# Polysaccharide decomposition by *Trichoderma*

## Contents



## Plant cell wall degradation

The glycosyl hydrolases involved in plant polysaccharide degradation – such as cellulases, xylanases, and pectinases - and some of the associated accessorial enzymes (e.g. copper-dependent cellulose monooxygenases) and their evolution has recently been described for nine *Trichoderma* spp. in detail (Druzhinina *et al.*, 2018), and these data will therefore not be repeated here.

Concerning the accessory enzymes (Levasseur *et al.*, 2013), we identified 62 – 87 to be (some putatively though) involved plant polysaccharide degradation. Among them, three auxiliary enzymes of family AA9 (representing copper-dependent lytic polysaccharide monooxygenases, (LPMOs (Ferreira *et al.*, 2015)), have already been described in detail in *T. reesei* (Kubicek & Kubicek, 2016) and orthologs are present in all other *Trichoderma* species. Of the remaining 12 families so far incepted, eight are also present in all *Trichoderma* spp. Families AA2 and AA4, which comprise lignin peroxidases and vanillin alcohol oxidases, are absent from *Trichoderma* which is consistent with the fact that *Trichoderma* is unable to degrade lignin. However, *Trichoderma* spp. contain AA7 glucooligosaccharide oxidases, which underlines the importance of oxidative glucose catabolism as reported above, and also AA8 iron reductases. The latter consist of a cytochrome domain (protoheme IX) of spectral class b, and have implicated into cellulose degradation by Fenton chemistry (Eastwood *et al.*, 2011). This mechanism has so far been described only for brown rot fungi (Cragg *et al.*, 2015), but data suggesting the occurrence of this mechanism also in *T. reesei* and *N. crassa* have recently been presented (Schmoll *et al.*, 2012, Bischof *et al.*, 2013).

With regards to the deacetylation of hemicelluloses, all *Trichoderma* spp. contain members of the CE5 family which comprises - although not exclusively - acetylxylan esterases. Interestingly, CE8 pectin methylesterases are absent from species of section Longibrachiatum but all *Trichoderma* spp. contain a single copy of the CE15 4-O-methyl-glucuronyl methylesterase.

### Numbers of carbohydrate esterases, auxiliary activities and polysaccharide lyases in *Trichoderma*

| Enzyme type | Family | Enzyme | *Trichoderma* spp. * | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | R | L | C | P | H | G | F | V | A | M | S | U |
| Carbhydrate esterases | CE1 | acetyl xylan esterase, feruloyl esterase and others | 5 | 5 | 5 | 5 | 7 | 7 | 7 | 7 | 6 | 5 | 6 | 6 |
| | CE1/2 | | 1 | | | | 1 | | | 2 | 2 | 2 | 2 | 2 |
| | CE1/3 | | 1 | | | | 1 | | | 2 | 2 | 2 | 2 | 2 |
| Auxiliary activities | AA3 | GMC oxidoreductases | | | | | 1 | 1 | | | | | | |
| | AA3/2 | glucose- and aryl-alcohol oxidase | 9 | 9 | 9 | 9 | 16 | 16 | 15 | 14 | 9 | 9 | 9 | 9 |
| | AA3/3 | alcohol oxidase | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| | AA5/1 | glyoxal oxidase | | | | | | | | 1 | 1 | 1 | 1 | 1 |
| | AA6 | 1,4-benzoquinone reductase | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | AA7 | gluco- or chitooligosaccharide oxidase | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 |
| | AA8 | iron reductase | 1 | 1 | 1 | 1 | 3 | 2 | 2 | 6 | 5 | 3 | 4 | 3 |
| | AA9 | lytic cellulose monooxygenase | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 |
| | AA11 | lytic chitin monooxygenase | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 |
| | AA12 | pyrroloquinoline quinone-dependent oxidoreductase | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | | 1 | |
| | PL1/4 | pectate lyase | | | | | | | | | 1 | 1 | | 1 |
| | PL20 | endo-β-1,4-glucuronan lyase | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Polysaccharide lyases | PL7/4 | ß-mannuronate and alginate lyase | 2 | 2 | 2 | 2 | 4 | 4 | 4 | 3 | 3 | 3 | 3 | 3 |
| | PL8 | hyaluronate lyase | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Total** | | | 33 | 31 | 31 | 31 | 47 | 44 | 41 | 50 | 41 | 37 | 39 | 38 |

*Trichoderma one-letter code: P, T. parareesei; R, T. reesei, L, T. longibrachiatum, C, T. citrinoviride, V, T. virens, H, T. harzianum; F. T. afroharzianum, G, T. guizhouense; A, T. atroviride; M, T. gamsii; S, T. asperellum; U, T. hamatum*

Carbohydrate binding modules (CBM) are generally believed to function by concentrating the respective partner enzyme at their dedicated substrates and so enhance the catalytic efficacy, what is particularly important for the degradation of refractory substrates (Boraston *et al.*, 2004). However, although CBMs have been defined as modules within the primary structures of carbohydrate-active enzymes, this paradigm is now diluted by several findings of independent CBM proteins (Charnock *et al.*, 2002, Flint *et al.*, 2004, Vaaje-Kolstad *et al.*, 2005, Abbott *et al.*, 2008, Abramyan & Stajich, 2012). The CAZyme database currently hosts 81 CBM families, of which 12 are found in *Trichoderma* (Figure 8A). Four of them (CBM1, CBM13, CBM43 and CBM66) are found as domains in cellulolytic and hemicellulolytic enzymes. CBM1 is present in most of the cellulases, several hemicellulases, but also some of the acetyl esterases, one of the AA9 enzymes, the expansin-like protein swollenin and the protein CIP1 (whose function in cellulose depolymerisation has not yet been revealed), illustrating its importance for efficient cellulose breakdown. In addition, CBM42 domains, reported to bind to α-L-arabinofuranoside residues, are present in the GH54 α-L-arabinofuranosidases (Ribeiro *et al.*, 2010).

The CBM66 domain has been shown in bacteria to bind to nonreducing terminal fructose residues in fructans (Cuskin *et al.*, 2012), but in *Trichoderma* it is associated with GH43 α-L-arabinofuranosidases. This link between arabinan and fructan degradation is difficult to explain because these two polymers do not occur in close vicinity or association. However, the CBM66 domain has high similarity to the laminin G domains, which serve as receptors for various structures including α-dystroglycans (Givant-Horwitz *et al.*, 2005). We therefore consider it likely that the CBM66 domains in fungal α-L-arabinofuranosidases bind to a different structure than fructosides. Two independent CBM66 proteins (one only in species of section *Longibrachiatum*) are also present in the *Trichoderma* genomes, but both lack a signal peptide. Their function is therefore intracellular.

Finally, *Trichoderma* contains 3-4 CBM13 proteins that are supposed to bind xylan (Leskinen *et al.*, 2005). In *Aspergillus* spp. CBM13 domains occur in α–D-galactosidases, but the *Trichoderma* CBM13 proteins occur as individual proteins. Like the CBM66 proteins, they also lack a signal peptide and thus cannot act outside of the cells, and their true function needs further investigation.

## Fungal cell wall polysaccharide degradation

Fungal cell walls are made up by proteins and polysaccharides, chitin and ß-1,3/1,6-glucans thereby comprising the highest amount. The cell wall is subject to turnover during growth and development, and fungi consequently possess a variety of chitinases and ß-glucanases. In addition, *Trichoderma* as a mycoparasite needs these enzymes for digestion of host cell walls too. It is therefore not surprising that chitinases and ß-glucanases, as already reported earlier [12, 16] represent the CAZymes that are most abundant in *Trichoderma* (Figure 7).

Intriguingly, the GH18-B and GH18-C chitinases (which are distinguished by the presence of a CBM1 cellulose-binding module and of CBM18/CBM50 (=LysM) chitin binding modules, respectively; (Hartl *et al.*, 2012)) show a strong difference between the three *Trichoderma* sections and clades (Figure 8). The combination of one or more CBM18 and CBM50 is unique to species of HV, and may serve to increase the overall affinity to the polymer (Abramyan & Stajich, 2012). The addition of a CBM1 module to chitinases of subgroup 18-B has been shown to enhance binding and chitinase activity on insoluble chitin, which is probably due to the fact that three aromatic amino acids (W, F and Y) of CBM1, known to be required for binding to cellulose, are also required for binding to chitin (Limon *et al.*, 2001).

Thirteen CBM50 domains – but none of the CBM18 domains - also occur as independent proteins, consist of up to 5 tandem copies and were most abundant in HV. Expression of these independent CBM modules has been shown in the case of CBM50 and *T. atroviride* (Gruber *et al.*, 2011).

2-4 genes encoding CE4 chitin deacetylases were also found (see above), of which two contain 2 and 3, respectively, CBM18 chitin binding modules that flank the catalytic domain. Acetyl esterases containing CBM18 modules have so far only been reported for the amphibian pathogen *Batrachochytrium dendrobatidis* (Liu & Stajich, 2015), but a BLASTP analysis shows that they are present in several other Sordariomycetes too. They deacetylate chitin to chitosan, which can lead to the formation of a chitosan layer on top of chitin (Cord-Landwehr *et al.*, 2016), thus protecting the cell wall against either its own chitinases and those from competing or host organisms, and prevent the recognition of the fungus by plants. In another fungus, *Pochonia chlamydospora*, chitosanases were demonstrated to be important for its nematophagous activity (Aranda-Martinez *et al.*, 2016).

Finally, all *Trichoderma* spp. possess 3-4 genes encoding proteins of family AA11 that comprises lytic chitin monooxygenase, thereby completing the tools for chitin degradation. None of them however contains an X278 domain, as described for the enzyme from *A. oryzae* (Hemsworth *et al.*, 2014), or another chitin binding domain (CBM18, CBM50).

## Degradation of α-glucans

In contrast to the degradation of plant cell wall polysaccharides, the ability of *Trichoderma* to degrade starch and other α-linked polysaccharides has not been systematically studied yet. Starch is the major energy storage of plants, and is composed of two distinct glucose polymers: amylose, comprising essentially unbranched α-(1→4)-linked glucose residues, and the larger and branched amylopectin, produced by the formation of α-(1→6) linkages

between adjoining straight glucan chains on an α-(1→4) backbone (Miao *et al.*, 2015). Despite the simplicity of this chemical structure, amylopectin and amylose molecules are organized radially in a supramolecular assembly thereby forming water insoluble granules that vary in size and crystallinity (Buleon *et al.*, 1998). This renders them poorly accessible to the active sites of amylolytic enzymes. To degrade starch, the GH inventory of *Trichoderma* displays several GH13 (subfamily 1 and 40, respectively) α-amylases and GH31 α-glucosidases, two GH15 glucoamylases and a single GH133 amylo-α-1,6-glucosidase, of which 3, 2, 4 and 1, respectively, occur in the *Trichoderma* core genome. Two of the GH15 and one of the GH13, all belong to the core genes, contain carbohydrate binding domains (see below).

In addition, a single GH4 α-glucosidase occurs in species of the *T. harzianum* complex. GH4 has so far been found only in bacterial and archaeal taxa (Hall *et al.*, 2009), but a BLASTP search revealed that an orthologue occurs also in *Talaromyces stipitatus* and *Aspergillus sydowii* with higher similarity and lower E-value than from anaerobic bacteria of the phylum *Chloroflexi*, which are frequently found in anaerobic environments containing plant biomass (Tian *et al.*, 2017). It is therefore thinkable that some Eurotiales obtained this gene from bacteria by HGT, from which is was subsequently laterally transferred to *Trichoderma*. Despite of the fact that the closest neighbours have been annotated as α–galactosidases, a function of the fungal GH4 enzyme is difficult to predict because this GH family contains α-glycosidases and 6-phospho-α-and -β-glycosidases (Hall *et al.*, 2009). However, a function in the degradation of phosphorylated glycosides is less likely because the GH4 protein contains a signal peptide and is therefore secreted.

Three of the starch degrading enzymes also contained CBMs, i.e. CBM48 (that binds amorphous starch like glycogen (Janecek *et al.*, 2011)) which is present in one GH13, and a granular starch binding CBM20 (Nekiunaite *et al.*, 2016) that occurs in both GH15 glucoamylases. This illustrates the importance of glucamylase in the attack of the initially recalcitrant granular starch. Another starch-binding CBM - CBM21 (Chou *et al.*, 2006) - occurred only as individual protein.

Interestingly, members of the recently discovered AA13 family that encodes a lytic starch monooxygenase (Vu *et al.*, 2014), and which are present in most Sordario- and Eurotiomycetes (data not shown), were not found in *Trichoderma*.

## Degradation of uronic acid polymers

Cleavage of glycosidic bonds by ß-elimination is an important mechanism in the decomposition of uronic acid polymers and has been well demonstrated for pectin (Sutherland, 1995). It is performed by polysaccharide lyases (PL). The presence of pectate lyases in *Trichoderma* has recently been described (Druzhinina *et al.*, 2018). However, *Trichoderma* also possesses PL7 alginate lyases, PL8 hyaluronate lyases, and a PL20 endo-ß-1,4-glucuronan lyase (se above). Alginates are components of the in the cell walls of brown algae, and present in bacterial biofilms, whereas hyaluronic acid is a key component of the envelope of epithelial cells in mammals. The true substrate specificity of any of these enzymes has not yet been investigated but their presence suggests that *Trichoderma* may have the capability of degrading the envelope of algal and mammalian cells.

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