New BIOTECHNOLOGY 67 (2022) 32–41

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Full length Article

Glycoside Hydrolase family 30 harbors fungal subfamilies with distinct polysaccharide specificities

Xinxin Li [a], Dimitrios Kouzounis [b], Mirjam A. Kabel [b], Ronald P. de Vries [a,*], Adiphol Dilokpimol [a]

[a] Fungal Physiology, Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology, Utrecht University, Uppsalalaan 8, 3584 CT, Utrecht, the Netherlands
[b] Laboratory of Food Chemistry, Wageningen University & Research, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Glycoside Hydrolase family 30
Fungal subfamily
β-(1→6)-glucanase
β-(1→6)-galactobiohydrolase
β-(1→4)-xylobiohydrolase
Endoxylanase

ABSTRACT

Efficient bioconversion of agro-industrial side streams requires a wide range of enzyme activities. Glycoside Hydrolase family 30 (GH30) is a diverse family that contains various catalytic functions and has so far been divided into ten subfamilies (GH30_1-10). In this study, a GH30 phylogenetic tree using over 150 amino acid sequences was contructed. The members of GH30 cluster into four subfamilies and eleven candidates from these subfamilies were selected for biochemical characterization. Novel enzyme activities were identified in GH30. GH30_3 enzymes possess β-(1→6)-glucanase activity. GH30_5 targets β-(1→6)-galactan with mainly β-(1→6)-galactobiohydrolase catalytic behavior. β-(1→4)-Xylanolytic enzymes belong to GH30_7 targeting β-(1→4)-xylan with several activities (e.g. xylobiohydrolase, endoxylanase). Additionally, a new fungal subfamily in GH30 was proposed, i.e. GH30_11, which displays β-(1→6)-galactobiohydrolase. This study confirmed that GH30 fungal subfamilies harbor distinct polysaccharide specificity and have high potential for the production of short (non-digestible) di- and oligosaccharides.

Introduction

Glycoside hydrolases (GHs) are ubiquitously present in nature. They are critical for the efficient degradation of plant biomass in various industrial applications, such as in food and biofuel production, and the pulp and paper industries [1]. GHs are divided into different families based on their amino acid sequence similarities in the Carbohydrate-Active enZyme (CAZy) database [2]. Glycoside Hydrolase family 30 (GH30) is a diverse but understudied family, containing enzymes from bacteria, fungi and animals. Recently, GH30 has gained increased attention because of the discovery of fungal β-(1→4)-xylobiohydrolases [3,4]. In addition to this new activity, the GH30 enzyme family also contains β-glucocerebrosidases, β-glucosidases, reducing end β-(1→4)-exoxylanases, β-(1→4)-endoxylanases, β-(1→6)-galactanases, β-(1→6)-glucanases, and β-fucosidases (http://www.cazy.org/GH30_characterized.html) [2,5].

GH30 was previously divided into ten subfamilies (GH30_1-10) based on phylogenetic analysis [5–7] with fungal candidates in only GH30_3, 5 and 7 (Fig. 1, Table 1). In GH30_3, four fungal enzymes have been previously characterized, all showing β-(1→6)-glucanase activity [8–11]. In GH30_5, only two fungal enzymes have been characterized. These β-(1→6)-galactanases hydrolyze between galactosyl moieties in type II arabinogalactan [12,13]. In contrast to GH30_3 and 5, which showed similar activities within the subfamily, the nine characterized enzymes from GH30_7 hydrolyze β-(1→4)-xylan with diverse activities (Table 1). These activities include β-(1→4)-endoxylanases, β-(1→4)-xylobiohydrolases and reducing end β-(1→4)-exoxylanases.

Based on current knowledge, fungal GH30 enzymes could be used to depolymerize different polysaccharides and generate 'short' non-digestible di- and oligosaccharides, e.g. β-(1→6)-glucobiose/glucooligosaccharides, β-(1→6)-galactobiose/galactooligosaccharides, and β-(1→4)-xylobiose/xylooligosaccharides, which are of particularly interest in food and feed industries [14,15]. Non-digestible oligosaccharides (NDOs) have been reported to exhibit prebiotic potential by

Abbreviations: ABF, α-L-arabinofuranosidase; AGU, α-glucuronosidase; AG-II, arabinogalactan II; BeWX, Beech wood xylan; GAAG-II, Gum Arabic arabinogalactan II; GH, Glycoside Hydrolase; GlcA-X2, glucuronyl-xylobiose; GlcA-X3, glucuronyl-xylotriose; LWAG-II, Larch wood arabinogalactan II; MeGlcA, 4-*O*-methylglucuronic acid; MGX, 4-*O*-methyl-D-glucurono-D-xylan; ML, maximum-likelihood; NDO, non-digestible oligosaccharide; WB, wheat bran; WAX, wheat arabinoxylan; XOS, xylooligosaccharides.

* Corresponding author.
E-mail address: r.devries@wi.knaw.nl (R.P. de Vries).

https://doi.org/10.1016/j.nbt.2021.12.004
Received 12 April 2021; Received in revised form 19 December 2021; Accepted 19 December 2021
Available online 21 December 2021

selectively stimulating the growth and activity of beneficial bacteria in the colon and promote the health of the host [16]. However, these enzymes can also be used in other biotechnology applications. For example, β-(1→6)-glucanases from GH30_3 could be included in antifungal products due to their fungal cell wall degrading capabilities [17, 18], while β-(1→4)-xylanolytic enzymes from GH30_7 enzymes can be applied for biomass saccharification for the production of biofuels and fine chemicals [19].

In this study, to verify the functional specificity of these identified fungal GH30 subfamilies and deepen understanding of the fungal members in GH30, a GH30 phylogenetic tree was constructed using 161 amino acid sequences from bacterial, fungal and animal species, from which eleven candidates covering different fungal subfamilies were selected for biochemical characterization using polysaccharides and crude plant biomass. This revealed a new fungal subfamily in GH30. After discussion with curators from the CAZy Database, this is now established as GH30_11, which displays β-(1→6)-galactobiohydrolase activity. In addition, biochemical characterization revealed that the enzymes from different GH30 subfamilies exhibited distinct substrate specificities. Furthermore, to investigate the catalytic mechanism of GH30 fungal enzymes, the potential residues involved in substrate-protein interactions were analyzed by homology modelling, from which some possible amino acid residues affecting substrate specificity were highlighted. In conclusion, this study facilitates the industrial use of fungal GH30 enzymes, as the knowledge of different fungal subfamilies will help to select optimal candidates for the desired

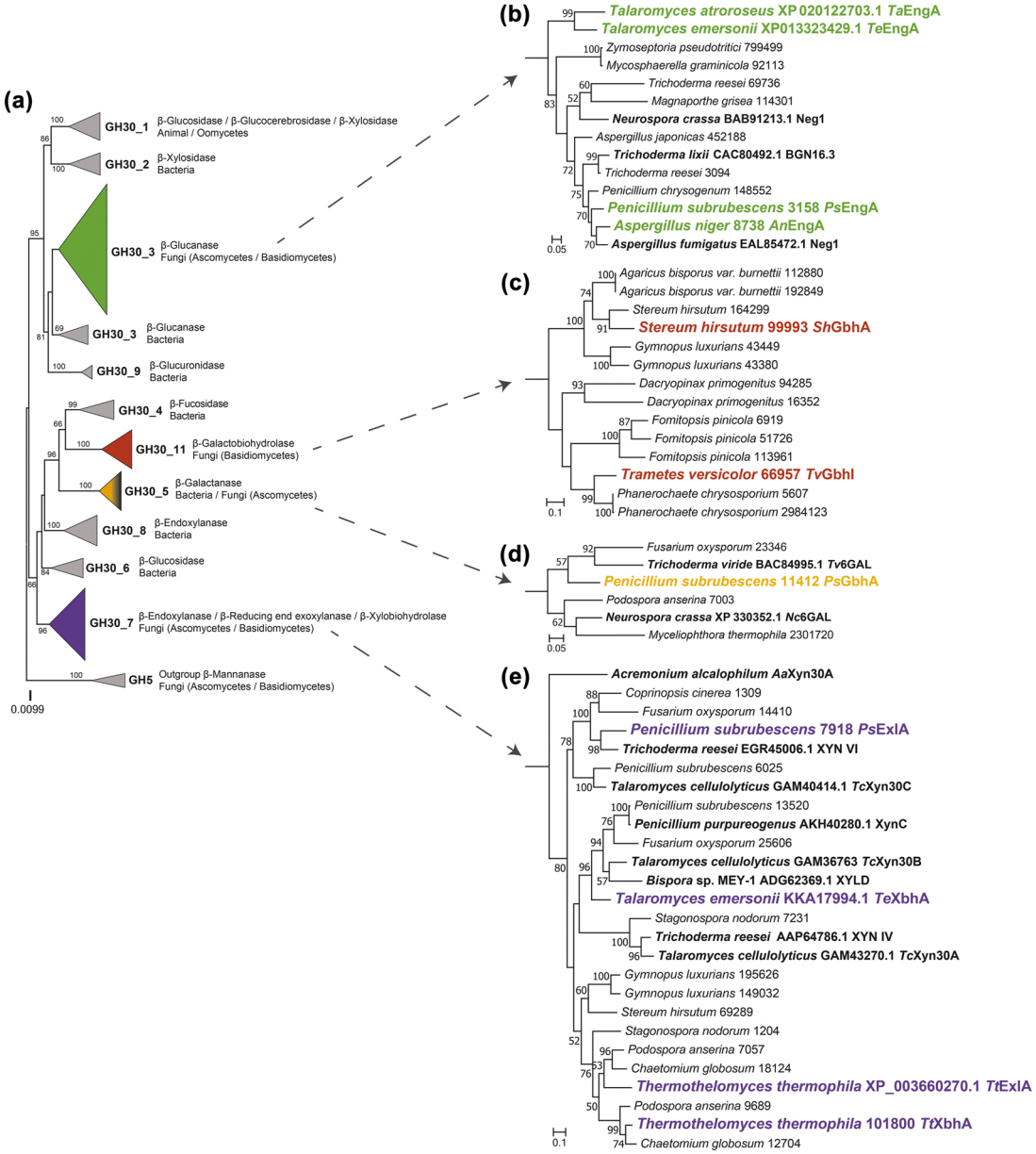

Fig. 1. Phylogenetic relationship among GH30 members from fungi, bacteria, and animals based on their amino acid sequences. The phylogenetic analysis was performed as previously reported [22]. The main subfamilies were collapsed. Statistical support for phylogenetic grouping was estimated by 500 bootstrap re-samplings, only the bootstrap above 50 % were shown on the clades. Five mannanases from GH5 were used as an outgroup. GH30_10 containing bacterial xylobiohydrolases was identified by Crooks et al. [7], which is not shown in the current GH30 tree. (a) Whole tree; (b) GH30_3; (c) GH30_11; (d) GH30_5; (e) GH30_7. Selected candidates are highlighted and the characterized enzymes are in boldface. The full phylogenetic tree can be found in Supplementary Figure S1.

application, be that for the production of specific short NDOs or for other biorefinery process.

Materials and methods

Materials

β-(1→6)-Glucan (Pustulan) was from Carbosynth Ltd. (Berkshire, UK). Beech wood xylan (BeWX) was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Wheat bran (WB) was from Wageningen Mill (Wageningen, the Netherlands). β-(1→3),(1→4)-Glucan (from Barley), insoluble wheat arabinoxylan (WAX, P-WAXYI, from wheat flour), α-L-arabinofuranosidase (ABF, E-AFASE, GH51 from *Aspergillus niger*), α-glucuronosidase (AGU, E-AGUBS, GH67 from *Geobacillus stearothermophilus*) and oligosaccharides were from Megazyme (Bray, Ireland). β-(1→4)-Glucan (Avicel®PH-101), β-(1→3)-glucan (from *Euglena gracilis*), β-(1→3),(1→6)-glucan (Laminarin), Larch wood arabinogalactan II (LWAG-II), Gum Arabic arabinogalactan II (GAAG-II), pectin (from potato, apple and esterified citrus), 4-*O*-methyl-D-glucurono-D-xylan (MGX) and other chemicals were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Bioinformatics and homology modelling

The fungal sequences from JGI Mycocosm (https://mycocosm.jgi.doe.gov/mycocosm/home), the sequences from characterized GH30 enzymes in CAZy database (http://www.cazy.org/), the additional sequences of bacteria and animals obtained by BLASTP from the non-redundant sequence database (https://www.ncbi.nlm.nih.gov/) were used for phylogenetic analysis (a list of fungal species is shown in Supplementary Table S1). The secretory signal peptides were detected using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The amino acid sequences without predicted signal peptides were aligned using MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/) and visualized using Easy Sequencing in Postscript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/index.php) [20,21]. The phylogenetic analysis was performed using maximum likelihood (ML) implemented in the Molecular Evolutionary Genetic Analysis software version 7 (MEGA7) as described previously [22] with 95 % partial deletion and the Poisson correction distance of substitution rates [23]. Statistical support for phylogenetic grouping was estimated by 500 bootstrap re-samplings. Only bootstrap values above 50 % are shown in the tree. Homology models were generated using the Homology detection and structure prediction by HMM-HMM comparison (HHpred, https://toolkit.tuebingen.mpg.de/tools/hhpred) [24]. The structure of *Tc*Xyn30B from *Talaromyces cellulolyticus* (PDB ID: 6KRN) [25] was used as a template. The quality of the models were validated as described previously [26]. To compare the putative catalytic residues, all models were superimposed on *Tc*Xyn30B complexed with glucuronyl-xylobiose (GlcA-X2) ligand (PDB ID: 6KRN) [25] and visualized by PyMOL 2.3.5 (Schrödinger, Inc., New York, NY). In addition, the structure of *Erwinia chrysanthemi Ec*Xyn30A complexed with glucuronyl-xylotriose (GlcA-X3) ligand (PDB ID: 2Y24) [27] was also used to define the putative subsites (–1 to –3) in the catalytic domain.

Table 1
Substrate specificity of characterized enzymes and selected candidates from fungal subfamilies of GH30.

Fungal species	Gene Name	Enzyme Name	Accession no.	Known activity	Linkage	Mainly active polysaccharides	SF	Reference
Aspergillus fumigatus	*neg1*	Neg1	EAL85472.1	Glucanase	β-(1→6)	β-(1→6)-glucan	3	[8]
Trichoderma harzianum	N.A.	BGN16.3	CAC80492.1	Glucanase	β-(1→6)	β-(1→6)-glucan	3	[9]
Neurospora crassa	*neg1*	Neg1	BAB91213.1	Glucanase	β-(1→6)	β-(1→6)-glucan	3	[10]
Penicillium subrubescens	*engA*	*Ps*EngA	jgi|Pensub1|3158	Glucanase	β-(1→6)	β-(1→6)-glucan	3	This study
Aspergillus niger	*engA*	*An*EngA	jgi|Aspni|8738	Glucanase	β-(1→6)	β-(1→6)-glucan	3	This study
Talaromyces atroroseus	*engA*	*Ta*EngA	XP020122703.1	Glucanase	β-(1→6)	β-(1→6)-glucan	3	This study
Talaromyces emersonii [a]	*engA*	*Te*EngA	XP013323429.1	Glucanase	β-(1→6)	β-(1→6)-glucan	3	This study
Lentinula edodes	*lepus30a*	*Le*Pus30A	BAK52530.1	Glucanase	β-(1→6)	β-(1→6)-glucan	3	[11]
Stereum hirsutum	*gbhA*	*Sh*GbhA	jgi|Stehi1|99993	Galactobiohydrolase	β-(1→6)	Type II arabinogalactan	11	This study
Trametes versicolor	*gbhI*	*Tv*GbhI	jgi|Trave1|66957	Galactobiohydrolase	β-(1→6)	Type II arabinogalactan	11	This study
Neurospora crassa	*Nc6GAL*	*Nc*6GAL	XP330352.1	Galactanase	β-(1→6)	Type II arabinogalactan	5	[13]
Penicillium subrubescens	*gbhA*	*Ps*GbhA	jgi|Pensub1|11412	Galactobiohydrolase	β-(1→6)	Type II arabinogalactan	5	This study
Trichoderma viride	*Tv6GAL*	*Tv*6GAL	BAC84995.1	Galactanase	β-(1→6)	Type II arabinogalactan	5	[38]
Talaromyces cellulolyticus	*xyn30C*	*Tc*Xyn30C	GAM40414.1	Endoxylanase	β-(1→4)	Glucurono- / arabinoxylan	7	[48]
Bispora sp. MEY-1	*xylD*	XYLD	ADG62369.1	Endoxylanase	β-(1→4)	Glucurono- / arabinoxylan	7	[50]
Penicillium purpureogenum [b]	*xynC*	XynC	AKH40280.1	Endoxylanase	β-(1→4)	Glucurono- / arabinoxylan	7	[51]
Trichoderma reesei	*XYN VI*	XYN VI	EGR45006.1	Endoxylanase	β-(1→4)	Glucuronoxylan	7	[40]
Penicillium subrubescens	*exlA*	*Ps*ExlA	jgi|Pensub1|7918	Endoxylanase	β-(1→4)	Glucuronoxylan	7	This study
Trichoderma reesei	*xyn4*	XYN IV	AAP64786.1	Endoxylanase / reducing end exoxylanases	β-(1→4)	Rhodymenan / glucuronoxylan	7	[52]
Talaromyces cellulolyticus	*xyn30A*	*Tc*Xyn30A	GAM43270.1	reducing end exoxylanases	β-(1→4)	Glucurono- / arabinoxylan	7	[53]
Acremonium alcalophilum	*AaXyn30A*	*Aa*Xyn30A	N.A.	Xylobiohydrolase	β-(1→4)	Rhodymenan / glucuronoxylan	7	[3]
Thermothelomyces thermophila [c]	*xbhA*	*Tt*XbhA	jgi|Spoth2|101800	Xylobiohydrolase	β-(1→4)	Glucuronoxylan	7	This study
Talaromyces emersonii	*xbhA*	*Te*XbhA	KKA17994.1	Xylobiohydrolase	β-(1→4)	Glucuronoxylan	7	This study
Talaromyces cellulolyticus	*xyn30B*	*Tc*Xyn30B	GAM36763	Endoxylanase / xylobiohydrolase	β-(1→4)	Glucuronoxylan	7	[39]
Thermothelomyces thermophila	*exlA*	*Tt*ExlA [d]	XP003660270.1	Endoxylanase / xylobiohydrolase	β-(1→4)	Glucuronoxylan	7	This study

N.A., not available. SF, subfamily.
[a] The homotypic synonym*: Rasamsonia emersonii.*
[b] The homotypic synonym*: Talaromyces purpureogenus.*
[c] The homotypic synonym*: Myceliophthora thermophila.*
[d] Formerly named TtXyn30A [4].

Cloning, protein production and purification of the selected candidates

The selected genes (Fig. 1, Table 1) without predicted signal peptides and introns were codon optimized and synthesized into pPicZαA plasmid (Genscript Biotech, Leiden, the Netherlands), which was then transformed into *Escherichia coli* DH5α (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) and subsequently transformed into *Pichia pastoris* strain X-33 (Invitrogen) according to the manufacturer's recommendation. The positive colonies were selected as described earlier [22].

The selected P. pastoris transformants were grown and induced for the production of enzyme as described previously [28]. Culture supernatants were harvested (8000 × *g*, 4 °C, 20 min). One portion was filtered (0.22 mm; Merck), aliquoted and stored at −20 °C (as crude enzyme). The other part was purified by ÄKTA FPLC System (GE Life Sciences, Uppsala, Sweden). Crude enzymes were loaded onto a HisTrap FF 1 mL column (Cytiva, Marlborough, USA) equilibrated with 20 mM HEPES, 0.4 M NaCl, 20 mM imidazole, pH 7.5, and eluted using a linear gradient of 22−400 mM imidazole in buffer mentioned above at a flow rate of 1.0 mL/min. Fractions containing enzyme were collected, concentrated and buffer-exchanged to 20 mM HEPES (pH 7.0) using 10 kDa cut-off ultrafiltration units Amicon (Millipore, Bedford, MA). All purification steps were performed at 4 °C [26]. The concentration of purified protein was evaluated as described earlier [22].

Enzyme activity assays

Activity screening using crude enzymes

The activity assays of crude enzymes were performed in 600 μL reaction mixtures containing 500 μL 1% (w/v) of the substrates (Fig. 2 and Supplementary Table S2) in 50 mM NaOAc buffer (pH 5.5) and 100 μL crude enzymes at 30 °C, 110 rpm for 16 h. The culture supernatant from *P. pastoris* harboring pPicZαA plasmid without insertion was used as a negative control for crude enzymes.

Activity assays using purified enzymes

The activity of the purified enzymes was assayed using 3 mg/mL of the selected polysaccharides in 50 mM NaOAc (pH 5.5) incubated with 6 μg/mL purified enzyme. HEPES buffer was used as a negative control for purified enzymes. The reaction was performed at 30 °C, 110 rpm, for up to 16 h, and aliquots of 500 μL were removed from the mixtures at different time points (5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 6 h and 16 h) for time course analysis.

To validate the activity of GH30_3 enzymes towards β-glucans with different linkages, 3 mg/mL β-glucans in 50 mM NaOAc (pH 5.5) were incubated with 6 or 60 μg/mL purified enzyme, respectively, at 30 °C for 16 h.

To investigate the effect of xylan substitutions on GH30_7 enzyme activity, MGX and WAX were used as representative substrates for 4-*O*-

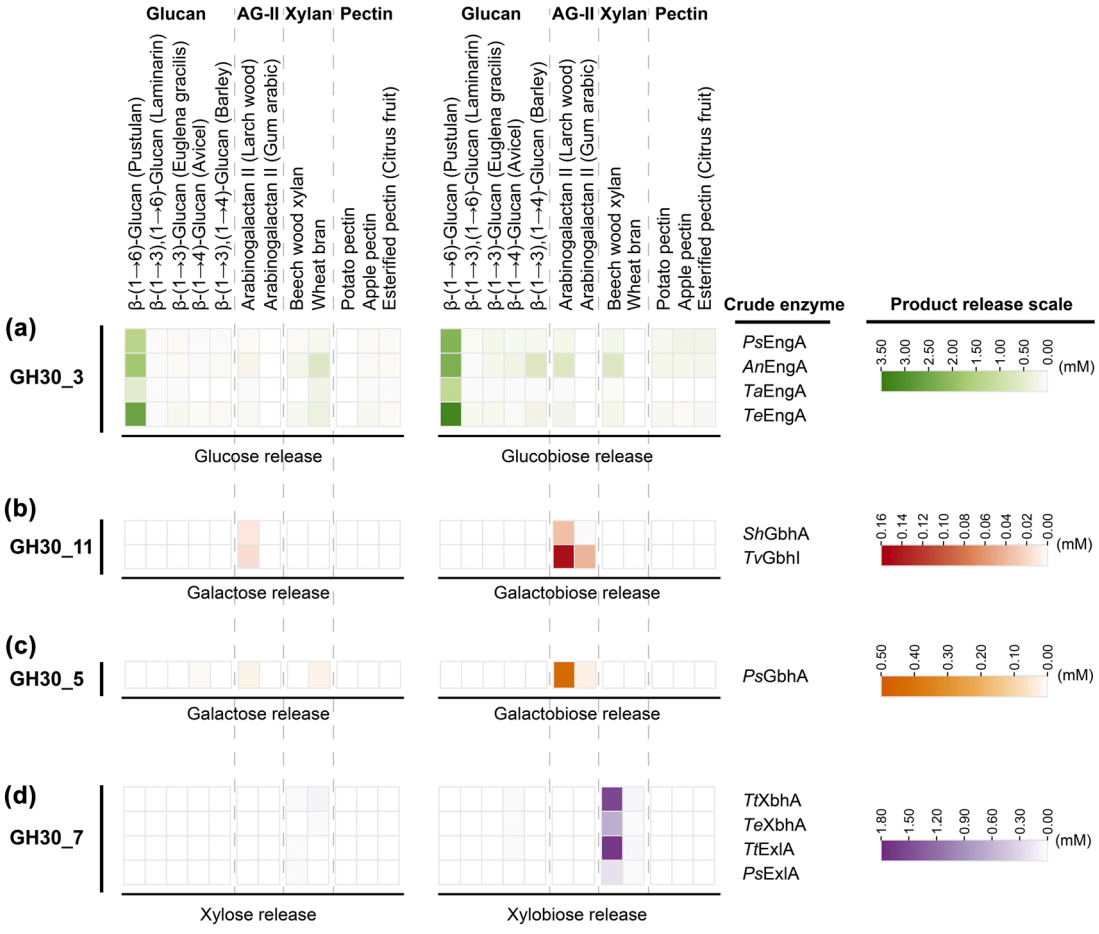

Fig. 2. Mono- and di-saccharides released from different substrates by crude GH30 enzymes. Hydrolysis was performed at 30 °C for 16 h. Saccharide release was detected by HPAEC-PAD. AG-II, arabinogalactan type II. (a)-(d) the type and amount of specific products released from different substrates by crude enzymes of each subfamily. The amount of glucobiose, galactobiose and xylobiose released was calculated based on cellobiose, β-(1→6)-galactobiose, and β-(1→4)-xylobiose standard curves, respectively. All assays were performed in duplicate.

methylglucuronic acid (MeGlcA) substituted and arabinofuranose substituted xylans, respectively [29]. 3 mg/mL MGX and WAX were incubated with 0.17 mg/mL AGU and ABF, respectively, in 50 mM NaOAc (pH 5.5) and 0.02 % NaN_3 at 30 °C, 110 rpm, for 72 h. The reaction was stopped by heating at 95 °C for 10 min. These are referred to as pre-treated substrates. For the activity assay, the reaction mixtures containing 550 µL 3 mg/mL MGX, WAX or pre-treated substrates and 50 µL purified GH30_7 enzymes (equal to approximately 3 µg protein) were incubated under the above-mentioned conditions.

Mono- and oligosaccharide analysis

All mentioned reactions were stopped by heat inactivation at 95 °C for 10 min and used for HPLAE-PAD analysis. The reaction mixtures were centrifuged (14,000 × *g*, 4 °C, 15 min) and the supernatant was diluted 10-fold in Ultrapure water (18.2 MΩ cm; Elga PURELAB flex, Marlow, UK) prior to the analysis. The mono- and oligosaccharide release was quantified using HPAEC-PAD (Dionex ICS-5000+ system) (Thermo Fisher Scientific, Sunnyvale, CA) equipped with a CarboPac PA1 (250 mm × 4 mm i.d.) column (Thermo Fisher Scientific) with different profiles according to [26] and [30], respectively. 5–250 µM different monosaccharides (glucose, galactose, arabinose, xylose) and disaccharides (cellobiose, β-(1→6)-glucobiose, β-(1→6)-galactobiose, β-(1→4)-xylobiose) were used as standards for identification and quantification. Total sugar composition of the used substrates was determined according to [31].

The released glucuronic acid was quantified using HPAEC-PAD in the same way as for monitoring monosaccharide release. The chromatographic separation was performed with a multi-step gradient according to [26] using the following procedure: 0–20 min: 18 mM NaOH, 20–30 min: 0–40 mM NaOH and 0–400 mM sodium acetate, 30–35 min: 40–100 mM NaOH and 400 mM to 1 M sodium acetate, 35–40 min: 100 mM NaOH and 1 M to 0 M sodium acetate, 10 min: 18 mM NaOH (20 °C; flow rate: 0.30 mL/min).

Results and discussion

Fungal GH30 enzymes belong to four subfamilies

Phylogenetic analysis of GH30 updated the fungal subfamilies included in the CAZy database (Fig. 1a and Supplementary Fig. S1) (http://www.cazy.org/GH30_characterized.html). Fungal members clustered into the previously defined GH30_3, 5 and 7 and also into the newly classified GH30_11 subfamily (this work). In the GH30 phylogenetic tree (*the order of description is based on the location of subfamilies in the tree*), GH30_3 members were further divided into two main clades based on different kingdoms, i.e. fungi and bacteria. The fungal clade was predicted to possess β-(1→6)-glucanase (ENG) activity based on earlier characterization [8–11] (Fig. 1a and b). The fungal GH30_11 shares the same node with the bacterial GH30_4, which suggests that they were more related to each other than to the other subfamilies. However, the sequence alignment showed low amino acid sequence identity (<28 %) between members from GH30_11 and GH30_4, indicating that they might differ in catalytic function. GH30_11 contained only sequences from basidiomycetes (Fig. 1c), but no characterized enzyme from this subfamily has been reported until now. A previous study found two β-D-fucosidases in GH30_4 [32]. Similar to GH30_3, members of GH30_5 were classified into two clades (fungi and bacteria). However, the fungal clade of GH30_5 only contained sequences from ascomycetes (Fig. 1d). The fungal and bacterial GH30_5 showing similar activity (β-(1→6)-galactanase) shared more than 50 % amino acid sequence identity [12,13,33]. GH30_7 contained sequences from both ascomycetes and basidiomycetes (Fig. 1e) and the characterized enzymes showed several xylanolytic activities (Table 1).

To evaluate different fungal subfamilies systematically and compare their activities and substrate specificity, eleven candidates from these four subfamilies were selected for biochemical characterization: four from GH30_3, two from the newly established GH30_11, one from GH30_5, and four from GH30_7.

Different fungal subfamilies target different substrates

The activity of culture supernatants was first screened towards several substrates (Fig. 2 and Supplementary Table S2): β-glucans with different linkages, arabinogalactan II (AG-II), and xylan and pectin from different sources. The screening results showed that selected candidates exhibited different linkage specificities, with GH30_3 candidates acting on glucosyl linkages, GH30_11 and GH30_5 candidates hydrolyzing galactosyl linkages, and GH30_7 candidates breaking xylosyl linkages. The corresponding mono- and di-saccharides released from hydrolyzing the tested substrates by different candidates are shown in Fig. 2.

GH30_3 candidates were active towards various substrates, but showed a preference for β-(1→6)-glucan (Fig. 2a). Their major disaccharide product was β-(1→6)-glucobiose, whereas minor products, e.g. β-(1→4)-glucobiose, were released from other substrates. The release of these saccharides from xylans and pectins were presumably due to the presence of small amounts of glucan or cellulose in those samples.

GH30_11 and GH30_5 candidates were mainly active against AG-II, especially Larch wood arabinogalactan II (LWAG-II), and released more β-(1→6)-galactobiose than monomeric galactose (Fig. 2b and c). AG-II consists of a β-(1→3)-galactan backbone substituted with β-(1→6)-galactooligosaccharides, whereas the galactans in pectin consist mainly of β-(1→4)-linkages [34–36]. The results suggested that GH30_11 and GH30_5 enzymes target β-(1→6)-galactan, which agreed with the previous reports of GH30_5 [12,13]. In addition, *Ps*GbhA also released a small amount of galactose from wheat bran (WB, Suppl. Table S2), which might be due to the presence of AG-II in WB [37].

GH30_7 were only active on xylan substrates, i.e. Beech wood xylan (BeWX) and WB, and showed a high preference for BeWX (Fig. 2d). The candidates from GH30_7 all released xylobiose as the major product from BeWX, while the production of other xylooligosaccharides (XOS) was enzyme dependent. This is discussed in more detail below.

Based on the initial screening results of the crude enzymes, β-(1→6)-glucan, LWAG-II and BeWX were selected as the main polysaccharides for more detailed characterization of candidate enzymes from GH30_3, GH30_11&GH30_5, and GH30_7, respectively.

Purified GH30_3 enzymes possess β-(1→6)-glucanase activity

The purified GH30_3 enzymes were not only tested on β-(1→6)-glucan as a substrate, but also on four other β-glucans with different linkages to further explore their linkage specificity. Unlike the crude GH30_3 candidates, which could hydrolyze β-glucans with different linkages, the purified GH30_3 candidates were solely active towards β-(1→6)-glucan. The main product was β-(1→6)-glucobiose followed by glucose and β-(1→6)-glucooligosaccharides (Fig. 3a). The release of β-(1→6)-glucobiose and glucose was also detected from β-(1→3),(1→6)-glucan after increasing the enzyme concentration 10-fold (data not shown). This showed that GH30_3 enzymes only have trace or unspecific activity towards other linkages. These results agreed with previous studies showing that *T. harzianum* BGN16.3 from ascomycete clade [9] and *L. edodes Le*Pus30A from basidiomycete clade [11] were mainly active towards β-(1→6)-glucan and had much lower activity towards β-(1→3),(1→6)-glucan. *Te*EngA was the most active GH30_3 enzyme tested here, and considerable release of β-(1→6)-glucobiose was observed within 1 h, which was then further hydrolyzed to glucose (Fig. 3b and c).

Purified GH30_11 and GH30_5 enzymes possess β-(1→6)-galactobiohydrolase activity

LWAG-II was selected as the main substrate for the purified GH30_11 and GH30_5 enzymes, which released only β-(1→6)-galactobiose from

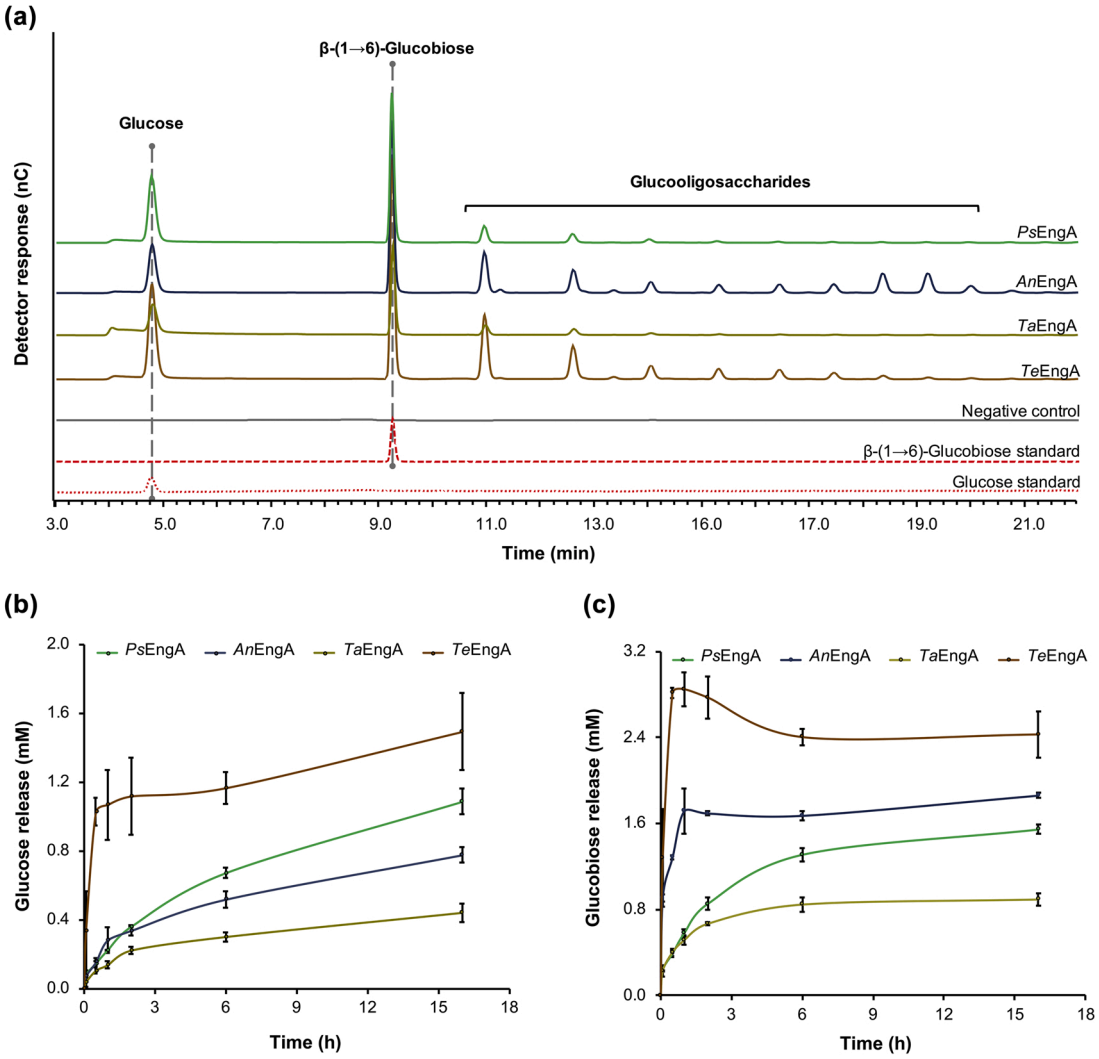

Fig. 3. Mono- and oligo-saccharides released from β-(1→6)-glucan by purified GH30_3 enzymes. (a) HPAEC-PAD profile of saccharide released in 16 h; (b) the amount of glucose released from β-(1→6)-glucan; (c) the amount of β-(1→6)-glucobiose released from β-(1→6)-glucan. Hydrolysis was performed in NaOAc (pH 5.5) at 30 °C for up to 16 h. All assays were performed in duplicate.

LWAG-II (Fig. 4a). Note that low amounts of galactose and arabinose were already present in LWAG-II (Fig. 4b), and no increased levels of these monosaccharides were detected after 16 h incubation. Hence, a specific β-(1→6)-galactobiohydrolase activity was concluded for these enzymes. For GH30_5, two fungal enzymes, i.e. *T. viride Tv*6GAL [38] and *N. crassa Nc*6GAL [13] have been previously characterized. Both of these enzymes showed endo β-(1→6)-galactanase activity, of which *Tv*6Gal released β-(1→6)-galactobiose as the major product from pre-treated arabinogalactan-protein from radish, while *Nc*6GAL specifically targeted β-(1→6)-galactooligosaccharides and released mainly β-(1→6)-galactobiose. No fungal GH30_11 enzyme has been previously characterized. Based on these results, β-(1→6)-galactobiohydrolase activity should be considered in the evaluation of fungal GH30_11 and GH30_5.

Of the GH30_11 and GH30_5 enzymes tested, *Sh*GbhA was the most active and released 2- and 4-fold higher amounts of β-(1→6)-galactobiose than *Tv*GbhI and *Ps*GbhA, respectively (Fig. 4c). In addition, *Sh*GbhA continuously released β-(1→6)-galactobiose over a period of 16 h, whereas *Ps*GbhA and *Tv*GbhI reached a saturation level after 1 h. This suggests different substrate specificity or stability among these enzymes. GH30_11 and GH30_5 enzymes (Fig. 4c) showed a limited ability to hydrolyse LWAG-II. This might be related to the structure of LWAG-II, which consists of a β-(1→3)-galactan backbone with β-(1→6)-galactooligosaccharide side chains. The side chains are highly variable in length and some are further substituted by arabinofuranose, which could affect the enzyme activity [36].

Purified GH30_7 enzymes possess xylanolytic activity with different product profiles

BeWX degradation revealed various activities of GH30_7 enzymes

BeWX was selected as the substrate for purified GH30_7 enzymes. *Tt*XbhA and *Te*XbhA released almost exclusively xylobiose from BeWX, while *Ps*ExlA released different MeGlcA substituted XOS (Fig. 5a), indicating that they possess xylobiohydrolase and endoxylanase activities, respectively. The product pattern of *Tt*XbhA and *Te*XbhA in this study differed slightly from the previously reported xylobiohydrolases (e.g. *Tt*Xyn30A, *Tc*Xyn30B, *Aa*Xyn30A), although they all released xylobiose as the major product from BeWX after a long time incubation. *Tt*XbhA and *Te*XbhA could release high-purity xylobiose from BeWX,

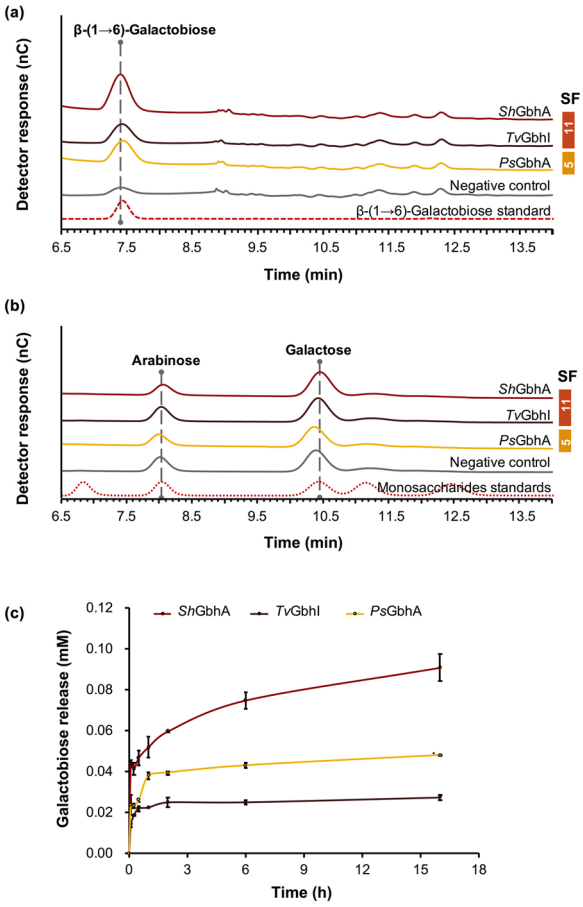

Fig. 4. Mono- and oligo-saccharides released from Larch wood arabinogalactan II (LWAG-II) by purified GH30_11 and GH30_5 enzymes. (a) HPAEC-PAD profile of galactooligosaccharides released from the hydrolysis of LWAG-II. Hydrolysis was performed at 30 °C for 16 h. (b) Monosaccharides released from the hydrolysis of LWAG-II; (c) the amount of β-(1→6)-galactobiose released. Hydrolysis was performed in NaOAc (pH 5.5) at 30 °C for up to 16 h. All assays were performed in duplicate. SF, subfamily.

whereas other oligosaccharides (e.g. × 4, X5) and a set of MeGlcA substituted XOS were also detected from the BeWX hydrolysis with *Tt*Xyn30A, *Tc*Xyn30B, and *Aa*Xyn30A [3,4,39]. *Tt*Xyn30A and *Tc*Xyn30B were reported to be bifunctional MeGlcA appendage-dependent xylobiohydrolases/endoxylanases [3,39], while AaXyn30A is a more strict fungal xylobiohydrolase [4]. AaXyn30A also shows slight endoxylanase activity, which was likely to be the result of excessive enzyme loading in the experimental setup [4,7].

*Ps*ExlA showed a similar hydrolytic product pattern to *T. reesei* XYN VI [40], both of which resembled the bacterial endoxylanases from GH30_8 [27,41–43]. *Tt*ExlA predominantly released xylobiose, but also different (MeGlcA substituted) XOS (Fig. 5a), similar to the earlier report [4], which shows that it is an endoxylanase with xylobiohydrolase activity. Among the four tested enzymes, *Tt*XbhA was the most suitable for xylobiose production as it released the highest amount of xylobiose (Fig. 5b).

Considering the different product profiles from BeWX (glucuronoxylan) hydrolysis, the effect of MeGlcA substitution on GH30_7 activity was investigated. Given the low level of MeGlcA substitutions in BeWX (mole ratio MeGlcA:Xyl = 1:15, [44]), 4-*O*-methyl-D-glucurono-D-xylan (MGX; mole ratio MeGlcA:Xyl = 1:5 [45]) was used instead of BeWX in this experiment. GH67 α-glucuronosidase (AGU) from *Geobacillus stearothermophilus* could partially hydrolyze the α-1,2-glycosidic bond between MeGlcA and terminal non-reducing xylosyl residues of xylooligosaccharides and xylan [46]. MGX contains approximately 0.1 mg total uronic acid per mg polysaccharide. Only around 8% MeGlcA (of the total uronic acid) was released from MGX by GH67 AGU. The comparison of the amount of xylobiose released from AGU-treated and untreated MGX revealed that the activity of *Te*XbhA was most affected. For this GH30_7 enzyme, a decrease in the yield of xylobiose was observed with the AGU-treated MGX, while the activity of the other enzymes was only slightly or not affected (Fig. 5d). It is not understood how such a minor decrease in the MeGlcA substitution could decrease the yield of xylobiose for TeXbhA. Further studies are required to address the fungal GH30_7 functional requirements for the MeGlcA and the yield of xylobiose.

WAX degradation confirmed the BeWX preference of GH30_7 enzymes

To validate the activity of GH30_7 enzymes towards different types of xylan, they were also analyzed using wheat arabinoxylan (WAX) as a substrate. *Tt*XbhA released around 10-fold less xylobiose from WAX than from BeWX, while the other enzymes showed only trace amounts or no xylobiose release from WAX (Fig. 5b and c). This agreed with most of the previously characterized enzymes from GH30_7, which also showed much lower activity against WAX than BeWX [3,4,40,39]. This could be due to the high arabinosyl substitution of WAX, which might hinder the binding of these enzymes to WAX.

To confirm the effect of arabinosyl substitution towards GH30_7 activity, WAX was treated with GH51 α-arabinofuranosidase (ABF) from *A. niger*, which partially hydrolyses mono- arabinosyl substitutions at *O*-3 and di- arabinosyl substitutions at *O*-2 and *O*-3 from WAX [47]. WAX contains about 0.38 mg arabinose per mg polysaccharide, of which around 30 % arabinose was released after incubation with GH51 ABF. The removal of arabinosyl substitution in WAX improved the release of xylobiose by all enzymes (Fig. 5e). *Tt*XbhA released 3-fold more xylobiose from ABF-treated WAX, and the other GH30_7 enzymes could also release detectable amount of xylobiose. These results indicate that the high degree of arabinosyl substitution hinders the accessibility of the enzyme to the xylan backbone.

Differences in the catalytic region of GH30_7 affect their substrate specificities

To investigate the catalytic mechanism of GH30 fungal enzymes, homology modelling analysis of selected candidates was used in this study. Currently, there is no structure available with >35 % amino acid sequence similarity to be used as a reliable template for the homology modeling of GH30_3, GH30_11 and GH30_5 enzymes (Supplementary Table 3). Hence, only the homology models of the enzymes from GH30_7 were created (Supplementary Table 3 and Fig. S3). *T. cellulolyticus Tc*Xyn30B (Supplementary Fig. S3a, PDB ID: 6KRN) [25] was used as a template for homology models of GH30_7 enzymes (Supplementary Fig. S3b-e). The putative subsites –1 to –3 are predicted based on the structure of *Tc*Xyn30B as well as the *E. chrysanthemi Ec*Xyn30A (Supplementary Fig. S3f; PDB ID: 2Y24) [27].

A comparison of the putative catalytic amino acids of GH30_7 enzymes showed that the residues in subsite –1 are highly conserved, while those in subsites –2 and –3 are less conserved (Table 2). Instead of the -3 subsite of *Ps*ExlA, a short loop was observed with *Tt*XbhA, *Te*XbhA and *Tt*ExlA (Supplementary Figs. S2 and S3b-e). This loop forms a steric barrier close to the catalytic site to accommodate two xylosyl residues, at subsites –1 and –2, explaining the xylobiose release [3,39]. Within the loop, two amino acids were reported to possibly contribute to xylobiohydrolase activity, i.e. N93 in *Tc*Xyn30B (corresponding to D88 of *Tt*XbhA, D78 of *Te*XbhA, and D78 of *Tt*ExlA) and W101 in *Aa*Xyn30A (corresponding to F89 of *Tt*XbhA, F79 of *Te*XbhA, and H79 of *Tt*ExlA) (Supplementary Figs. S2 and S3) [3,25].

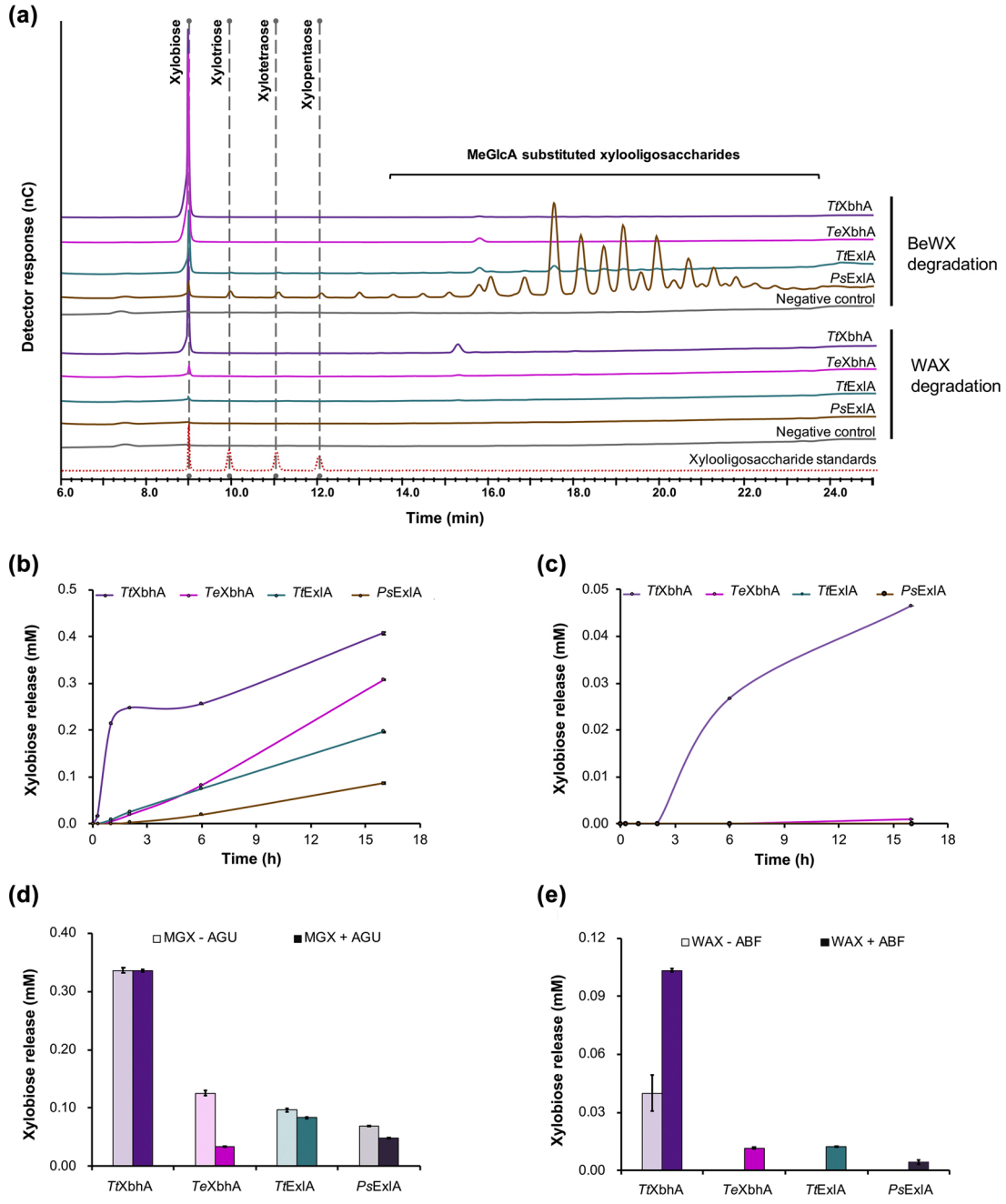

Fig. 5. Mono- and oligo-saccharides released from Beech wood xylan (BeWX) and wheat arabinoxylan (WAX) by purified GH30_7 enzymes. (a) HPAEC-PAD profile from the hydrolysis of BeWX (top) and WAX (bottom). Hydrolysis was performed at 30 °C for 16 h. (b) The amount of β-1→4-xylobiose released from BeWX; (c) the amount of β-(1→4)-xylobiose released from WAX. Hydrolysis was performed in NaOAc (pH 5.5) at 30 °C for up to 16 h. (d) The amount of β-(1→4)-xylobiose released from 4-*O*-methyl-D-glucurono-D-xylan treated with or without α-glucuronidase (MGX+/−AGU); (e) the amount of β-(1→4)-xylobiose released from WAX treated with or without arabinofuranosidase (WAX+/−ABF). All assays were performed in duplicate. The annotation was based on standards and previous research [49].

Conclusions

A combination of phylogenetic analysis and biochemical characterization of enzymes have led to the discovery of a new fungal subfamily in GH30, GH30_11, which displays β-(1→6)-galactobiohydrolase activity. The combined approach also helped to identify novel enzyme activities and confirmed that different fungal subfamilies harbored enzymes with distinct substrate specificities. However, while the different subfamilies act on different polysaccharides, they all mainly release 'short' non-digestible di- and oligosaccharides, which could be of interest in the food and feed industries. These findings contribute to understanding the fungal GH30 subfamily and facilitate industrial applications of fungal GH30 enzymes.

Funding

This work was partly supported by the China Scholarship Council (grant no: 201803250066).

Table 2
Putative subsite residues of the GH30_7 enzymes based on the comparison of homology models and reported structures[a].

Enzyme name	PDB ID	Ligand	Catalytic site	Putative subsite –1	Putative subsite –2	Short loop (putative subsite –3)
Template						
*Tc*Xyn30B (GH30_7)	6KRN	GlcA-X2	E202, E297	W141, N201, E202, Y279, E297, L301	F44, R46, Y209, W341, E345, S347, T349, **S351**	T91, S92, **N93**, **L94**, M95, N96
*Ec*Xyn30A (GH30_8)	2Y24	GlcA-X3	E165, E253	W113, N164, E165, E253	R293, W55, Y295, S258, Y255, W289	–
Models						
*Tt*XbhA	–	GlcA-X2	E198, E290	W137, N197, E198, Y272, E290, L294	F41, R43, Y205, W333, Q337, P341, T343, **E345**	N86, S87, **D88**, **F89**, M90, N91
*Te*XbhA	–	GlcA-X2	E187, E281	W126, N186, E187, Y263, E281, L285	F29, R31, Y194, W324, E328, G330, S332, **S334**	I76, K77, **D78**, **F79**, M80, N81
*Tt*ExlA	–	GlcA-X2	E188, E278	W127, N187, E188, Y260, E278, L282	F32, R34, Y195, W321, Q325, G327, T329, **S331**	S76, S77, **D78**, **H79**, M80, V81
*Ps*ExlA	–	GlcA-X2	E178, E270	W117, N177, E178, Y252, E270, E274	F29, R31, Y185, W315, E319, Q323, S325, **S327**	–

[a] The amino acids which could affect the substrate specificity are in boldface.

Author contributions

RPdV conceived and supervised the overall project. RPdV and AD selected the enzyme candidates from GH30 phylogenetic tree and designed the experiment. MAK and DK recommended and provided some substrates for experiment. XL and AD conducted the experiments. XL, DK and AD analyzed the data. XL and AD wrote the original draft. All authors commented on the manuscript.

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.

Acknowledgements

XL gratefully acknowledges the China Scholarship Council Scholarship.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2021.12.004.

References

[1] Shrivastava S. Industrial applications of glycoside hydrolases. Singapore: Springer; 2020. https://doi.org/10.1007/978-981-15-4767-6.
[2] Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 2014;(42):D490–495. https://doi.org/10.1093/nar/gkt1178.
[3] Šuchová K, Puchart V, Spodsberg N, Krogh KBM, Biely P. A novel GH30 xylobiohydrolase from *Acremonium alcalophilum* releasing xylobiose from the non-reducing end. Enzyme Microb Technol 2020;134:109484. https://doi.org/10.1016/j.enzmictec.2019.109484.
[4] Katsimpouras C, Dedes G, Thomaidis NS, Topakas E. A novel fungal GH30 xylanase with xylobiohydrolase auxiliary activity. Biotechnol Biofuels 2019;12:120. https://doi.org/10.1186/s13068-019-1455-2.
[5] St John FJ, González JM, Pozharski E. Consolidation of glycosyl hydrolase family 30: a dual domain 4/7 hydrolase family consisting of two structurally distinct groups. FEBS Lett 2010;584:4435–41. https://doi.org/10.1016/j.febslet.2010.09.051.
[6] Sakurama H, Kishino S, Uchibori Y, Yonejima Y, Ashida H, Kita K, et al. β-Glucuronidase from *Lactobacillus brevis* useful for baicalin hydrolysis belongs to glycoside hydrolase family 30. Appl Microbiol Biotechnol 2014;98:4021–32. https://doi.org/10.1007/s00253-013-5325-8.
[7] Crooks C, Bechle NJ, St John FJ. A new subfamily of Glycoside Hydrolase Family 30 with strict xylobiohydrolase function. Front Mol Biosci 2021;8:749. https://doi.org/10.3389/fmolb.2021.714238.
[8] Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 2005;438:1151–6. https://doi.org/10.1038/nature04332.
[9] Montero M, Sanz L, Rey M, Monte E, Llobell A. BGN16. 3, a novel acidic β-1, 6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413. FEBS J 2005;(272):3441–8. https://doi.org/10.1111/j.1742-4658.2005.04762.x.
[10] Oyama S, Yamagata Y, Abe K, NAKAJIMA T. Cloning and expression of an endo-1, 6-β-D-glucanase gene (neg1) from *Neurospora crassa*. Biosci Biotechnol Biochem 2002;66:1378–81. https://doi.org/10.1271/bbb.66.1378.
[11] Konno N, Sakamoto Y. An endo-β-1, 6-glucanase involved in *Lentinula edodes* fruiting body autolysis. Appl Microbiol Biotechnol 2011;91:1365–73. https://doi.org/10.1007/s00253-011-3295-2.
[12] Okemoto K, Uekita T, Tsumuraya Y, Hashimoto Y, Kasama T. Purification and characterization of an endo-β-(1→6)-galactanase from *Trichoderma viride*. Carbohydr Res 2003;338:219–30. https://doi.org/10.1016/s0008-6215(02)00405-6.
[13] Takata R, Tokita K, Mori S, Shimoda R, Harada N, Ichinose H, et al. Degradation of carbohydrate moieties of arabinogalactan-proteins by glycoside hydrolases from *Neurospora crassa*. Carbohydr Res 2010;345:2516–22. https://doi.org/10.1016/j.carres.2010.09.006.
[14] Meyer TSM, Miguel AM, Fernández DER, Ortiz GMD. Biotechnological production of oligosaccharides-applications in the food industry. In: Eissa AA, editor. Food production and industry. Rijeka: Intech; 2015. p. 25–78. https://doi.org/10.5772/60934.
[15] Patel S, Goyal A. Functional oligosaccharides: production, properties and applications. World J Microb Biot 2011;27:1119–28. https://doi.org/10.1007/s11274-010-0558-5.
[16] Swennen K, Courtin CM, Delcour JA. Non-digestible oligosaccharides with prebiotic properties. Crit Rev Food Sci Nutr 2006;46:459–71. https://doi.org/10.1080/10408390500215746.
[17] Lorito M, Peterbauer C, Hayes CK, Harman GE. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology 1994;140:623–9. https://doi.org/10.1099/00221287-140-3-623.
[18] Kottom TJ, Hebrink DM, Jenson PE, Gudmundsson G, Limper AH. Evidence for proinflammatory β-1, 6 glucans in the *Pneumocystis carinii* cell wall. Infect Immun 2015;83:2816–26. https://doi.org/10.1128/IAI.00196-15.
[19] Polizeli M, Rizzatti A, Monti R, Terenzi H, Jorge JA, Amorim D. Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 2005;67:577–91. https://doi.org/10.1007/s00253-005-1904-7.
[20] Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript server. Nucl Acids Res 2014;42:320–4. https://doi.org/10.1093/nar/gku316.
[21] Gouet P, Robert X, Courcelle E. ESPript/ENDscript: extracting and rendering sequence and 3D information from atomic structures of proteins. Nucl Acids Res 2003;31:3320–3. https://doi.org/10.1093/nar/gkg556.
[22] Li X, Griffin K, Langeveld S, Frommhagen M, Underlin EN, Kabel MA, et al. Functional validation of two fungal subfamilies in carbohydrate esterase family 1 by biochemical characterization of esterases from uncharacterized branches. Front Bioeng Biotechnol 2020;8:694. https://doi.org/10.3389/fbioe.2020.00694.
[23] Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016;33:1870–4. https://doi.org/10.1093/molbev/msw054.
[24] Söding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucl Acids Res 2005;33:244–8. https://doi.org/10.1093/nar/gki408.
[25] Nakamichi Y, Watanabe M, Matsushika A, Inoue H. Substrate recognition by a bifunctional GH30-7 xylanase B from *Talaromyces cellulolyticus*. FEBS Open Bio 2020;10:1180–9. https://doi.org/10.1002/2211-5463.12873.
[26] Cocono Linares N, Dilokpimol A, Stålbrand H, Mäkelä MR, de Vries RP. Recombinant production and characterization of six novel GH27 and GH36

α-galactosidases from *Penicillium subrubescens* and their synergism with a commercial mannanase during the hydrolysis of lignocellulosic biomass. Bioresour Technol 2020;295:122258. https://doi.org/10.1016/j.biortech.2019.122258.
[27] Urbániková Ľ, Vršanská M, Mørkeberg Krogh KB, Hoff T, Biely P. Structural basis for substrate recognition by *Erwinia chrysanthemi* GH30 glucuronoxylanase. FEBS J 2011;278:2105–16. https://doi.org/10.1111/j.1742-4658.2011.08127.x.
[28] Dilokpimol A, Mäkelä MR, Mansouri S, Belova O, Waterstraat M, Bunzel M, et al. Expanding the feruloyl esterase gene family of *Aspergillus niger* by characterization of a feruloyl esterase, FaeC. N Biotechnol 2017;37:200–9. https://doi.org/10.1016/j.nbt.2017.02.007.
[29] Karlsson EN, Schmitz E, Linares-Pastén JA, Adlercreutz P. Endo-xylanases as tools for production of substituted xylooligosaccharides with prebiotic properties. Appl Microbiol Biotechnol 2018;102:9081–8. https://doi.org/10.1007/s00253-018-9343-4.
[30] Mäkelä MR, Dilokpimol A, Koskela SM, Kuuskeri J, de Vries RP, Hilden K. Characterization of a feruloyl esterase from *Aspergillus terreus* facilitates the division of fungal enzymes from Carbohydrate Esterase family 1 of the carbohydrate-active enzymes (CAZy) database. Microb Biotechnol 2018;11:869–80. https://doi.org/10.1111/1751-7915.13273.
[31] Broxterman SE, van Erven G, Schols HA. The solubility of primary plant cell wall polysaccharides in LiCl-DMSO. Carbohydr Polym 2018;200:332–40. https://doi.org/10.1016/j.carbpol.2018.07.083.
[32] Yoshida S, Park DS, Bae B, Mackie R, Cann IKO, Nair SK. Structural and functional analyses of a glycoside hydrolase family 5 enzyme with an unexpected β-fucosidase activity. Biochemistry 2011;50:3369–75. https://doi.org/10.1021/bi200222u.
[33] Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M, et al. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Proc Natl Acad Sci USA 2001;98:12215–20. https://doi.org/10.1073/pnas.211433198.
[34] Sørensen SO, Pauly M, Bush M, Skjøt M, McCann MC, Borkhardt B, et al. Pectin engineering: modification of potato pectin by in vivo expression of an endo-1, 4-β-D-galactanase. Proc Natl Acad Sci USA 2000;97:7639–44. https://doi.org/10.1073/pnas.130568297.
[35] Wefers D, Flörchinger R, Bunzel M. Detailed structural characterization of arabinans and galactans of 14 apple cultivars before and after cold storage. Front Plant Sci 2018;9:1451. https://doi.org/10.3389/fpls.2018.01451.
[36] Fujita K, Sakamoto A, Kaneko S, Kotake T, Tsumuraya Y, Kitahara K. Degradative enzymes for type II arabinogalactan side chains in *Bifidobacterium longum subsp. longum*. Appl Microbiol Biotechnol 2019;103:1299–310. https://doi.org/10.1007/s00253-018-9566-4.
[37] Loosveld A, Maes C, Van Casteren W, Schols H, Grobet P, Delcour J. Structural variation and levels of water-extractable arabinogalactan-peptide in European wheat flours. Cereal Chem 1998;75:815–9. https://doi.org/10.1094/CCHEM.1998.75.6.815.
[38] Kotake T, Kaneko S, Kubomoto A, Haque MA, Kobayashi H, Tsumuraya Y. Molecular cloning and expression in *Escherichia coli* of a *Trichoderma viride* endo-beta-(1→6)-galactanase gene. Biochem J 2004;377:749–55. https://doi.org/10.1042/BJ20031145.
[39] Nakamichi Y, Fouquet T, Ito S, Watanabe M, Matsushika A, Inoue H. Structural and functional characterization of a bifunctional GH30-7 xylanase B from the filamentous fungus *Talaromyces cellulolyticus*. J Biol Chem 2019;294:4065–78. https://doi.org/10.1074/jbc.RA118.007207.
[40] Biely P, Puchart V, Stringer MA, Mørkeberg Krogh KB. *Trichoderma reesei* XYN VI–a novel appendage-dependent eukaryotic glucuronoxylan hydrolase. FEBS J 2014;281:3894–903. https://doi.org/10.1111/febs.12925.
[41] Sakka M, Tachino S, Katsuzaki H, van Dyk JS, Pletschke BI, Kimura T, et al. Characterization of Xyn30A and Axh43A of *Bacillus licheniformis* SVD1 identified by its genomic analysis. Enzyme Microb Technol 2012;51:193–9. https://doi.org/10.1016/j.enzmictec.2012.06.003.
[42] Valenzuela SV, Diaz P, Pastor FJ. Modular glucuronoxylan-specific xylanase with a family CBM35 carbohydrate-binding module. Appl Environ Microbiol 2012;78:3923–31. https://doi.org/10.1128/AEM.07932-11.
[43] Padilha IQ, Valenzuela Mayorga SV, Grisi T, Díaz Lucea P, de Araújo D. Pastor Blasco FIJ. A glucuronoxylan-specific xylanase from a new *Paenibacillus favisporus* strain isolated from tropical soil of Brazil. Int Microbiol 2014;17:175–84. https://doi.org/10.2436/20.1501.01.220.
[44] Teleman A, Tenkanen M, Jacobs A, Dahlman O. Characterization of *O*-acetyl-(4-*O*-methylglucurono) xylan isolated from birch and beech. Carbohydr Res 2002;337:373–7. https://doi.org/10.1016/S0008-6215(01)00327-5.
[45] Hurlbert JC, Preston IIIJF. Functional characterization of a novel xylanase from a corn strain of *Erwinia chrysanthemi*. J Bacteriol 2001;183:2093–100. https://doi.org/10.1128/JB.183.6.2093-2100.2001.
[46] Golan G, Shallom D, Teplitsky A, Zaide G, Shulami S, Baasov T, et al. Crystal structures of *Geobacillus stearothermophilus* α-glucuronidase complexed with its substrate and products: mechanistic implications. J Biol Chem 2004;279:3014–24.
[47] McCleary BV, McKie VA, Draga A, Rooney E, Mangan D, Larkin J. Hydrolysis of wheat flour arabinoxylan, acid-debranched wheat flour arabinoxylan and arabino-xylo-oligosaccharides by β-xylanase, α-L-arabinofuranosidase and β-xylosidase. Carbohydr Res 2015;407:79–96. https://doi.org/10.1016/j.carres.2015.01.017.
[48] Nakamichi Y, Fujii T, Fouquet T, Matsushika A, Inoue H. GH30-7 endoxylanase C from the filamentous fungus *Talaromyces cellulolyticus*. Appl Environ Microbiol 2019;85. https://doi.org/10.1128/AEM.01442-19. e01442-19.
[49] Martínez PM, Appeldoorn MM, Gruppen H, et al. The two *Rasamsonia emersonii* α-glucuronidases, ReGH67 and ReGH115, show a different mode-of-action towards glucuronoxylan and glucuronoxylo-oligosaccharides. Biotechnol Biofuels 2016;9:105. https://doi.org/10.1186/s13068-016-0519-9.
[50] Luo H, Yang J, Li J, Shi P, Huang H, Bai Y, et al. Molecular cloning and characterization of the novel acidic xylanase XYLD from *Bispora* sp. MEY-1 that is homologous to family 30 glycosyl hydrolases. Appl Microbiol Biotechnol 2010;86:1829–39. https://doi.org/10.1007/s00253-009-2410-0.
[51] Espinoza K, Eyzaguirre J. Identification, heterologous expression and characterization of a novel glycoside hydrolase family 30 xylanase from the fungus *Penicillium purpurogenum*. Carbohydr Res 2018;468:45–50. https://doi.org/10.1016/j.carres.2018.08.006.
[52] Tenkanen M, Vršanská M, Siika-aho M, Wong DW, Puchart V, Penttilä M, et al. Xylanase XYN IV from *Trichoderma reesei* showing exo-and endo-xylanase activity. FEBS J 2013;280:285–301. https://doi.org/10.1111/febs.12069.
[53] Nakamichi Y, Fouquet T, Ito S, Matsushika A, Inoue H. Mode of action of GH30-7 reducing-end xylose-releasing exoxylanase A (Xyn30A) from the filamentous fungus *Talaromyces cellulolyticus*. Appl Environ Microbiol 2019;85:e00552–00619. https://doi.org/10.1128/AEM.00552-19.