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Penicillium subrubescens adapts its enzyme production to the composition of plant biomass

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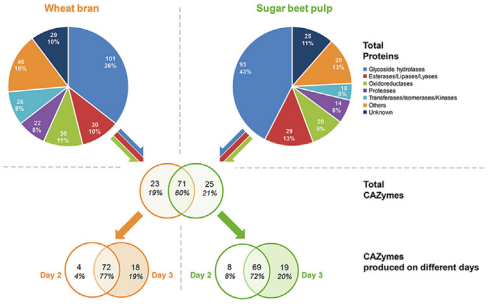
GRAPHICAL ABSTRACT



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ABSTRACT

Penicillium subrubescens is able to degrade a broad range of plant biomass and it has an expanded set of Carbohydrate Active enzyme (CAZyme)-encoding genes in comparison to other *Penicillium* species. Here we used exoproteome and transcriptome analysis to demonstrate the versatile plant biomass degradation mechanism by *P. subrubescens* during growth on wheat bran and sugar beet pulp. On wheat bran *P. subrubescens* degraded xylan main chain and side residues from Day 2 of cultivation, whereas it started to degrade side chains of pectin in sugar beet pulp prior to attacking the main chain on Day 3. In addition, on Day 3 the cellulolytic enzymes were highly increased. Our results confirm that *P. subrubescens* adapts its enzyme production to the available plant biomass and is a promising new fungal cell factory for the production of CAZymes.

1. Introduction

Plant biomass is one of the most abundant, valuable and sustainable polymeric substrates, and is currently the most suitable resource for renewable bioenergy and biochemical production. Plant biomass consists mainly of lignocellulose that is rich in polysaccharides (cellulose, hemicelluloses and pectin) and lignin (complex aromatic polymer) (Aguilar et al., 2013; Sánchez & Cardona, 2008). To be able to efficiently use such complex material, it needs to be degraded and/or modified. Different methods have been developed for the conversion of lignocellulosic biomass. However, the most environmentally sustainable way relies on the use of enzymes. An assortment of carbohydrate-

Abbreviations: CAZy, Carbohydrate Active enzyme; GH, glycoside hydrolase; CE, carbohydrate esterase

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active and lignin-modifying enzymes is required to efficiently and completely depolymerize lignocellulosic biomass. Filamentous fungi are excellent producers of plant cell wall degrading enzymes, and some species can also fine-tune their enzyme production specifically to the target biomass (Mäkelä et al., 2014; Rytioja et al., 2014; van den Brink & de Vries, 2011). Fungal enzymes have been used in a variety of industries, e.g. biofuels, biochemicals, food, feed, pulp and paper, textile, pharmaceutical and cosmetic (Himmel et al., 2007). However, only a handful of species have been developed as fungal cell factories, e.g. *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei* and *Myceliophthora thermophila*. This is partly due to the narrow substrate range, low enzyme production level, low secretion capacity, toxin production, and poor growth behavior under standard fermentation conditions that makes many other fungi unattractive for industrial applications (Benocci et al., 2017).

Penicillium subrubescens is a saprobic species belonging to section *Lanata-diversicata* of the genus *Penicillium*, which has several distinctive morphological features (Mansouri et al., 2013). The species showed significant promise as its plant cell wall-degrading enzyme production levels and saccharification abilities were similar to that of the industrial species *A. niger* (Mäkelä et al., 2016). Recently, genomic analysis of *P. subrubescens* revealed an expanded set of Carbohydrate Active enzyme (CAZyme)-encoding genes in comparison with related *Penicillium* species, but more similar in general numbers to *A. niger* and other aspergilli (Peng et al., 2017). This expansion does not occur across all CAZy families, but is rather specific to hemicellulose, pectin and inulin degrading enzymes, occurring particularly in glycoside hydrolase (GH) family GH1, GH11, GH12, GH29, GH32, GH36, GH43, GH51, GH54, GH62 and GH67, as well as carbohydrate esterase (CE) family CE8 (Peng et al., 2017).

Understanding how CAZymes are produced by *P. subrubescens* during growth on different plant biomass is critical to develop this fungus as a novel fungal cell factory. Previously, we reported the extracellular enzyme activity and saccharification abilities of *P. subrubescens* cultures grown on wheat bran and sugar beet pulp (Mäkelä et al., 2016). These two feedstocks differ in their carbohydrate composition. The major polysaccharides present in wheat bran are cellulose and (arabino)xylan, whereas those in sugar beet pulp are cellulose, pectin and xyloglucan (Benoit et al., 2015). To gain insight into how *P. subrubescens* degrades plant biomass, we report a comparative exoproteome and transcriptome analysis of *P. subrubescens* cultures grown on wheat bran and sugar beet pulp in this study.

2. Materials and methods

2.1. Fungal culture conditions

To prepare the substrates for cultivation, wheat bran and sugar beet pulp were ground using Ultra Centrifugal Mill ZM 200 with 12-tooth-rotor (RETSCH GmbH, Haan, Germany). The final particle size is less than 40 μm. *Penicillium subrubescens* FBCC1632/CBS132785 was cultivated (10^6 spore/mL) in minimal medium (de Vries et al., 2004) supplemented with 1% wheat bran or 1% sugar beet pulp at 25 °C, 250 rpm. The culture broth was collected every 24 h over the course of three days and centrifuged at 14,000 rpm, 4 °C, 5–10 min, and stored at −20 °C prior to trichloroacetic acid (TCA)/acetone protein precipitation for exoproteome analysis. For transcriptomic analysis, the 3-day old mycelium was harvested as described in Dilokpimol et al., 2017, prior to RNA extraction.

2.2. RNA extraction and sequencing

2.2.1. RNA extraction

RNA was extracted from 3-day old cultures grown on wheat bran or sugar beet pulp as previously described in Peng et al., 2017, using TRIzol reagent (Invitrogen/Thermo Fischer Scientific, Carlsbad, CA) and purified by NucleoSpin RNAII (Macherey-Nagel, Düren, Germany). The quality of RNA samples was verified using Fragment Analyser (Advanced Analytical Technologies, Thermo Fisher Scientific, Ankeny, IA).

2.2.2. RNA sequencing

RNA sequencing was performed by GenomeScan (Leiden, the Netherlands) using the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA). The Illumina data analysis pipeline RTA v1.18.64 and Bcl2fastq v1.8.4 were used for image analysis, base calling, and quality check. FASTQFilter v2.05 (in-house tool, GenomeScan) was used for adapter trimming and quality filtering. Approximately 40 million reads of 125 bp per sample on average were obtained after data filtering. 92.5% of the filtered reads mapped to the genome of *Penicillium subrubescens* FBCC1632/CBS132785 from DOE Joint Genome Institute (JGI) (https://mycocosm.jgi.doe.gov/Pensub1/Pensub1.home.html) (Peng et al., 2017) using Bowtie2 (Langmead et al., 2009). The gene expression level was measured and normalized as fragments per kilo base of transcript per million mapped reads (FPKM) (Trapnell et al., 2010). DESeq2 was used for transcript differential expressions (Love et al., 2014).

2.3. Exoproteome analysis by mass spectrometry

2.3.1. Trichloroacetic acid/acetone protein precipitation

All steps were performed at 4 °C unless specified otherwise. Two mL of culture broth was centrifuged at 2500 × g for 30 min. The supernatant was collected by centrifugation 37500 × g for 1 h. Two volumes of ice cold 20 mM dithiothreitol (DTT) in 20% TCA/80% acetone were mixed with the supernatant. The mixture was left on ice for 1 h prior to centrifugation at 3200 × g for 30 min. The precipitated protein pellet was washed with 15 mL iced cold 20 mM DTT in 80% acetone and incubated at −20 °C overnight. The solution mixture was centrifuged at 3200 × g for 30 min. The pellet was resuspended in 100 μL 0.25% Anionic Acid Labile Surfactant (AALS) (Protea Biosciences, Morgantown, WV) in 200 mM ammonium bicarbonate pH 7.8. The protein concentration was determined using the RCDC Protein Assay (Bio-Rad, Hercules, CA).

2.3.2. Trypsin digestion and LC-MSMS analysis

Five μg of total protein were digested with trypsin in-solution and analyzed by high pressure liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (Ozturkoglu Budak et al., 2014). Five μL of resuspended peptides spiked with trypsin digested bovine serum albumin (BSA) at a concentration of 4 μmol/μL as internal standard were injected into a 10 mm × 0.75 μm AQUASIL C18 microcapillary column coupled with a Velos LTQ-Orbitrap mass spectrometer (Thermo-Fisher, San Jose, CA). Proteome Discoverer 1.4 software using the precursor ion quantification workflow with precursor ion peak area value-based protein abundance calculation was used to identify proteins and peptides. MS/MS data was matched to the *P. subrubescens* FBCC1632/CBS132785 protein sequence database from the Joint Genome Institute. BSA was used as an internal standard as a mean to normalize the detected signals of the different injections. The area values of individual proteins were determined from BSA internal standard and expressed as a fold value. Functional annotations were assigned based on the IPR descriptions and the signal peptide was predicted using SignalP from JGI.

2.4. Orthologous mapping

Orthologous mapping was performed as described in Peng et al., 2018.

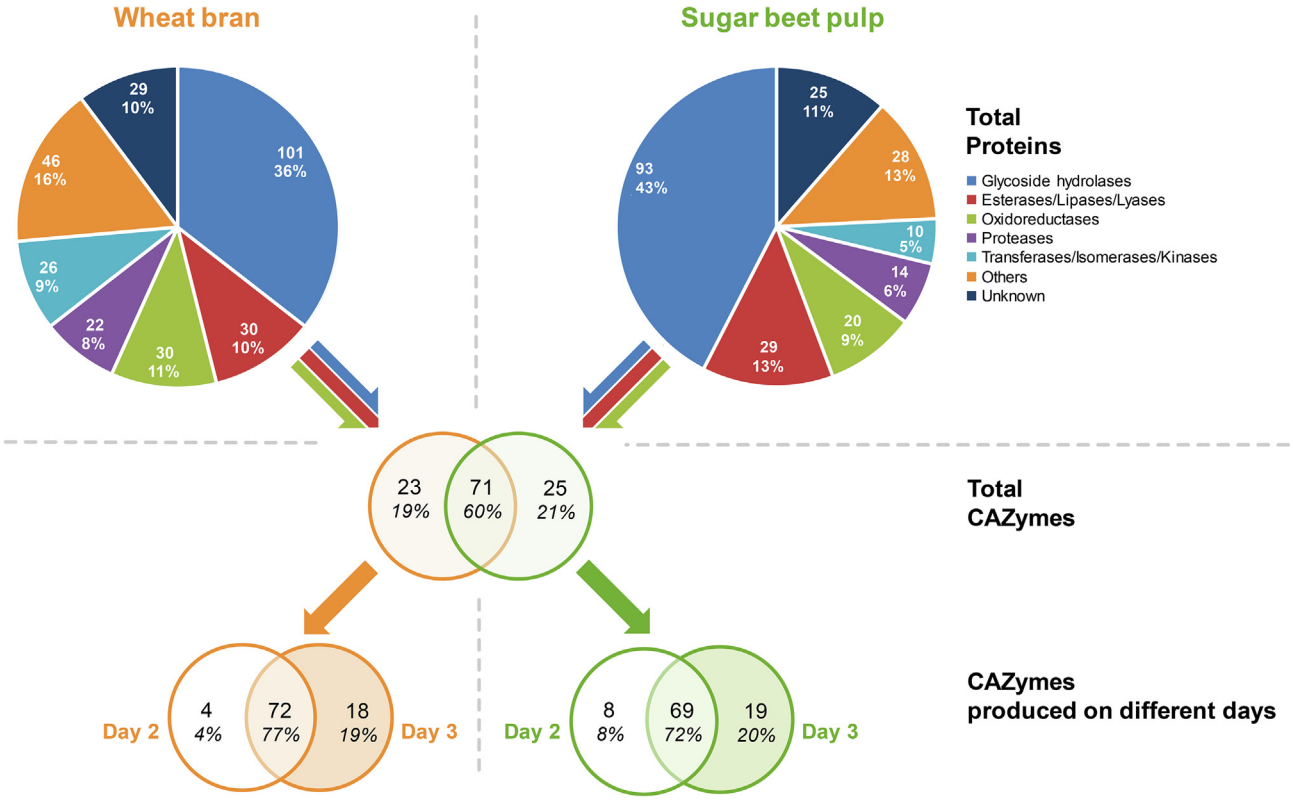

Fig. 1. Distribution of extracellular proteins showing an overlap as well as differential protein secretion by *P. subrubescens* grown on wheat bran or sugar beet pulp. Numbers in the diagram represent amount and percentage of proteins/enzymes detected in each category.

3. Results and discussion

3.1. CAZymes are the main extracellular enzymes produced by P. subrubescens grown on biomass

Identification of extracellular proteins during the growth of *P. subrubescens* on different biomass offered a detailed insight into the secreted plant biomass degrading enzymes by this fungus as well as its potential degradation mechanism. The culture broths from *P. subrubescens* grown in wheat bran or sugar beet pulp medium for two and three days were filtered and the supernatants were collected. The samples from the first day were not included for proteomic analysis, because the protein amount was too low for a reliable analysis. In total, 321 proteins were detected in the extracellular proteome, of which 254 and 194 were detected in cultures grown in wheat bran and sugar beet pulp, respectively (Fig. 1). Functional classification of the identified proteins showed that glycoside hydrolases (GHs) were the major secreted enzymes (36% and 43%, respectively) on either substrate. Together with esterases, lipases, lyases and oxidoreductases they made up over 50% of all detected proteins. Proteases, transferases, isomerases and kinases were minor components (< 20%). The remaining proteins were other functions and proteins of unknown function.

CAZymes play a major role in plant biomass degradation, and on either substrate approximately 100 CAZymes were secreted by *P. subrubescens* covering 40 CAZy families, but specific sets of enzymes were produced on each biomass (Fig. 2). For simplicity, we grouped the main enzymes which can catalyze the hydrolysis of the same polysaccharide together, i.e. xylan-, pectin-, cellulose-, xyloglucan-, inulin- & starch-, and β-glucan-, mannan- & arabinogalactan-protein (AGP) degrading enzymes (Figs. 2-4). The accessory enzymes that can hydrolyze multiple substrates are divided into two main groups: 1) the enzymes that can hydrolyze both pectin (side chain) and hemicelluloses (e.g. α-L-arabinofuranosidase, β-galactosidase) were referred to as the Hemicellulose + Pectin group, and 2) the enzymes that can hydrolyze different hemicelluloses but not pectin (e.g. β-glucosidase/β-xylosidase, acetylesterases) were referred to as the Hemicellulose group. *P. subrubescens* clearly uses different approaches to degrade the two plant biomasses (Fig. 2), indicating an ability to modify the produced enzymes to match the composition of the substrate. This is a phenomenon that has also been observed in some other fungi (Benocci et al., 2019; Berka et al., 2011; Mäkelä et al., 2017).

3.2. P. subrubescens uses different approaches and adapts enzyme production to degrade different biomasses

During degradation of wheat bran, *P. subrubescens* produced mainly xylanolytic enzymes (> 75% of the total CAZymes detected as determined from exoproteome data) targeting both main chain and side residues on Day 2 (Fig. 2 and Fig. 3, Xylan (main chain) and Xylan (side residue) groups). On Day 3, a higher production level of CAZymes targeting cellulose, starch and inulin (> 40% in total CAZyme signal) was observed, even though the xylanolytic enzymes still represented the major part of the proteins (almost 50%). Wheat bran contains a small amount of fructose and fructose-related polysaccharides (Hemdane et al., 2016; Onipe et al., 2015; Saunders & Walker, 1969), which could explain the induction of inulin-degrading enzymes. However, these enzymes were mainly produced on Day 3, suggesting that they may be controlled by similar regulatory systems as starch- or cellulose-degrading enzymes.

This degradation approach is also found in Aspergilli (Benoit et al., 2015), a sister genus of *Penicillium*. Our results provided molecular support for the previous report in which the activities of *endo*-β-1,4-xylanase, α-L-arabinofuranosidase and β-xylosidase were shown to be very high when growing *P. subrubescens* on wheat bran (Mäkelä et al., 2016). In addition, when compared to published proteomes of other Penicillia, *Penicillium funiculosum*, *Penicillium oxalicum* and *Penicillium*

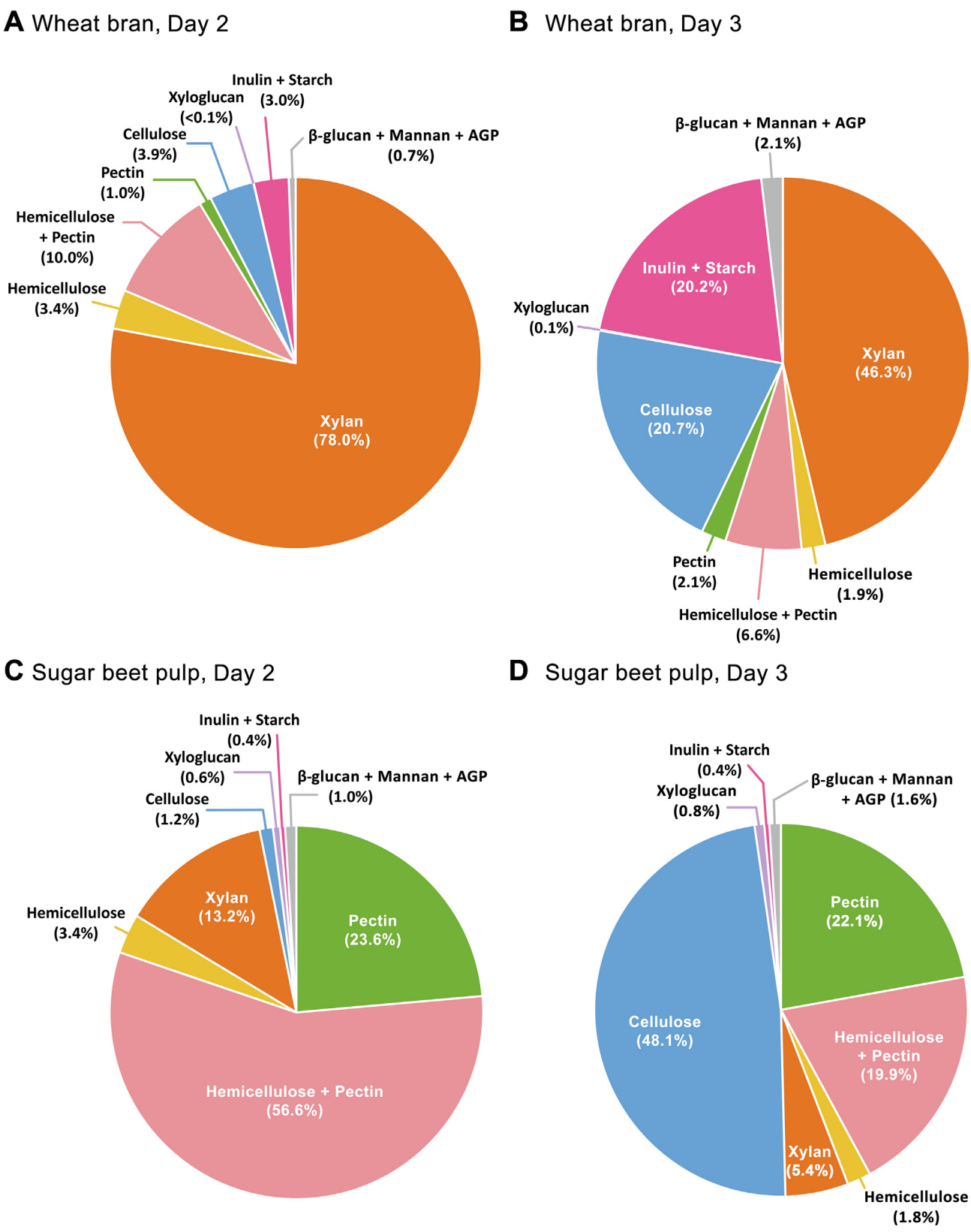

Fig. 2. Distribution of CAZymes produced by *P. subrubescens* (grouped based on activity targeting main polysaccharide components) grown on wheat bran or sugar beet pulp for two and three days as analyzed by exoproteomics.

decumbens (Liu et al., 2013; Ogunmolu et al., 2015; Song et al., 2016), the main cellulose-degrading enzymes detected were quite similar. In contrast, *endo*-xylanases from GH10 and GH30 were the main xylanases produced by *P. subrubescens* and *P. oxalicum* (Song et al., 2016), whereas *P. funiculosum* and *P. decumbens*, mainly produced GH11 xylanases (Liu et al., 2013; Ogunmolu et al., 2015). This could be explained by the closer relationship between *P. subrubescens* and *P. oxalicum* than to the other Penicillia, based on phylogenetic analysis, even

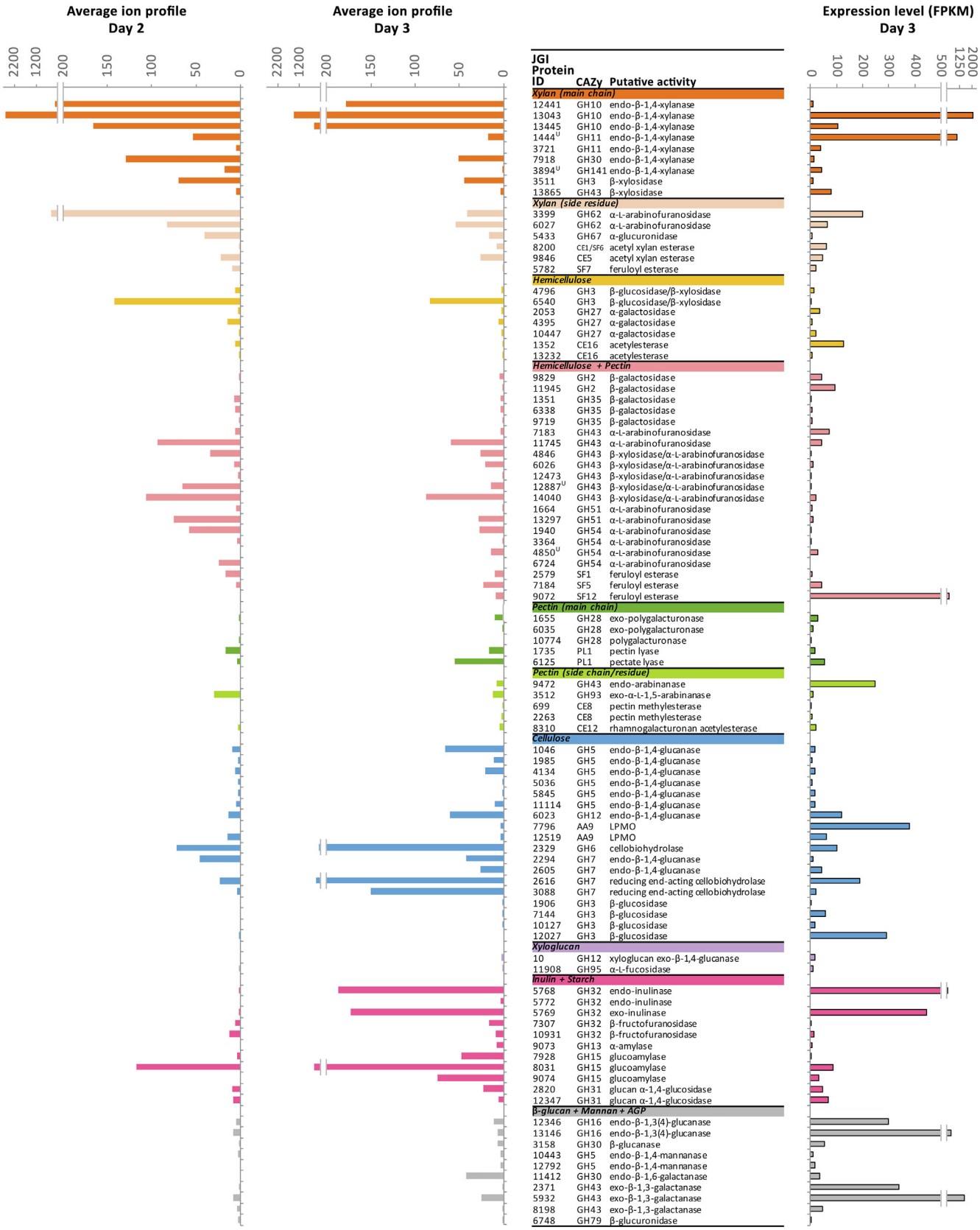

Fig. 3. Distribution of CAZymes in the exoproteome of *P. subrubescens* grown in liquid minimal medium containing 1% wheat bran for two and three days, and their transcriptome profile on Day 3. Orange, light orange, yellow, light pink, green, light green, blue, purple, pink and gray indicate xylan- (main chain), xylan- (side residue), hemicellulose, hemicellulose + pectin, pectin- (main chain), pectin- (side chain), cellulose-, xyloglucan-, inulin- & starch-, and β-glucan-, mannan- & arabinogalactan-protein (AGP) degrading enzymes. U indicates the protein/gene that has no orthologs in related species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

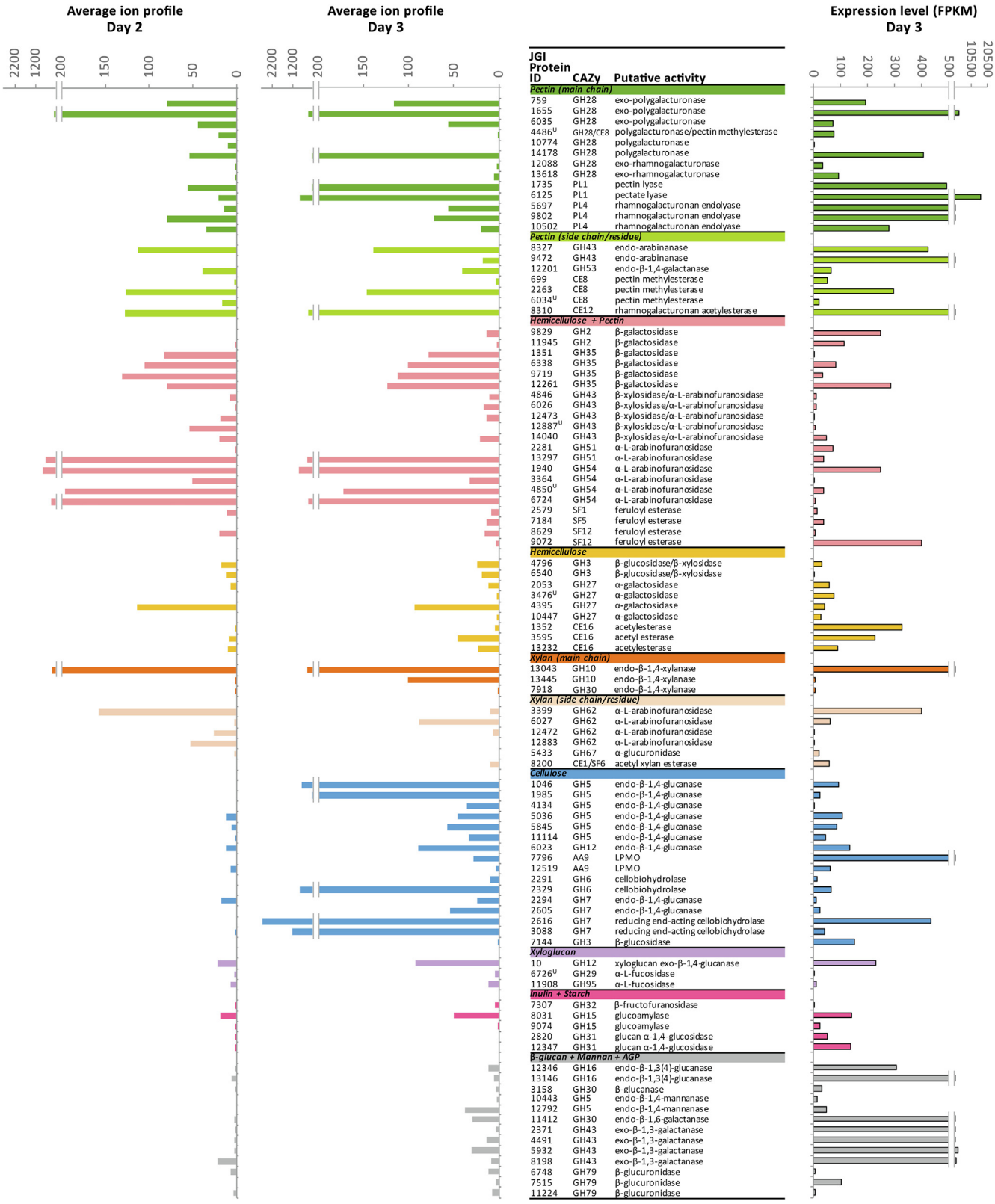

Fig. 4. Distribution of CAZymes in the exoproteome of *P. subrubescens* grown in liquid minimal medium containing 1% sugar beet pulp for two and three days, and their transcriptome profile on Day 3. Green, light green, light pink, yellow, orange, light orange, blue, purple, pink and gray indicate pectin- (main chain), pectin- (side chain), hemicellulose + pectin, hemicellulose, xylan- (main chain), xylan- (side residue), cellulose-, xyloglucan-, inulin- & starch-, and β-glucan-, mannan- & arabinogalactan-protein (AGP) degrading enzymes. U indicates the protein/gene that has no orthologs in related species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

though *P. subrubescens* has a significantly higher number of CAZymes than *P. oxalicum* (Peng et al., 2017). Previously, it was shown that *P. subrubescens* is an efficient inulin degrader (Mansouri et al., 2013). Five putative inulinases from GH32 were highly produced on wheat bran on Day 3 together with starch- and cellulose-degrading enzymes, whereas sugar beet pulp cultivation resulted in the production of low levels of starch degrading enzymes and poor production of a single inulinase (see below). In contrast, cellulolytic enzymes were highly produced on the Day 3 in sugar beet pulp. This indicates that GH32 genes may be controlled by the same regulator as amylolytic genes, but different from cellulolytic genes. Even though wheat bran also contains small amounts of fructose and fructose-containing di- and polysaccharides such as sucrose and fructans (Hemdane et al., 2016; Onipe et al., 2015; Saunders & Walker, 1969), these predicted inulinases only increased their levels on Day 3, indicating that fructose and fructose-containing saccharides may not act as the main inducers unless they are less preferred carbon sources. Furthermore, no inulinase was reported among the main proteins detected from *P. funiculosum*, *P. oxalicum* and *P. decumbens*; thus confirming that the inulinase production by *P. subrubescens* is unique to this species.

Towards degradation of sugar beet pulp *P. subrubescens* tended to degrade side chains of pectin before attacking the pectin main chain and cellulose (Fig. 4). During growth on sugar beet pulp, pectin- and pectin/hemicellulose-degrading enzymes (~80%, in particular debranching ones) were the major CAZymes produced by *P. subrubescens* on Day 2 (Fig. 4 in Pectin (side chain/residue) and Hemicellulose + Pectin groups). Enzymes acting on the pectin main chain became more pronounced on Day 3 (Fig. 4). Interestingly, the cellulolytic enzymes almost reached 50% of all produced CAZymes on Day 3, similar to those in wheat bran, suggesting that cellulose is a secondary substrate for *P. subrubescens*. In addition, four putative α-L-arabinofuranosidases (Protein ID 13297 from GH51 and Protein IDs 1940, 4850 and 6724 from GH54) were among the highest CAZymes produced by *P. subrubescens* grown in sugar beet pulp on Day 2. These predicted α-L-arabinofuranosidases were different from those predominantly detected in wheat bran, supporting the premise that *P. subrubescens* produced specific set of enzymes targeting different polysaccharides. These results also suggest that some of the expanded gene families in this species allow it to respond to plant biomass with diverse enzyme sets. Overall, *P. subrubescens* is an effective pectin degrading fungus, because it produced an almost complete set of required pectin-degrading enzymes at high level including pectin/pectate lyases, *exo*-polygalacturonases, *endo*-polygalacturonase, and rhamnogalacturonan endolyases together with several pectic debranching enzymes (e.g. α-L-arabinofuranosidases, *endo*-arabinanases, β-galactosidases, *endo*-β-1,4-galactanase, α-L-fucosidase, and feruloyl esterases) (Fig. 4). Inulin- and starch-degrading enzymes were produced at low levels in sugar beet pulp.

To further explore the possible cellular machinery for plant biomass degradation of *P. subrubescens*, transcriptome analysis of Day 3 mycelium of *P. subrubescens* grown in wheat bran or sugar beet pulp was performed (Fig. 5). Transcriptomics confirmed the high expression level of genes encoding xylan-, cellulose-, starch- and inulin-degrading enzymes in wheat bran, as well as the high expression level of genes encoding pectin-degrading enzymes in sugar beet pulp. The genes encoding enzymes in Hemicellulose + Pectin and cellulose groups were different from the exoproteome data, which could indicate a delay in the detected upregulation of some of these genes between transcriptome and proteome profiles. The transcriptome also showed high expression levels of genes encoding enzymes degrading β-glucan and arabinogalactan-proteins in both wheat bran and sugar beet pulp, however their corresponding enzymes were barely detected in the proteomic analysis.

3.3. Putative regulators of plant biomass degrading enzymes from *P. subrubescens*

Expression of genes encoding plant biomass degrading enzymes occurs via a network of transcriptional regulators. The sensing of inducers starts a signaling pathway leading to the activation of transcriptional regulators (or transcription factors), which then regulate the production of plant biomass degrading enzymes (Benocci et al., 2017; de Vries & Visser, 2001). It has been suggested that the inducers (mono- and short oligosaccharides) are produced by hydrolysis of polysaccharides by small amounts of constitutively produced enzymes or scouting enzymes, which then trigger the transcriptional factors of the main hydrolytic response. Genes encoding putative transcription factors involved in plant biomass conversion were identified based on Benocci et al. (2017). Most of these were expressed at similar levels on either substrate (Table 1), which is as expected because even though transcription factors are essential for the regulation of gene expression, only small amounts are needed to modulate the regulatory network. In addition, several regulators are produced at constitutive levels and are controlled by post-transcriptional activation. We observed indications of a fine-tuning mechanism between the putative repressors (e.g. CreA, ACE1 and HCR-1 and GaaX) and the putative activators (e.g. CLR-1, CLR-2, McmA) for expression of genes encoding plant biomass degrading enzymes, possibly since the transcriptomic analysis was performed on the third day, at which point the fungus had already grown past the initial stage of plant biomass degradation.

3.4. A potential new fungal cell factory for novel CAZymes

P. subrubescens contains > 700 predicted CAZyme-encoding genes in its genome, which is approximately 20–50% higher than most other genome-sequenced Penicilli (MycoCosm, https://mycocosm.jgi.doe.gov/mycocosm/home) (Grigoriev et al., 2013; Peng et al., 2017). Furthermore, the CAZyme-encoding genes related to plant biomass degradation as well as enzyme production levels, and saccharification abilities were similar to that of the industrial species *A. niger* (Mäkelä et al., 2016). Based on the exoproteome data, *P. subrubescens* produced complete sets of enzymes required to degrade complex polysaccharides, e.g. xylan and pectin, even though only few unique CAZymes, having no orthologs to other related species, were produced (Figs. 3, 4). Characterization of some of the unique CAZymes in expanded families demonstrated that their corresponding genes are functional and not pseudogenes (Coconi Linares et al., 2020). The absence of some of them in our proteomics and transcriptomics data may indicate that they are produced under specific growth conditions not covered by our study. Based on its ability to produce specific CAZymes (Mäkelä et al., 2016), the unusually high number of CAZy genes in its genome (Peng et al., 2017) and its ease of cultivation and genetic modification (Salazar-Cerezo et al., 2020), *P. subrubescens* has a high potential to become a novel fungal enzyme factory, particularly for the production of plant biomass degrading enzyme cocktails.

4. Conclusion

Exoproteomic analysis has expanded our understanding of the different approaches used by *P. subrubescens* to degrade two common feedstocks. To degrade xylan, *P. subrubescens* attacked both the main chain and side residues at the same time, whereas to degrade pectin it attacked the branches first. Our study points to the potential of *P. subrubescens* as a novel fungal cell factory for production of lignocellulolytic enzymes. Our future studies will address the regulatory mechanisms underlying lignocellulolytic enzyme production by *P. subrubescens* to improve the strain as a cell factory for extracellular enzyme production.

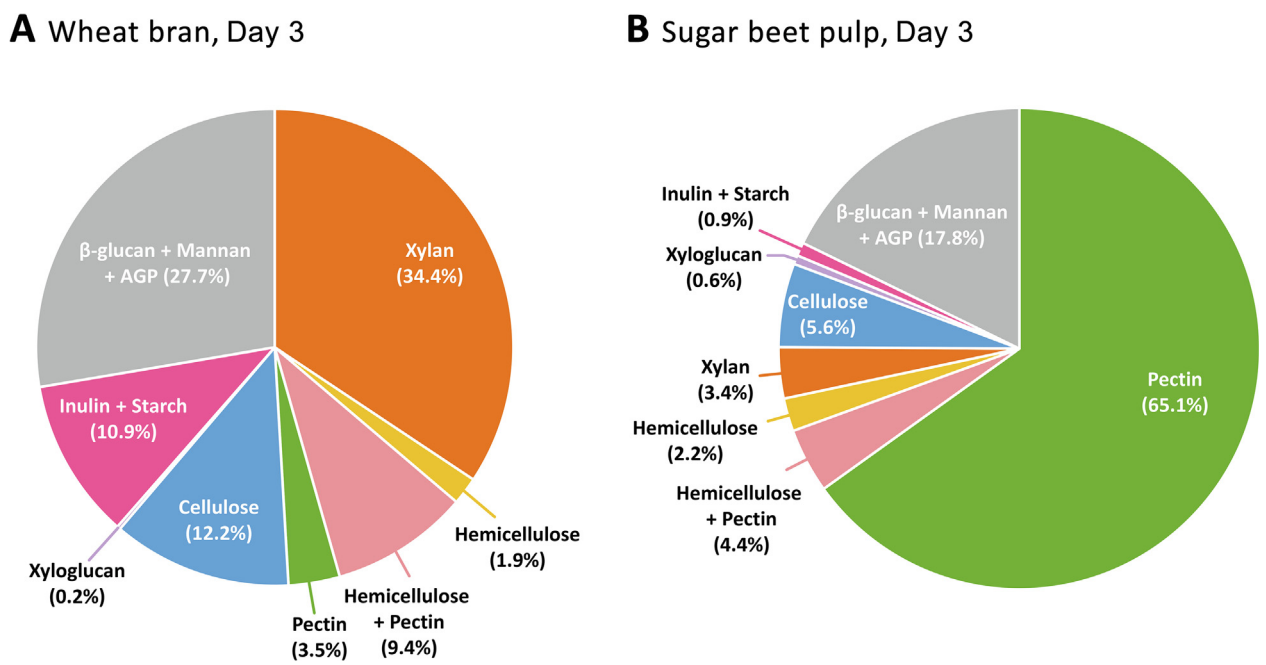

Fig. 5. Distribution of genes encoding CAZymes by *P. subrubescens* (grouped based on activity targeting main polysaccharide components) grown on wheat bran or sugar beet pulp for three days as analyzed by transcriptomics.

Table 1
Predicted regulators involved in plant biomass degradation in *P. subrubescens* with their expression level based on transcriptome analysis.

Transcription factor[a]	Accession number	Candidate in *P. subrubescens*	% identity	Expression level (FPKM)[b]Wheat bran	Sugar beet pulp
Cellulose, hemicellulose and pectin regulators					
XlnR/XLR1/XYR1	O42804	10486	73.9	43	48
AraR	A2QJX5	4479	75.0	49	35
ARA1	G4MNH3	–	–	–	–
CLR-1/ClrA	V5INA3	3548	60.4	78	**170**
CLR-2/ClrB/ManR	Q7SAJ9	12845	48.0	**144**	***215***
CreA/CRE1	AN6195	10721	85.0	***246***	***183***
ACE1 TacA (AceA like protein)	Q9P8W3 A0A0D6A8D5	3176	55.2 68.2	***362***	***358***
ACE2	Q96WN6	–	–	–	–
ACE3	Trire2|77513	7552	63.2	16	15
ClbR	I7HAQ2	2109	45.6	31	31
RhaR	A0A0A1I536	11774	77.8	18	43
GaaR	AN10548	6158	76.2	28	36
GaaX	AN10544	6160	80.7	36	***238***
GalR	AN10550	4479	59.0	49	35
GalX	AN10543	10184	42.5	**151**	**120**
HCR-1	Q7RXC0	388	69.2	**147**	**144**
McmA	AN8676	212	75.6	***319***	***405***
BglR/COL-26	Q1K639	10836	33.7	7	8
Starch and inulin regulators					
InuR	AN3835	9211	63.7	68	62
AmyR	Q9Y728	2819	80.5	27	20
MalR	AO090038000235	4551	42.0	67	54

[a] Based on Benocci et al. (2017)
[b] Genes among the top 10% are in bold and italics; genes among the top 20% are in bold.

CRediT authorship contribution statement

Adiphol Dilokpimol: Investigation, Formal analysis, Data curation, Visualization, Writing - original draft. **Mao Peng:** Formal analysis, Data curation, Visualization, Writing - review & editing. **Marcos Di Falco:** Formal analysis, Data curation, Writing - review & editing. **Thomas Chin A Woeng:** Formal analysis. **Rosa M.W. Hegi:** Formal analysis. **Zoraide Granchi:** Formal analysis, Data curation. **Adrian Tsang:** Resources, Funding acquisition, Writing - review & editing. **Kristiina S. Hildén:** Conceptualization, Writing - review & editing. **Miia R. Mäkelä:** Conceptualization, Writing - review & editing. **Ronald P. de Vries:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123477.

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