but the species of soluble sugars identified did vary significantly, mainly the appearing of trisaccharide (Fig. 2a). As shown in Fig. 2a, b, c, e, a large number of monosaccharide species were detected on day 2, indicating that a series of reduced sugar glycosidases (RSGs), such as α-glucosidases, β-xylosidases, and β-glucosidases, were secreted. When *T. lanuginosus* SD01 was grown on Mal or Cello for 2–3 days, several trisaccharide-related oligosaccharide bands appeared, suggesting that extracellular RSGs may also have glycosyl transferase activity (Fig. 2a, e).

Bands indicating the presence of soluble reducing sugars were not visible when *T. lanuginosus* SD01 was grown on Mal, Sta, or Cello for 4 days (Fig. 2a), indicating that soluble sugars produced by RSGs-degrading glycan could be efficiently assimilated and used for growth. In the genome of *T. lanuginosus*, no hydrolytic enzymes predicted to be related to cellulose degradation were identified. Therefore, it is likely that MCC could not be degraded and did not release reducing sugars, as shown in Fig. 2f. In Fig. 2c, it displayed that in the initial 2 days the amount of X3 (band 3), X4 (band 4) decreased and X2 (band 2), X1 (band 1) increased, which indicated that *T. lanuginosus* SD1 was able to secrete glycosidases to degrade large oligosaccharides into small oligosaccharides or monosaccharides to be utilized. The similar phenomenon was observed when Mal and cello were used as carbon sources (Figs. 2a

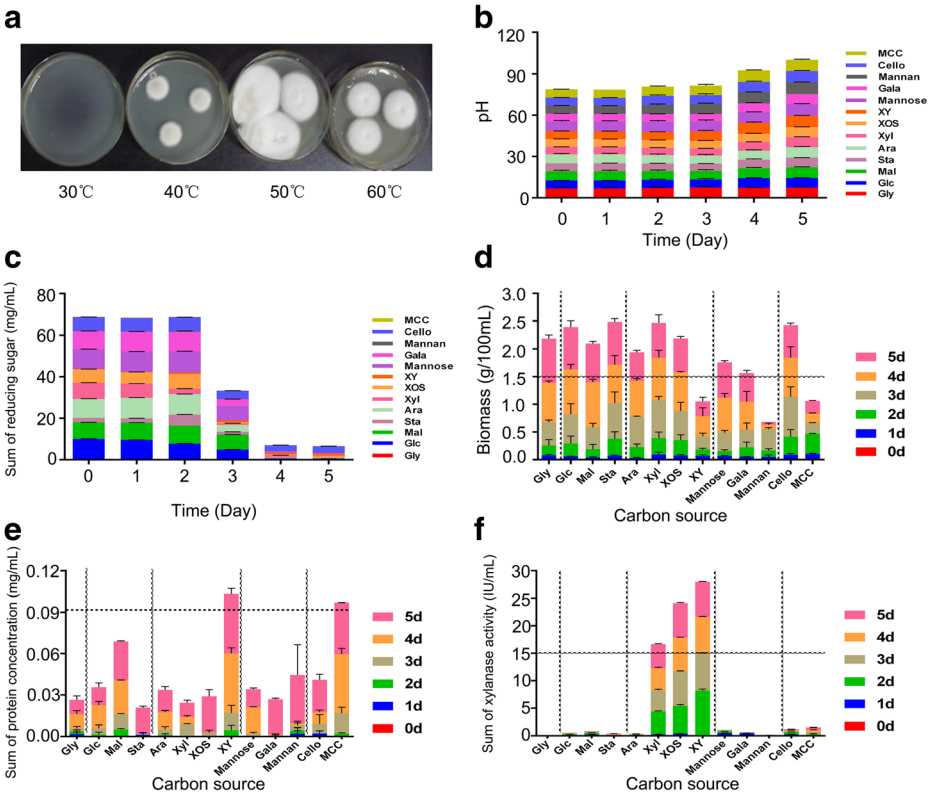

Fig. 1 Physiological and biochemical characteristics of *T. lanuginosus* grown on different carbon sources. **a** Colony diameters of *T. lanuginosus* SD01 cultured at different temperatures. **b** pH of *T. lanuginosus* SD01 crude enzymes over time. **c** *T. lanuginosus* SD01 crude enzymes reducing sugar content over time. **d** Dry biomass of *T. lanuginosus* SD01 mycelia over 5 days of cultivation. **e** Total protein concentration of *T. lanuginosus* SD01 crude enzymes. **f** Xylanase activity of *T. lanuginosus* SD01 crude enzymes. *Gly* glycerol, *Glc* glucose, *Mal* maltose, *Sta* starch, *Ara* arabinose, *Xyl* xylose, *XOS* xylooligosaccharide, *XY* xylan, *Gala* galactose, *Cello* cellobiose, *MCC* microcrystalline cellulose

and 2e). Endo-1, 4-β-Xylanase (EC: 3.2.1.8) could degrade corncob XY into unsubstituted XOS and substituted XOS (SXOS). [43] We respectively utilized 2 mL crude enzymes of *T. lanuginosus* SD01 grown on xylan for 0–5 days to degrade 0.1 g xylan substrate for 5 min and detected the product profiles with FACE. The results (Fig. S3) displayed that the total gray values of bands 4–6 between X1 and X3 were 1–2 times of the total gray value of the accumulated substituted XOS (SXOS) bands (bands 1–3). While only three SXOS bands (mainly X4–X6) were significantly accumulated in Fig 2d. Therefore, X1–X3 released by xylanase from xylan were rapidly assimilated and metabolized. Because predicted XY side chain degradation enzymes are absent in the genome of *T. lanuginosus* (Table S1), the accumulated oligosaccharide bands were likely SXOS. In order to identify the side chain of SXOS between X4 and X6, GH62 family AXH, which has been reported to cleave arabinofuranose from singly substituted xylopyranosyl backbone residues in wheat arabinoxylan, [44] were heterologously expressed, diluted, and used to degrade the XOS bands shown in Fig. 2d (Fig. S1). The gray value of band b

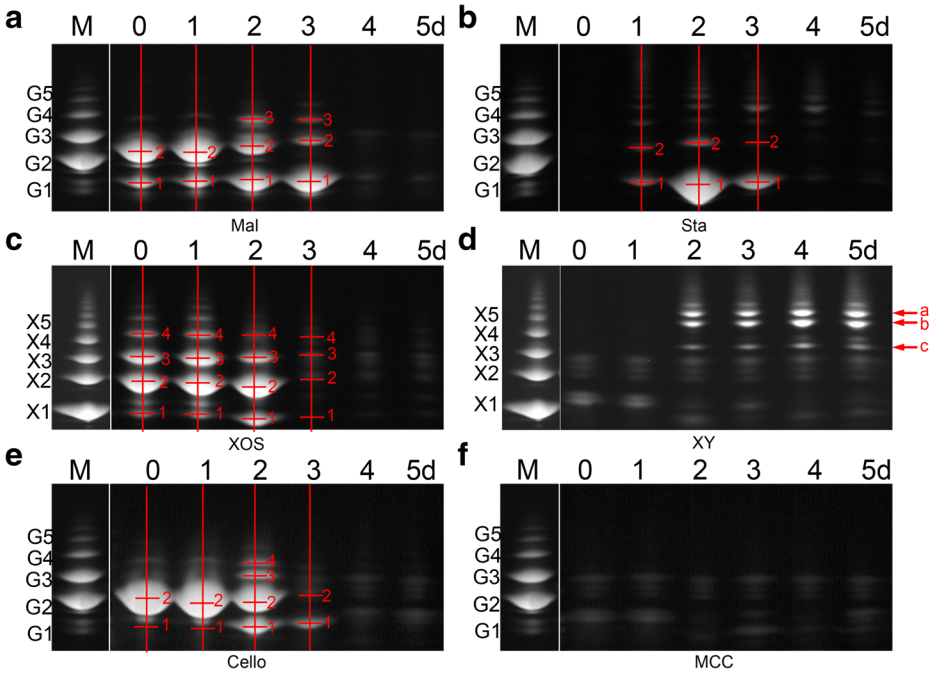

Fig. 2 Quantitative determination of extracellular reducing sugars by FACE. Carbon sources **a**–**f** represent maltose (Mal), starch (Sta), xylooligosaccharide (XOS), xylan (XY), cellobiose (Cello), and microcrystalline cellulose (MCC), respectively. Lane M represent standard marker. G1–G5 represent glucose, cellobiose, cellotriose, cellotetrose, and cellopentose, respectively. X1–X5 represent xylose, xylobiose, xylotriose, xylotetraose and xylopentaose, respectively. Numbers represent oligosaccharide bands. Red arrows represent substituted xylooligosaccharides (SXOS) that cannot be degraded by *T. lanuginosus* SD01

decreased from 94.6 ± 16.2 to 36.4 ± 3.4 as the AXH concentration increased, indicating that band b was degraded by AXH. Therefore, band b likely represents ara-substituted XOS. However, the intensity of band a did not change significantly as the concentration of AXH increased, suggesting that band a represented XOS species with more complicated side chains, such as acetyl and ferulic acid. These SXOS cannot be used by *T. lanuginosus* SD01 but they do have the potential for using as prebiotics and as inducers of enzyme secretion by other microorganisms such as *Paenibacillus* spp. and *Prevotella* spp.. [45, 46]

Analysis of Glycoside Hydrolases Secreted by *T. lanuginosus* SD01 Using Zymogram and LC-MS/MS

The genome of *T. lanuginosus* contains 61 glycoside hydrolase genes and five lytic polysaccharide monooxygenase (LPMO) genes, which is fewer than in *Aspergillus niger*, *Trichoderma reesei*, or *Penicillium oxlicum* (Tables S1, S2, and S3). Protein content measurements revealed that *T. lanuginosus* SD01 secreted few proteins and that the highest protein concentration was only 0.103 ± 0.003 mg/mL for XY (Fig. 1e). The protein species were relatively simple, and only six distinct protein bands were detected using SDS-PAGE (Fig. 3a). This is far fewer than in *A. niger, T. reesei*, or *P. oxalicum* [25].

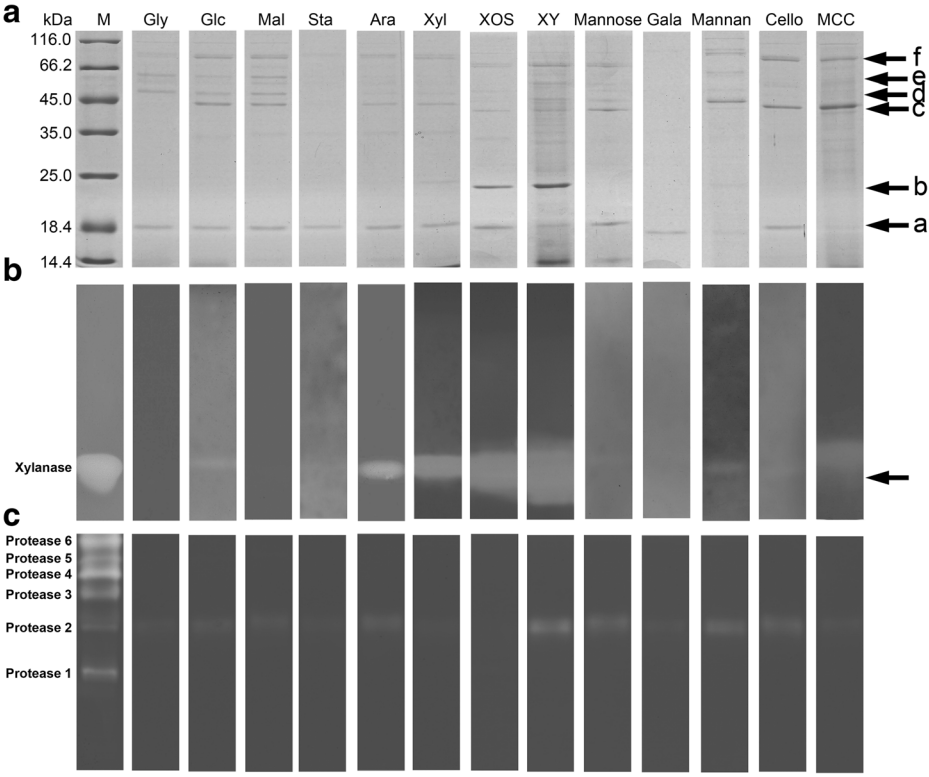

Fig. 3 Characterization of proteins, xylanase, and metalloprotease in the secretome of *T. lanuginosus* SD01 on day 4. **a** Proteins in the secretome of *T. lanuginosus* SD01 grown on different carbon sources assessed by SDS-PAGE. **b** Xylanase zymograms showing that xylanase was induced by different carbon sources. **c** Gelatin zymograms showing that metalloprotease was induced by different carbon sources

At least five protein bands including four bands with MWs above 45 kDa and one band with a MW of ~ 18 kDa were detected when *T. lanuginosus* SD01 was grown on almost all carbon sources. However, the type and concentration of proteins varied with the carbon source (Fig. 3a). Consistently, the highly expressed proteins (> 3%) in the secretome identified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) included four proteins with MWs between 45 and 120 kDa, one protein with a MW of approximately 25 kDa, and one protein with a MW of approximately 18 kDa (Fig. 4a). Classification of proteins involved in biomass degradation (Table 1) suggested that 13 proteins had a relative expression level above 1%, and 9 RSGs belonging to GH1, GH3, GH17, GH20, GH31, GH36, GH38, and GH64 families were highly expressed, including α-glucosidase (g5655.t1), β-hexosaminidase (g3593.t1), endo-β-1, 3-glucosidase (g4265.t1), β-1, 3-glucosidase (g330.t1), glucan endo-β-1, 3-glucosidase (g4137.t1), α-mannosidase (g2600.t1), α-galactosidase (g2448.t1), and β-Glucosidase (g2281.t1 and g850.t1). These results indicate that *T. lanuginosus* SD01 degrades soluble oligosaccharides into monosaccharides by secreting large numbers of RSGs, which may help the fungus to efficiently utilize oligosaccharides and oligopeptides in saprophytic habitats for their growth.

In contrast to the direct assimilation of monosaccharides, diverse enzymes were required for the degradation of Sta, mannan, and MCC. The relative expression level of amylases, including GH15 family glucoamylase (g1543.t1) and GH31 family α-glucosidase (g5655.t1), was generally

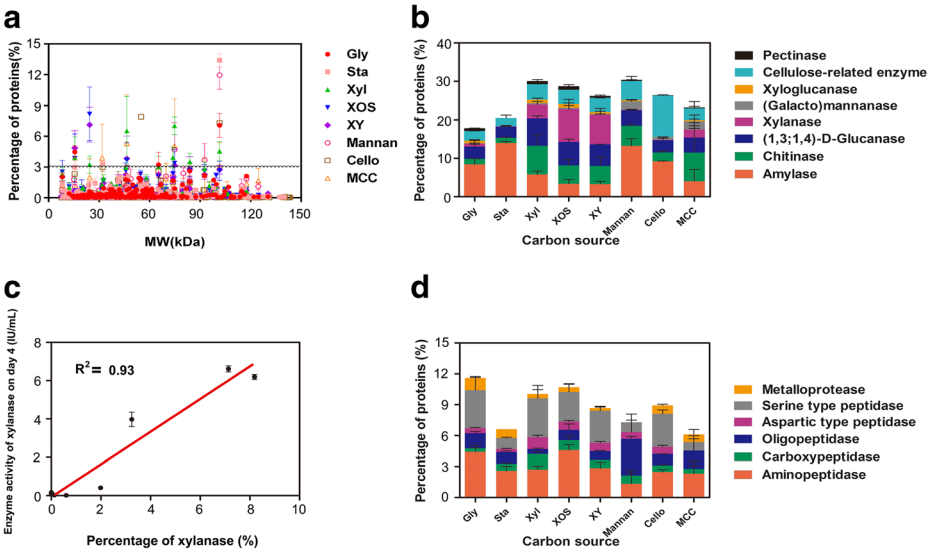

Fig. 4 Classification of proteins identified in the secretome of *T. lanuginosus* SD01 using LC-MS/MS. **a** Cluster analysis of all identified proteins based on MW and relative expression. **b** Relative expression level of biomass hydrolysis-related proteins when *T. lanuginosus* SD01 was grown on different carbon sources for 4 days. **c** Correlation analysis of xylanase relative expression and xylanase activity. **d** Relative expression of proteases when *T. lanuginosus* SD01 was grown on different carbon sources for 4 days

high (Fig. 4b) (Table 1). The highest expression of α-glucosidase (13.40 ± 0.64%) was detected on Sta (Table 1), indicating that *T. lanuginosus* SD01 could quickly utilize Sta. Similarly, chitinases and β-(1, 3; 1, 4)-D-glucanases were detected on all carbon sources (Fig. 3b), which may be related to the presence of yeast cell walls in the media. Chitinase and β-(1, 3; 1, 4)-D-glucanases belonged to GH18 and GH17 families, respectively. β-1, 3-glucosidases (g4265.t1 and g330.t1) were also annotated as glycosyltransferases (Table 1).

Mannan induced GH38 α-mannosidase (g2600.t1) and GH36 α-galactosidase (g2448.t1) but not main chain-degrading enzymes, indicating that *T. lanuginosus* SD01 was able to utilize mannan-oligosaccharides for growth but could not efficiently degrade complete mannan. In the genome of *T. lanuginosus*, no genes encoding cellulolytic enzymes are predicted but five LPMOs and eight β-glucosidase genes are annotated (Table S3). However, the relative expression of LPMOs was very low under all conditions and Cello mainly induced GH1 β-glucosidase (g2281.t1). These results indicate that *T. lanuginosus* SD01 can rapidly utilize Cello.

With respect to the degradation of XY, SDS-PAGE results indicated that a visible protein band (band b) with a MW of approximately 25 kDa was induced when Xyl, XOS, or XY were used as substrates (Fig. 3a). Similarly, only one xylanase band was detected on the xylanase zymogram (Fig. 3b). In addition, the MW of the only endo-β-1, 4-xylanase (g4601.t1) detected by LC-MS/MS was 24.4 kDa and its expression level was highly correlated with enzymatic activity on day 4 ($R^2 = 0.93$) (Fig. 4c). Therefore, the band b, shown in Fig. 2a, is endo-β-1, 4-xylanase, which may have good properties, such as thermostability, salt tolerance, and wide range of pH optima, as reported previously. [11] The expression levels of endo-β-1, 4-xylanase increased significantly on Xyl, XOS, and XY substrates and was highest on the XOS and XY substrate (8.19 ± 2.60% and 7.14 ± 1.71%) (Fig. 4b and Table 1), indicating that xylanase could be specifically induced by XOS and XY.

Table 1 Relative quantity of biomass degradating related proteins in the secretome of *T. lanuginosus* SD01

Substrate	Enzyme activity	CAZy family	Gene name	Accession number	MW (kDa)	Signal peptide	Percentage of secretome (%)	
							Gly	Sta
Amylase								
	Glucoamylase	GH15	g1543.t1	gi|61658242	66.6	Y	*1.27 ± 0.33*	0.56 ± 0.22
	α-glucosidase	GH31	g5655.t1	gi|170295863	101.6	Y	*7.08 ± 1.18*	*13.40 ± 0.64*
	α-glucosidase	GH31	g5709.t1	gi|74844417	108.6	Y	0.05 ± 0.01	
Chitinase								
	Class V chitinase ChiB1	GH18	g835.t1	gi|74663646	46.5	Y	0.75 ± 0.04	0.68 ± 0.16
	Chitinase	GH18	g5595.t1	gi|68989433	42.2	N	0.26 ± 0.02	0.38 ± 0.17
	Chitinase 3	GH18	g1072.t1	gi|1168933	63.8	Y	0.05 ± 0.01	
	β-hexosaminidase	GH20	g3593.t1	gi|47605402	69.76	Y	0.42 ± 0.07	0.26 ± 0.08
Hemicellulase								
(1,3;1,4)-β-D-Glucan	Exo-β-1,3-glucanase (Exg1)	GH5	g3796.t1	gi|242802088	46	Y	0.05 ± 0.01	0.05 ± 0.00
	Exo-β-1,3-glucanase	GH55	g5651.t1	gi|1352399	95.5	Y	0.28 ± 0.12	
	Endo-β-(1,3;1,4)-glucanase	GH16	g1129.t1	gi|238490310	58.2	N		0.23 ± 0.05
	Endo-β-1,3-glucanase	GH17	g5765.t1	gi|74673195	68.8	N		0.05 ± 0.00
	Endo-β-1,3-glucanase Engl1	GH81	g2486.t1	gi|159130650	106.1	Y		
	β-1,3-1,4-Glucanase	GH16	g2390.t1	gi|301070474	33.3	Y		
	Glucanase	GH5	g6172.t1	gi|70986924	54.3	N		
	Endo-β-1,3-glucosidase[a]	GH17	g4265.t1	gi|70985687	45.9	Y	*1.78 ± 0.58*	*1.91 ± 0.50*
	β-1,3-Glucosidase[a]	GH17	g330.t1	gi|557722836	32.6	Y	0.93 ± 0.012	0.72 ± 0.23
	Glucan endo-1,3-β-glucosidase	GH64	g4137.t1	gi|242776524	45	N	0.06 ± 0.02	
Xylan	Endo-β-1,4-xylanase	GH11	g4601.t1	gi|3915307	24.4	Y	0.60 ± 0.96	
	β-1,4- Xylosidase	GH43	g3706.t1	gi|321150563	38.1	N	0.16 ± 0.09	0.11 ± 0.05
(Galacto) mannan	Endo mannanase	GH76	g1864.t1	gi|395398579	52	Y		
	β-mannosidase B	GH2	g5848.t1	gi|597501007	97.8	N		0.08 ± 0.00
	β-mannosidase B-like protein	GH2	g2018.t1	gi|672796640	140.3	N		
	α-Mannosidase	GH38	g2600.t1	gi|119492509	124.7	N	0.10 ± 0.03	
	α-1,2-Mannosidase	GH47	g2295.t1	gi|242778318	58.1	Y		
		GH92	g1159.t1	gi|242775262	84.5	Y		
	α-Galactosidase	GH36	g2448.t1	gi|67902302	82.1	Y		
Xyloglucan	β-Galactosidase	GH35	g62.t1	gi|32448796	104.7	Y	0.66 ± 0.098	

Table 1 (continued)

Substrate	Enzyme activity	CAZy family	Gene name	Accession number	MW (kDa)	Signal peptide	Percentage of secretome (%)	
							Gly	Sta
	α-1,6-Xylosidase	GH31	g2019.t1	gi|119480271	85	N		
Cellulose-related enzyme	LPMO	AA9	g1217.t1	gi|599157709	36.2	Y	0.14 ± 0.05	0.26 ± 0.03
		AA9	g721.t1	gi|67526107	29.2	Y	0.13 ± 0.03	0.05 ± 0.00
		AA9	g3708.t1	gi|21263647	100.7	Y		
		AA9	g1505.t1	gi|67526107	28.9	Y		
		AA10	g4320.t1	gi|70986442	23	Y		
	β-Glucosidase	GH1	g2281.t1	gi|303387574	55	N		*1.23 ± 0.00*
		GH3	g850.t1	gi|304651073	92.7	Y	*2.17 ± 0.25*	0.30 ± 0.06
		GH3	g3163.t1	gi|662522738	90.8	Y		
		GH3	g1554.t1	gi|212535192	92.1	N		
		GH17	g3626.t1	gi|121805794	55	Y		0.76 ± 0.04
Pectinase								
Polygalacturonan	Endopolygalacturonase	GH28	g291.t1	gi|67902680	39.1	Y	0.08 ± 0.00	
Rhamnogalacturon	β-Galactosidase	GH35	g62.t1	gi|32448796	104.7	N	0.66 ± 0.10	

Substrate	Percentage of secretome (%)					
	Xyl	XOS	XY	Mannan	Cello	MCC
Amylase						
	1.19 ± 0.43	0.69 ± 0.32	0.55 ± 0.28	*1.31 ± 0.55*	*1.67 ± 0.28*	
	4.56 ± 0.93	*2.65 ± 0.95*	*2.75 ± 0.43*	*11.96 ± 1.39*	*7.32 ± 0.20*	*3.11 ± 2.29*
					0.21 ± 0.04	
Chitinase						
	6.55 ± 3.34	*3.70 ± 1.68*	*3.84 ± 2.19*	*2.97 ± 0.22*	*1.22 ± 0.08*	*5.29 ± 4.76*
	0.11 ± 0.04	0.23 ± 0.06	0.14 ± 0.05		0.22 ± 0.06	0.19 ± 0.12
	0.12 ± 0.05		0.08 ± 0.02		0.1 ± 0.03	0.08 ± 0.07
	0.73 ± 0.44	0.83 ± 0.48	0.66 ± 0.05	*2.10 ± 0.10*	0.79 ± 0.20	*1.88 ± 1.49*
Hemicellulase						
(1,3;1,4)-β-D-Glucan					0.11 ± 0.04	

Table 1 (continued)

Substrate	Percentage of secretome (%)					
	Xyl	XOS	XY	Mannan	Cello	MCC
	1.19 ± 0.38	0.81 ± 0.23	0.98 ± 0.24	0.62 ± 0.09	0.09 ± 0.02	0.82 ± 0.70
	0.2 ± 0.08	0.16 ± 0.04	0.21 ± 0.00	0.13 ± 0.01	0.25 ± 0.03	
					0.05 ± 0.01	
	0.46 ± 0.14	0.18 ± 0.07	0.16 ± 0.04	0.56 ± 0.48		0.84 ± 0.7335757
	0.24 ± 0.06	0.12 ± 0.02	0.18 ± 0.08	0.42 ± 0.01		
		0.34 ± 0.25	0.07 ± 0.01			
	2.95 ± 0.08	*2.65 ± 0.76*	*2.25 ± 0.24*	*1.09 ± 0.17*	*1.87 ± 0.44*	0.52 ± 0.38
	1.89 ± 0.38	*1.71 ± 0.13*	*1.44 ± 0.01*	0.45 ± 0.16	0.82 ± 0.10	0.12 ± 0.06
	0.40 ± 0.22	0.26 ± 0.14	0.36 ± 0.05	0.97 ± 0.20		*1.21 ± 0.93*
Xylan	*3.24 ± 0.84*	*8.19 ± 2.60*	*7.14 ± 1.71*	0.19 ± 0.07		*1.99 ± 1.81*
	0.40 ± 0.06	0.35 ± 0.03	0.59 ± 0.02		0.07 ± 0.02	0.09 ± 0.04
(Galacto) mannan					0.07 ± 0.02	
					0.43 ± 0.03	
					0.11 ± 0.00	
		0.26 ± 0.18	0.12 ± 0.02	*1.12 ± 0.37*	0.09 ± 0.00	*1.65 ± 1.22*
			0.07 ± 0.01	0.17 ± 0.06		
					0.02 ± 0.00	
				1.00 ± 0.03	0.03 ± 0.00	0.09 ± 0.07
Xyloglucan	0.8 ± 0.31	0.80 ± 0.32	0.40 ± 0.25	0.31 ± 0.02	0.11 ± 0.00	
		0.19 ± 0.13	0.07 ± 0.01			
Cellulose-related enzyme	0.22 ± 0.12	0.23 ± 0.14	0.25 ± 0.00		0.34 ± 0.03	
	0.25 ± 0.06	0.15 ± 0.04	0.14 ± 0.05		0.19 ± 0.01	
					0.02 ± 0.00	
		0.25 ± 0.02	0.18 ± 0.08		0.13 ± 0.05	
	0.16 ± 0.06	0.10 ± 0.01	0.17 ± 0.02	0.32 ± 0.11		
		0.12 ± 0.02			*7.9 ± 0.19*	
	2.81 ± 0.68	*2.63 ± 0.62*	*2.27 ± 0.47*	*3.69 ± 1.62*	0.75 ± 0.24	*2.28 ± 1.66*
	0.17 ± 0.04	0.26 ± 0.11	0.21 ± 0.12			0.18 ± 0.15
					0.76 ± 0.14	
	0.35 ± 0.32	0.34 ± 0.01	0.54 ± 0.40	0.87 ± 0.45	0.65 ± 0.03	0.31 ± 0.24

Table 1 (continued)

Substrate	Percentage of secretome (%)					
	Xyl	XOS	XY	Mannan	Cello	MCC
Pectinase						
Polygalacturonan	0.11 ± 0.05	0.19 ± 0.01	0.07 ± 0.00			
Rhamnogalacturon	0.80 ± 0.31	0.80 ± 0.32	0.40 ± 0.25	0.31 ± 0.02	0.11 ± 0.00	

Italic entries represent quantitative values that are higher than 1%

[a] Proteins with glycosyltransferase activity by GO functional annotation

Table 2 Relative quantity of proteases in the secretome of *T. lanuginosus* SD01

Substrate	Enzyme activity	CAZy family	Gene name	Accession number	MW (kDa)	Signal peptide	Percentage of secretome (%)	
							Gly	Sta
Exopeptidase								
	Aminopeptidase							
	Zinc metalloprotease	M1	g194.t1	gi|342165089	98.03	N	*1.73 ± 0.16*	*1.00 ± 0.14*
	Aminopeptidase	M1	g2981.t1	gi|212540596	99.51	N	*1.33 ± 0.14*	0.44 ± 0.02
	Aspartyl aminopeptidase	M18	g2612.t1	gi|121805180	51.35	N	0.36 ± 0.012	0.05 ± 0
	Aminopeptidase	M24B	g2684.t1	gi|212535482	68.05	N		
	Prolidase pepP	M24B	g5234.t1	gi|242775923	52.27	N	0.17 ± 0.098	0.21 ± 0.02
	Aminopeptidase	M28	g1799.t1	gi|24279473	42.32	Y	*0.52 ± 0.13*	*0.89 ± 0.29*
	Pheromone maturation dipeptidyl aminopeptidase DapB	S9B	g424.t1	gi|212546617	99.01	N	0.31 ± 0.07	
	Carboxypeptidase							
	Glutamate carboxypeptidase Tre2	M28	g826.t1	gi|242780537	98.89	N		
	Glutamate carboxypeptidase	M28	g3667.t1	gi|242802192	85.51	N		
	Carboxypeptidase CpyA/Prc1	S10	g5392.t1	gi|121700286	61.34	Y	0.05 ± 0	
	Carboxypeptidase S1	S10	g1739.t1	gi|212535660	152.76	Y	0.46 ± 0.00	*0.68 ± 0.07*
	Pheromone processing carboxypeptidase Kex1	S10	g1953.t1	gi|599153237	92.9841356	Y		
	Oligopeptidase							
	Dipeptidyl peptidase	M49	g5149.t1	gi|242765819	79.43	N	*1.29 ± 0.16*	*1.14 ± 0.12*
	Dipeptidyl-peptidase 5	S9C	g2769.t1	gi|42558924	80.08	Y	0.18 ± 0.012	
	Serine protease	S28	g3330.t1	gi|13638618	90.60	Y		
Endopeptidase								
	Aspartic-type peptidase							
	Aspartic-type endopeptidase opsB	A1	g4767.t1	gi|169776867	55.93	Y		0.05 ± 0
	Aspartic-type endopeptidase (CtsD)	A1	g4472.t1	gi|242766368	51.27	N		
	Aspartic endopeptidase Pep1	A1	g2765.t1	gi|119478346	42.93	Y	0.47 ± 0.09	0.26 ± 0.03
	Yapsin	A1	g1092.t1	gi|212540820	52.43	Y		
	Serine type peptidase							
	Serine protease	S8	g5703.t1	gi|290749783	48.40	Y	0.19 ± 0.01	0.22 ± 0.06
	Tripeptidyl peptidase SED3	S53	g1667.t1	gi|320038277	65.30	Y	*3.23 ± 1.22*	*0.84 ± 0.03*
	Tripeptidyl-peptidase 1	S53	g2105.t1	gi|389640755	67.17	N	0.29 ± 0.02	
	Metalloprotease							

Table 2 (continued)

Substrate	Enzyme activity	CAZy family	Gene name	Accession number	MW (kDa)	Signal peptide	Percentage of secretome (%)	
							Gly	Sta
	Metallopeptidase MepB	M3	g4625.t1	gi|212543933	81.82	N	*0.89 ± 0.16*	*0.50 ± 0.02*
	ADAM family of metalloprotease ADM-B	M12B	g4813.t1	gi|242769596	84.5907482	Y		0.05 ± 0.00
	Peptidase	M20	g5001.t1	gi|358373474	47.86	Y	0.29 ± 0.012	0.34 ± 0.01

Substrate	Percentage of secretome (%)					
	Xyl	XOS	XY	Mannan	Cello	MCC
Exopeptidase						
	0.6 ± 0.17	*1.14 ± 0.20*	*0.86 ± 0.28*		*0.97 ± 0.21*	0.55 ± 0.18
	1.08 ± 0.39	*2.20 ± 0.26*	*0.99 ± 0.20*	0.38 ± 0.25	0.44 ± 0.10	0.63 ± 0.36
		0.33 ± 0.28	0.19 ± 0.03	*0.82 ± 0.01*	0.13 ± 0.03	0.67 ± 0.41
			0.07 ± 0.00			
		0.19 ± 0.08			0.19 ± 0.01	0.12 ± 0.11
	0.54 ± 0.16	*0.51 ± 0.10*	*0.53 ± 0.07*	*0.53 ± 0.28*	*0.60 ± 0.17*	0.07 ± 0.02
	0.48 ± 0.14	0.38 ± 0.10	0.21 ± 0.12		0.14 ± 0.01	0.27 ± 0.15
						0.09 ± 0.01
					0.02 ± 0.00	
			0.07 ± 0.00		0.04 ± 0.04	
	1.52 ± 0.25	*0.98 ± 0.37*	*0.76 ± 0.03*	*0.80 ± 0.51*	*0.54 ± 0.06*	0.36 ± 0.31
					0.05 ± 0.00	
	0.48 ± 0.08	*1.01 ± 0.57*	*0.67 ± 0.12*	*1.02 ± 0.30*	*0.83 ± 0.13*	0.54 ± 0.42
			0.28 ± 0.00	*2.87 ± 0.69*	0.21 ± 0.09	*1.24 ± 0.74*
					0.08 ± 0.03	
Endopeptidase						

Table 2 (continued)

Substrate	Percentage of secretome (%)					
	Xyl	XOS	XY	Mannan	Cello	MCC
		0.07 ± 0.03			0.17 ± 0.04	
					0.08 ± 0.01	
	1.13 ± 0.21	*0.74 ± 0.16*	*0.78 ± 0.14*		0.36 ± 0.03	
					0.07 ± 0.08	
	0.37 ± 0.10	0.29 ± 0.15	0.38 ± 0.10	0.47 ± 0.07	0.19 ± 0.03	0.27 ± 0.18
	3.02 ± 1.06	*2.09 ± 0.77*	*2.29 ± 0.06*	*1.13 ± 0.33*	*2.65 ± 0.33*	0.54 ± 0.46
	0.39 ± 0.15	0.51 ± 0.16	0.46 ± 0.20		0.36 ± 0.03	
	0.27 ± 0.21	0.22 ± 0.19	0.14 ± 0.05		*0.60 ± 0.11*	0.59 ± 0.38
		0.09 ± 0.06			0.03 ± 0.00	
	0.25 ± 0.18	0.27 ± 0.08	0.14 ± 0.12		0.19 ± 0.02	0.18 ± 0.10

Italic entries represent quantitative values that higher than 0.5%

Analysis of Proteases Secreted by *T. lanuginosus* SD01 by Zymogram and LC-MS/MS

T. lanuginosus SD01 utilizes organic nitrogen sources in the environment mainly by secreting proteases. In the genome of *T. lanuginosus*, 50 proteases, including 23 exopeptidases (nine aminopeptidases, seven carboxypeptidases, and seven oligopeptidases) and 27 endopeptidases (seven aspartic proteases, three serine proteases, five cysteine proteases, and 12 metalloproteases) have been annotated (Table S4). In the secretome of *T. lanuginosus* SD01, 25 proteases were detected, including 15 exopeptidases (seven aminopeptidases, five carboxypeptidases, and three oligopeptidases) and 10 endopeptidases (four aspartic proteases, three serine proteases, and three metal proteases) (Table 2). With regard to relative expression levels (Fig. 4d), exopeptidase expression was higher than endopeptidases. Most endopeptidases belonged to neutral or alkaline serine proteases and this was likely due to the neutral or alkaline saprophytic environment. In addition, the number and relative expression level of several acidic proteases, including aspartic-type proteases, were generally < 0.5% (except xyl, XOS, and XY).

Among the total proteases, the expression level of the serine endopeptidase S53 tripeptidyl peptidase SED3 (g1667.t1) was highest. This enzyme may degrade protein into oligopeptide fragments but its function requires further research. Although metalloproteinases are able to degrade gelatin, only one obvious band was detected on the gelatin zymogram (Fig. 3c). Among the highly expressed proteases, two M1 proteases (g2981.t1 and g194.t1) with similar molecular weights (99.5 and 98.0 kDa) were detected by LC-MS/MS (Table 2). Thus, it is possible that the protease band in the gelatin zymogram may represent the M1 metalloproteases g2981.t1 and g194.t1. The relative expression levels of 10 proteases were higher than 0.5%, and seven of these were exopeptidases containing M1, M18, and M28 aminopeptidases; S10 carboxypeptidase; and M49 and S9C oligopeptidases. The high expression level of exopeptidases suggests that *T. lanuginosus* SD01 is able to degrade oligopeptide fragments produced by other microorganisms in saprophytic habitats.

Analysis of Proteins Involved in Intracellular Metabolic Pathways by LC-MS/MS

Carbon sources transported intracellularly mainly metabolized via energy metabolic pathways. Hexoses can be directly metabolized by the glucolysis pathway (EMP), whereas pentoses are preferentially metabolized by the pentose phosphate pathway (PPP) with its metabolic intermediates being further metabolized by the glucolysis pathway and the tricarboxylic acid cycle (TCA) (Fig. 5). [47] ANOVA tests indicated that there were no significant differences in the levels of EMP, TCA, or PPP pathway proteins of *T. lanuginosus* SD01 grown on different carbon sources ($p = 0.4080$, $p = 0.5024$, and $p = 0.9890$, respectively; Table S5), which may maintain a stabilized intracellular metabolism for their quick proliferation. Similar results have been reported from intracellular proteomic analyses of *A. niger* and *T. reesei* (Fig. 5). [47–49]

Discussion

Environment-friendly and efficient industrial biotechnology has attracted widespread attention. Thermophiles have beneficial properties for widespread use in industrial biotechnology. [2, 6–8] Because the genome of *T. lanuginosus* is small and the extracellular enzymes are simple, these conditions are favorable for genetic manipulation. And *T. lanuginosus* can utilize XY

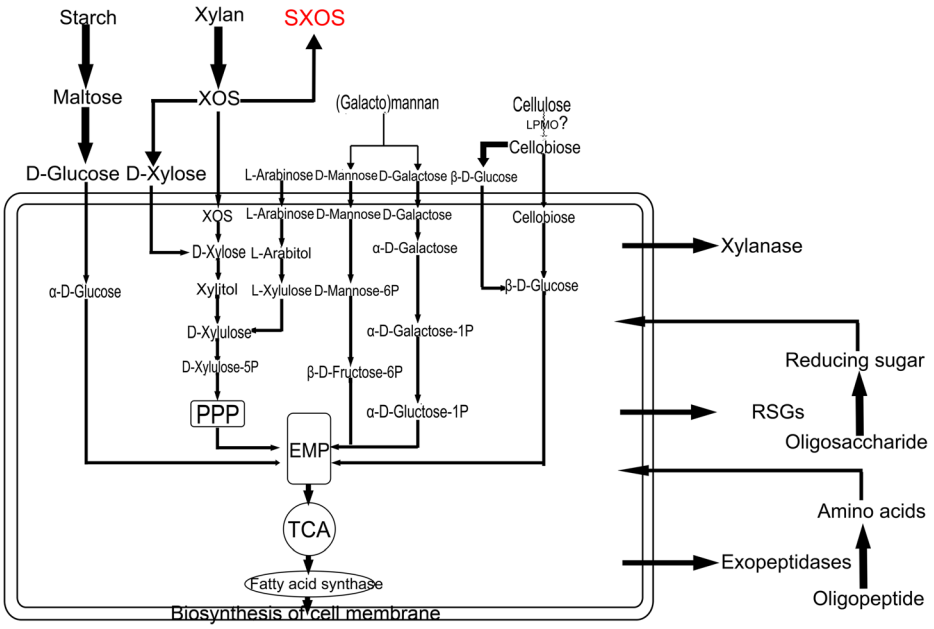

Fig. 5 Schematic diagram representing the degradation properties of different carbon sources and intracellular metabolism. Solid arrows indicate that the reaction can proceed for *T. lanuginosus* SD01. Dotted arrows indicate that the reaction could not proceed for *T. lanuginosus* SD01. Thick lines indicate the preferential substrates of *T. lanuginosus* SD01

from lignocellulose, thus, *T. lanuginosus* has the potential to be used as a new expression host which utilize cheap biomass materials producing valuable proteins. [11]

Secretome analysis suggested that *T. lanuginosus* SD01 secretes mainly GH1, GH3, GH17, GH20, GH31, GH36, GH38, and GH64 family RSGs and that these RSGs are induced starting on day 2. In addition, seven exopeptidases, belonging to M1, M18, M28, M49, S9C, and S10 families, were efficiently induced. The high expression levels of RSGs and exopeptidases, as well as the stability of intracellular metabolism, may help *T. lanuginosus* SD01 effectively degradate oligosaccharides and oligopeptides and dominate saprophytic habitats at high temperatures. And these thermophilic RSGs and exopeptidases could be widely applied in different fields, such as biomass hydrolysis, food processing, and medicine.

When XY was the carbon source, only one thermostable xylanase (g4601.t1) was secreted by *T. lanuginosus* SD01. The optimal reaction temperature of this xylanase was 70 °C (Fig. S2.). Our previous research and other previous studies have demonstrated the thermophilic nature of GH11 xylanase from *T. lanuginosus*. [50–52] This enzyme has widespread applications in winemaking and biomass degradation. Because *T. lanuginosus* SD01 does not secrete cellulases, crude enzymes could also reduce the pulp fiber loss and application costs of pulp bleaching. [53]

In the proteome of *T. lanuginosus* SD1, only xylan backbone degradation enzymes including one xylanase and xylosidase were detected and no genes were predicted to encode α-Arabinfuranosidease; therefore, xylan could just be degraded into SXOS with diverse side chains decorated xylan backbone, and the molecular weights of SXOS released by *T. lanuginosus* SD1 were between tetraose and hexaose. SXOS are functional sugars that are beneficial for health and can be used as prebiotics. It was previously reported that SXOS

intake of 2 to10 g per day lowers cholesterol and glucose levels in blood and improves immunity. Several previous studies have proved the advantage of XOS as an important prebiotics in promoting the healthy functioning of the intestinal system and regulating the content of blood cholesterol and glucose. [43, 54, 55] In addition, our research group has revealed that XOS was responsible for improving xylanase activity and shortening xylanase-production time in *A. niger*, [56] thus, we speculated the huge potential of SXOS as probiotics and enzyme inducers. What is more, XOS is developed as probiotics, mainly because that XOS cannot be degraded into monosaccharide and absorbed by the human body, and the SXOS produced by *T. lanuginosus* SD1 contains diverse side chains, which was more difficult to be degraded and absorbed by the human body in comparison with XOS without side chains. Thus, the SXOS produced by *T. lanuginosus* SD1 was more competitive as prebiotics. Moreover, SXOS are important food thickeners and are commonly used as bread making additives. [57] In industry, SXOS are typically prepared by hydrolyzing raw materials containing abundant XY, such as sugar cane bagasse, corncobs, rice husks, and wheat straw, with acid, alkaline, or enzymes. [43] However, the large amounts of monosaccharides and toxic complexes produced by the hydrolysates requires further separation and purification, which results in the loss of XOS and an increase in cost. When *T. lanuginosus* SD01 was cultured in 1% XY (*w*/*v*) medium for 3 days, 0.82 ± 0.01 mg/mL reducing sugars were detected in the media (Fig. 1c). Because unsubstituted XOS could be rapidly taken up, the reducing sugars detected in the media were SXOS. Therefore, *T. lanuginosus* SD01 could produce about 8.2% SXOS when cultured in corncob XY for 3 days. In the future, higher yields of SXOS could be achieved by further optimizing the culture conditions. The XY content in corncobs is very abundant, reaching up to 35−40%, [58]; thus, it is possible to prepare SXOS using corncobs by *T. lanuginosus* SD01.

Conclusions

In this research, an integrated functional-omics technology was established to systematically analyze the composition of extracellular enzymes when *T. lanuginosus* SD01 was grown on different carbon sources, and explore the potential for industrial applications. *T. lanuginosus* SD01 secretes abundant RSGs and exopeptidases in order to efficiently utilize readily available oligosaccharides and oligopeptides, allowing the fungus to quickly dominate saprophytic habitats at high temperatures. Additionally, xylanase secreted by *T. lanuginosus* SD01 can degrade the XY backbone into available sugars to support mycelia growth and xylanase crude enzyme can be directly used for pulp bleaching. Corncob XY can be converted into SXOS by *T. lanuginosus* SD01, and this feature could be used to produce high-value SXOS using XY-rich corncobs. *T. lanuginosus* SD01 has the potential to be used to produce thermostable xylanase and SXOS that can be easily separated by membranes in industrial production, while the mycelia can be used as microbial inoculants to accelerate maize straw processing. *T. lanuginosus* SD01 demonstrates clear potential in transforming inexpensive raw materials into high-value products through batch fermentation for industrial production.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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