ECFG15
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# Functional and biological importance of lytic polysaccharide monooxygenases (LPMO) and cellobiose dehydrogenase (CDh) in *Aspergillus nidulans*

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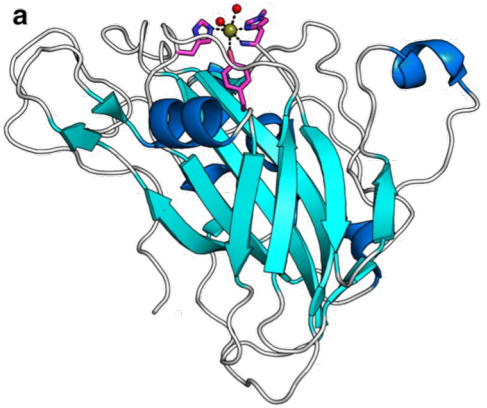
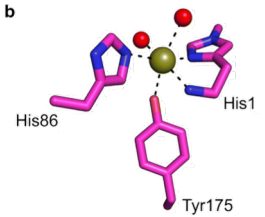
# LYTIC POLYSACCHARIDE MONOOXYGENASES

- Metalloenzymes which present a characteristic solvent exposed active-site oxidizing polysaccharide chains in a crystalline environment.
  - Aromatic and hydrophilic amino acids for interaction with substrate.
  - Exposed copper is held in place by a conserved “histidine brace” motif
    - Two conserved histidines, one of which is the N-terminal residue
- Reaction
  - Reduction of the copper by an external reductant (chemical or protein)
  - Reaction with either $O_2$ or $H_2O_2$ → powerful oxygen species
  - Hydroxylate the C1 or the C4 carbon in glycosidic bonds




Eijsink et al., 2019.
DOI: 10.1186/s13068-019-1392-0

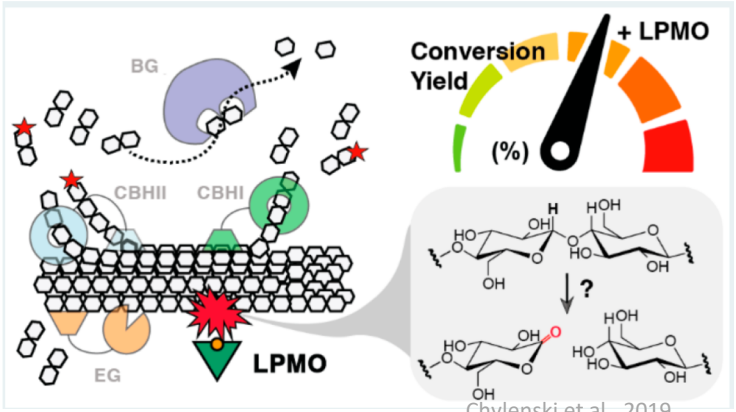


# LYTIC POLYSACCHARIDE MONOOXYGENASES

- The discovery of LPMOs profoundly changed the enzymatic conversion of polysaccharides, especially recalcitrant materials such as chitin and cellulose

- Scratch in the polymer,  providing additional ending points for other enzymes

- LPMO contribution → large if process conditions are adapted to the key determinants of LPMO activity (Müller et al., 2015)
  - Presence of electron donors and molecular oxygen.




Chylenski et al., 2019

# AA9 LPMO IN FUNGI

- Sequence-based classification system of the CAZy database places LPMOs into 7 families of the class ‘Auxiliary Activities’
  - families AA9-11 and AA13-16

- Family AA9 - identified across *Ascomycota* and *Basidiomycota*
  - Cellulose, glucans
  - Hemicellulose
  - Mixed

TABLE 4 Distribution of CAZyme-encoding genes in basidiomycetes and *Aspergillus* species[d] (Rytioja et al., 2014)

| Ecology | White rot | | | | | | | | | | | | | | | | Brown rot | | | | | | | | | | | | Litter-/Straw-decomposing/ Coprophilic | | | | | | Plant pathogen/ Parasitic | | | | ECM | | Yeast/ Mold | | *Aspergillus* species | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **CAZy family** | *A. subglabra* | *B. adusta* | *C. subvermispora* | *D. squalens* | *F. mediterranea* | *G. lucidum* | *Ganoderma* sp. | *H. irregulare* | *P. carnosa* | *P. chrysosporium* | *P. brevispora* | *P. ostreatus* | *P. strigosozonata* | *S. hirsutum* | *T. versicolor* | *S. commune*† | *B. botryosum** | *J. argillacea** | *C. puteana* | *Dacryopinax* sp. | *F. pinicola* | *G. trabeum* | *P. placenta* | *S. lacrymans* 7.9 | *W. cocos* | *A. bisporus* var. *bisporus* | *G. marginata* | *V. volvacea* | *C. cinerea* | *M. laricis-populina* | *P. graminis* | *U. maydis* | *T. mesenterica* | *L. bicolor* | *P. indica* | *C. neoformans* var. *grubii* | *R. glutinis* | *W. sebi* | *A. fumigatus* | *A. nidulans* | *A. niger* (ATCC1015) | *A. oryzae* |
| AA9 | 20 | 28 | 9 | 15 | 13 | 15 | 16 | 10 | 11 | 15 | 12 | 29 | 14 | 16 | 18 | 22 | 32 | 15 | 10 | 0 | 4 | 4 | 2 | 5 | 2 | 11 | 19 | 30 | 33 | 4 | 3 | 0 | 0 | 13 | 25 | 1 | - | 10 | 7 | 9 | 8 | 8 |

- Description of oxidative enzymes in Chytridiomycota and Cryptomycota → LPMOs were also present in early evolution (Lange et al., 2019)

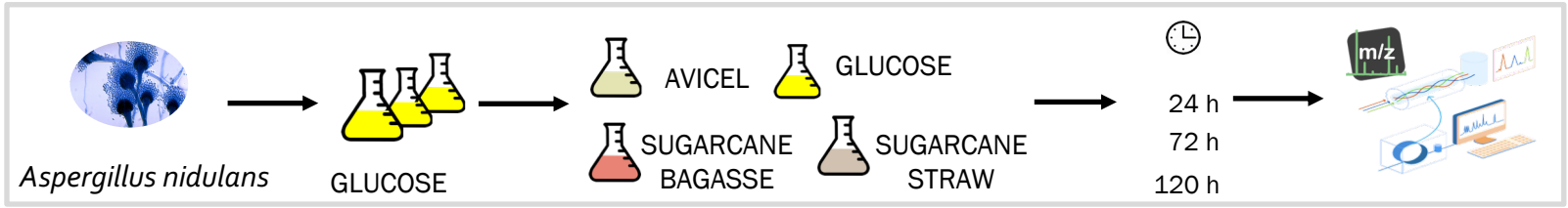
- Multiplicity of AA9 in fungal genomes is still not understood, little is known about the regulation of expression or biological functional

# *Aspergillus nidulans*

- 9 genes encoding for AA9 LPMOs
- Secretion of some of these enzymes has been verified by proteomics after cultivation with different substrates
- *An*LPMO9D (Jagadeeswaran et al., 2016)
  - Oxidizes C1
  - Cellulose and xylolgucan
- *An*LPMO9B (Jagadeeswaran et al., 2018)
  - Oxidizes C4
  - Cellulose and cellooligosaccharides

| Protein | CBM | Uniprot | Cultivation condition | | | | |
|---|---|---|---|---|---|---|---|
| | | | Starches | Sorghum stover | Delignified surgacane bagasse | Xylan from beechwood | Glucose |
| AnLPMO9A | | Q5BAP2 | X | | X | X | X |
| AnLPMO9B | CBM1 | Q5BCX8 | X | | | | |
| AnLPMO9C | | Q5AZ52 | X | X | X | | |
| AnLPMO9D | | Q5B8T4 | X | X | | | |
| AnLPMO9E | CBM1 | Q5AQA6 | | | | | |
| AnLPMO9F | | Q5B6H0 | | X | | | |
| AnLPMO9G | | Q5BEI9 | | X | | | |
| AnLPMO9H | | Q5B7G9 | | | | | |
| AnLPMO9I | | Q5AUY9 | | | | | |
| REFERENCE | | | Nekiunaite et al., 2016 | Saykhedkar et al., 2012 | Rubio et al., 2016 | | |

# TARGETS SELECTION


| Protein (n=301) | CAZy domain | Avicel | | | Bagasse | | | Glucose | | | Straw | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24h | 72h | 120h | 24h | 72h | 120h | 24h | 72h | 120h | 24h | 72h | 120h |
| *An*LPMO9F | AA9 | 24 | 16 | 11 | 15 | 11 | 13 | 0 | 0 | 0 | 22 | 8 | 11 |
| *An*LPMO9G | AA9 | 3 | 7 | 0 | 4 | 4 | 8 | 0 | 0 | 0 | 3 | 3 | 3 |
| *An*LPMO9D | AA9 | 0 | 0 | 0 | 30 | 5 | 5 | 0 | 0 | 0 | 3 | 0 | 0 |
| *An*LPMO9C | AA9 | 0 | 0 | 0 | 10 | 3 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| *An*LPMO9B | AA9/CBM1 | 0 | 0 | 0 | 5 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| *An*LPMO9A | AA9 | 0 | 0 | 0 | 2 | 0 | 0 | 7 | 2 | 0 | 0 | 0 | 0 |

# CLONING AND EXPRESSION

- Genes encoding *An*LPMOs -9C, -9F and -9G, and the CDH were cloned into the pEXPYR vector for expression controlled by the glucoamylase promotor
- Homologous expression

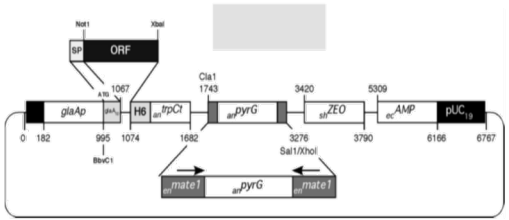
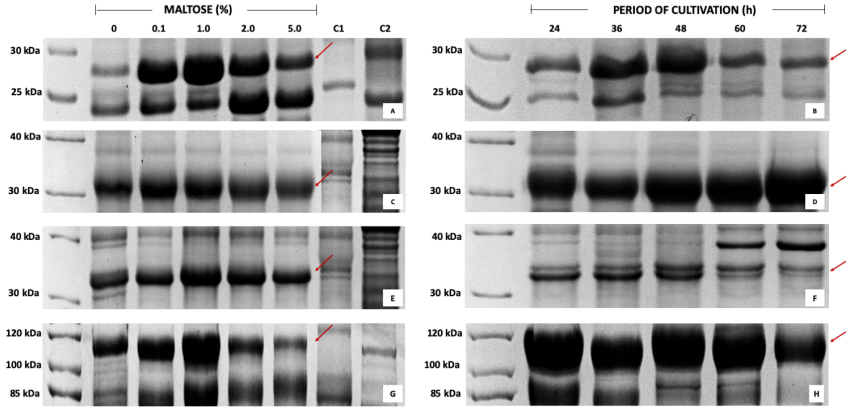
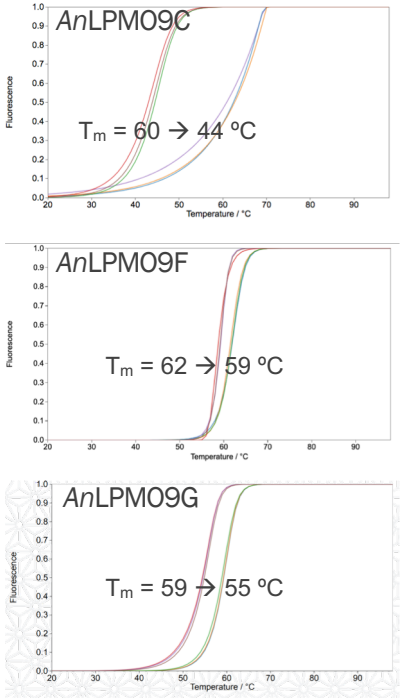



**Fig. 3 –** pEXPYR expression vector for high levels production of client proteins in fungi.



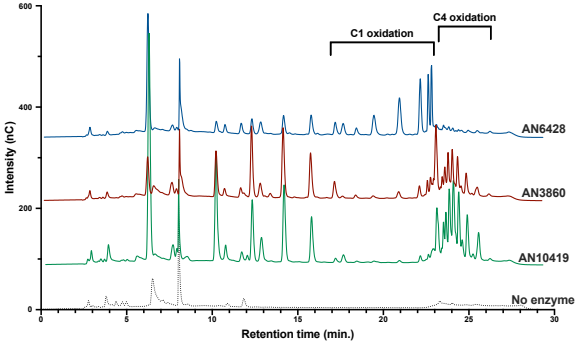
# LPMO CHARACTERIZATION

## DSF

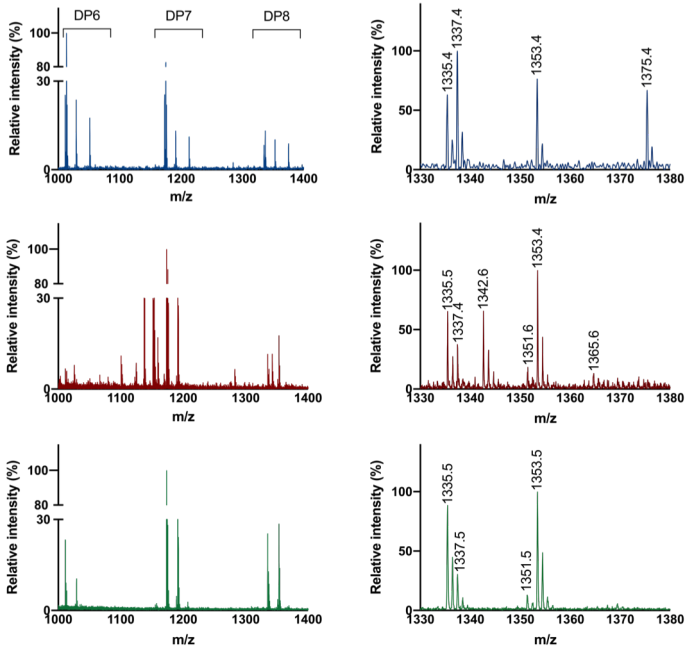



## Regioselectivity (PASC reactions)

### LC (HPAEC-PAD)




### MS MALDI TOF

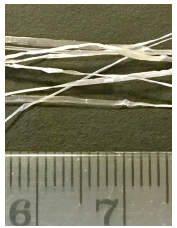


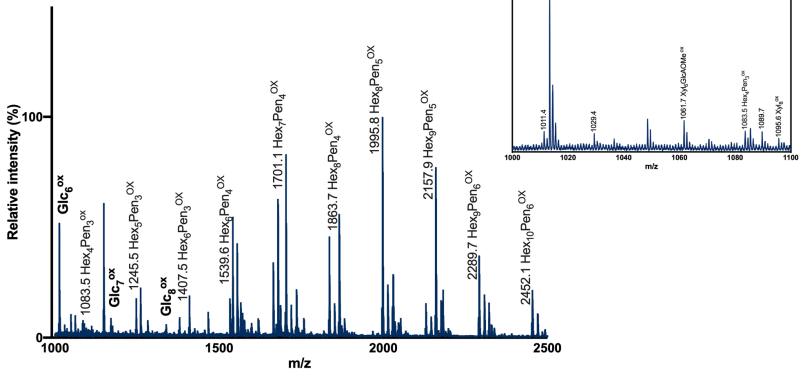
# SPECIFICITY FOR SUBSTRATE

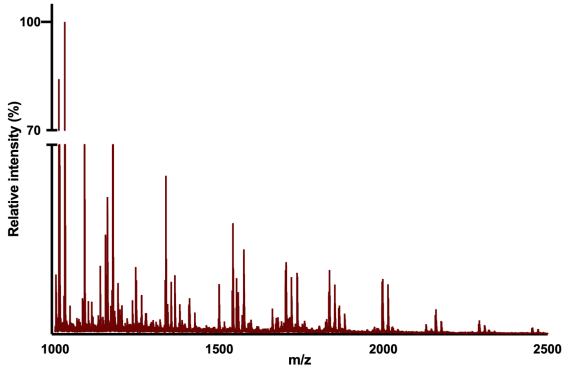
| Substrate | *A. nidulans* LPMOs | | |
|---|---|---|---|
| | *An*LPMO9C (C1) | *An*LPMO9F (C1/C4) | *An*LPMO9G (C1/C4) |
| Cellulosic | | | |
| PASC | + | + | + |
| Celery | + | + | + |
| Celloheaxose | - | - | - |
| Chitin (beta from squid pen) | - | - | - |
| Hemicellulosic | | | |
| Xylan from birchwood | - | - | - |
| Xyloglucan from tamarind | + | + | + |
| Beta-glucan from barley (β-1,3/1,4 mixed linkage) | - | + | + |
| Glucomannan (konjac) | - | - | + |
| Mannan from ivory nut | - | - | - |
| Mixed | | | |
| PASC + birchwood xylan | - | - | - |

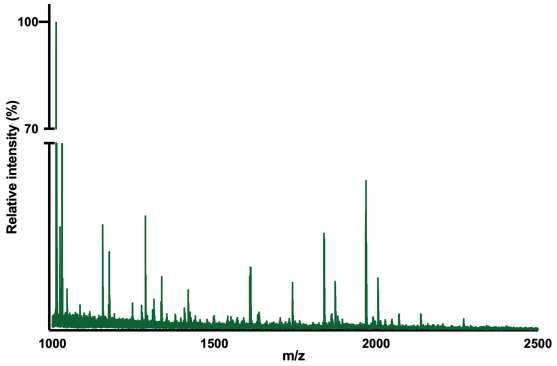
# SPECIFICITY FOR SUBSTRATE

Celery

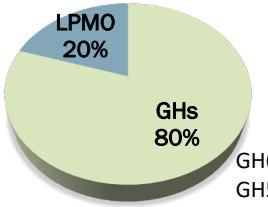




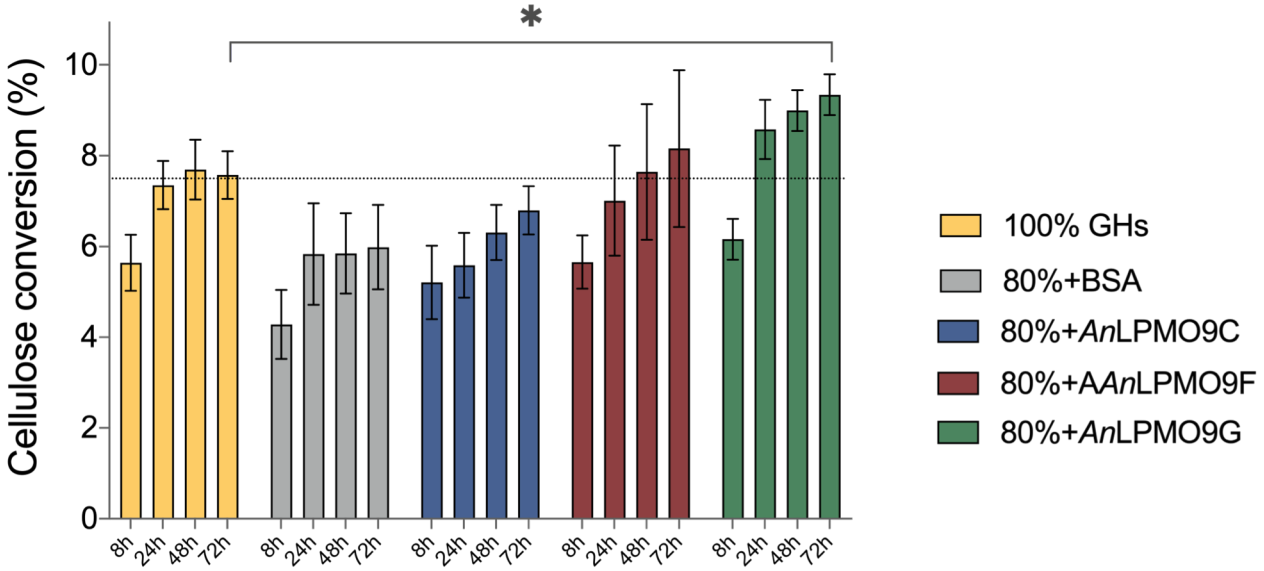



# AVICEL DEGRADATION

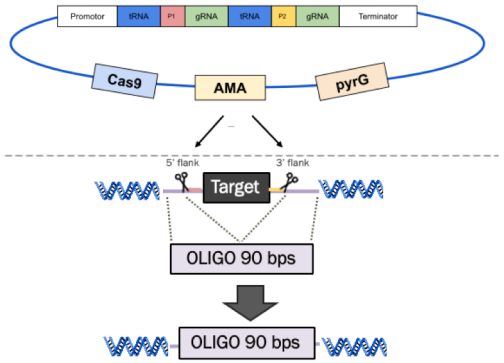



GH6 and GH7 – cellobiohydrolases +
GH5 endoglucanase + GH3 beta-glucosidase



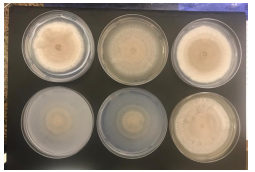
# DELETION AND MUTANTS CHARACTERIZATION

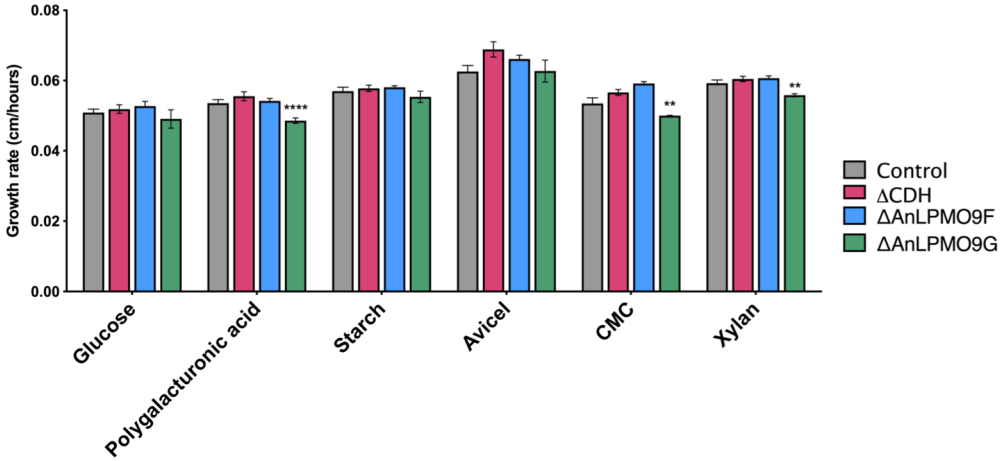
CRISPR/Cas9 system for Aspergilli (Nødvig et al.,.2018)



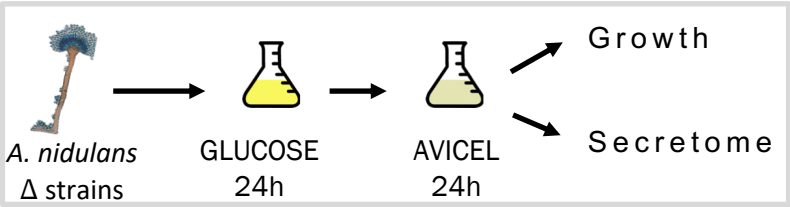

Growth on agar plates with different substrates

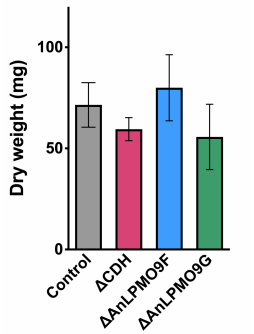
- ΔCDH
- Δ*An*LPMO9F
- ΔA*nL*PMO9G

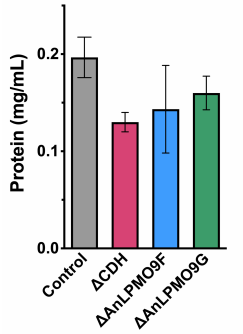


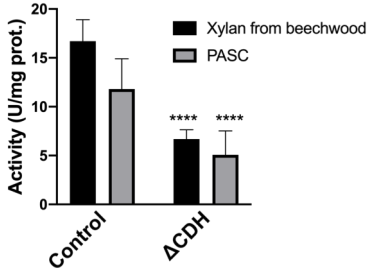


# MUTANTS CHARACTERIZATION

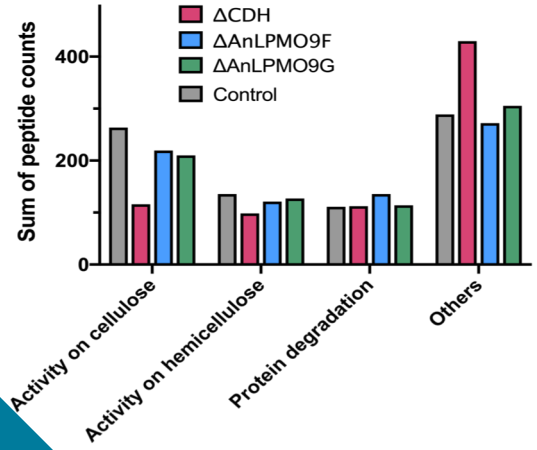



**MS Q-Tof**
(n=57)




| Role | Protein (ANOVA, p<0.05) | SP | Control | ΔCDH | ΔLPMO9F | ΔLPMOG |
|---|---|---|---|---|---|---|
| | | | Sum of peptide counts | | | |
| Cellulose degradation | AN2828 - Beta-glucosidase (bglL) | Y | 29.1 | 2.6 | 9.9 | 8.3 |
| | AN5176 - Cellobiohydrolase (cbhA) | Y | 18.8 | 0.0 | 42.4 | 23.9 |
| | AN3860 - Lytic polysaccharide monooxygenase (AnLPMO9F) | Y | 14.0 | 0.0 | 0.0 | 14.5 |
| Hemicellulose degradation | AN3613 - Endo-1,4-beta-xylanase (xlnA) | Y | 0.0 | 0.0 | 7.5 | 7.1 |
| Others | AN7349 - Alpha-1,3-glucanase (mutanase) (mutA) | Y | 0.0 | 0.0 | 8.5 | 0.0 |
| | AN7269 - Secondary metabolite biosynthetic process, fungal-type cell wall localization | N | 0.0 | 10.9 | 0.0 | 1.2 |
| | AN7657 - 1,3-beta-transglycosidase (gelA), predicted role in glucan processing | Y | 8.8 | 28.2 | 8.6 | 6.4 |
| | AN8969 - Lysozyme activity, peptidoglycan catabolic process | Y | 0.0 | 33.2 | 0.0 | 0.0 |
| | AN4055 - Acid phosphatase | Y | 47.1 | 82.1 | 60.1 | 42.8 |
| | AN7181 - Unknown protein | Y | 1.9 | 4.8 | 20.2 | 7.5 |
| Proteases | AN7962 - Deuterolysin-type metallo-proteinase (pepJ) | Y | 39.1 | 13.4 | 41.8 | 26.7 |
| | AN10351 - Metalloaminopeptidase activity | N | 0.0 | 4.3 | 5.4 | 0.0 |

Characterized

X

Total number

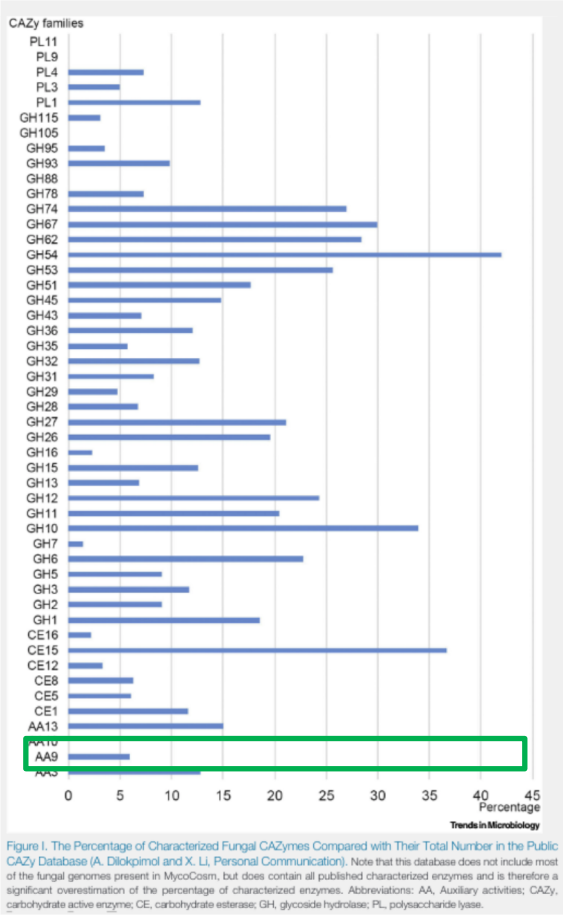



Figure I. The Percentage of Characterized Fungal CAZymes Compared with Their Total Number in the Public CAZy Database (A. Dilokpimol and X. Li, Personal Communication). Note that this database does not include most of the fungal genomes present in MycoCosm, but does contain all published characterized enzymes and is therefore a significant overestimation of the percentage of characterized enzymes. Abbreviations: AA, Auxiliary activities; CAZy, carbohydrate active enzyme; CE, carbohydrate esterase; GH, glycoside hydrolase; PL, polysaccharide lyase.

Ronald P. de Vries and Miia R. Mäkelä, 2020

# ACKNOWLEDGEMENTS


Andre R L Damasio (Supervision)

Institute of Biology

Department of Biochemistry and Tissue Biology

Laboratory of Enzymology and

Molecular Biology of Microorganisms (LEBIMO)

Paul H Walton (Supervision)

Department of Chemistry

York Structural Biology Laboratory (YSBL)

Technology Facility