1 **Review Article:**

2 # Xylan Degrading Enzymes from Fungal Sources

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12 **Abstract**

13 Fungi have the ability to degrade xylan as the major component of plant cell wall
14 hemicellulose. Fungi have evolved batteries of xylanolytic enzymes that concertedly act to
15 depolymerise xylan backbones decorated with variable carbohydrate branches. As an
16 alternative to acid extraction in industrial processes the combination of endo-1,4-β-xylanase
17 and β-xylosidase can reduce xylan to xylose. However, unlike chemical extraction procedures
18 enzyme systems can selectively hydrolyse α-L-arabinofuranosyl, 4-O-methyl-α-D-
19 glucuronopyranosyl, acetyl and phenolic branches, and therefore have the potential to
20 deconstruct hemicellulose whilst retaining desirable structural integrity and functionality. The
21 sources, structures and catalytic activities of fungal xylanolytic enzymes are reviewed and
22 discussed in the context of their biotechnological potential.

23 **Key words:** Hemicellulose, Xylan, B-xylosidase, Endo-xylanase, Xylose

24 **1. General Overview of Biological Processing**

25 Biological processing of plant biomass has become a significant concern in the quest for
26 renewable energy. The initial focus of research has been the characterization and
27 quantification of lignocellulosic constituents within plants species. Consequently several
28 extraction methods leading to the hydrolysis of cellulose and hemicellulose fractions from
29 agricultural lignocellulosic biomass have been adopted, which include the use of concentrated
30 acids, alkali, hydrogen peroxide, steam explosion, hot water treatment, CO_2 explosion and
31 organic solvent treatments [1]. However the use of such harsh physical and chemical
32 treatments for hydrolysis can result in problems during post-treatment processes, especially if
33 the solubilised sugar products are be recovered as high value-added products for use in
34 synthetic chemistry or in the food industry. Plant cell wall degrading enzymes of microbial
35 origin have therefore attracted industrial attention since they have the potential to replace
36 toxic chemical treatments in current use and create new functional ingredients. The catalytic
37 performance and formulation of functional enzyme cocktails for hydrolyzing plant biomass
38 together with associated methods for the efficient recovery of oligosaccharides has been the
39 subject of research to establish commercial feasibilities.

40 The field of bioconversion has evolved into a broad multidisciplinary research base that
41 focuses on the (i) enhancement of enzyme efficiency and specificity, (ii) genetic
42 improvement of organisms to produce tailored plant cell wall degrading enzymes, (iii)
43 engineering of strains that can efficiently utilize pentose sugars and (iv) ethanol tolerance.
44 The work contained in this review article summarises the current research into the utilization
45 of hemicellulose, in particular reports the molecular characteristics of xylanases and β-
46 xylosidases from fungal sources, for industrial applications.

47 **2. Composition of Plant Biomass**

48 Lignocellulosic materials are composed of cellulose, hemicellulose, pectin, proteins and an

49 aromatic polymer lignin. Cellulose is a generally a linear polymer constructed of D-glucose
50 subunits associated by β-1,4 glycosidic bonds that have a propensity to form microfibrils.
51 Long chains of fibrils of cellulose are interconnected by hydrogen bonding to make it
52 crystalline in nature. Plant cellulose is often embedded within a matrix containing
53 hemicellulose and lignin [2, 3].

54 Lignin is a heterogeneous complex of polyphenolic compounds synthesized from
55 phenylpropanoid units such as *p*-coumaryl, coniferyl, syringyl and sinapyl alcohols that are
56 connected by non-hydrolyzable C-C and aryl-ether linkages. In addition lignin is also
57 interconnected to cellulose and hemicellulose to form a physical barrier and provide cell
58 walls with structural integrity in order to resist microbial attack and oxidative stress [4, 5].

59 Hemicellulose has a heteropolymeric structure, which is comprised of a linear xylan
60 backbone with short lateral side chains of different sugars. Xylan is the most abundant
61 constituent of hemicellulose in annual plants and hardwood species, and is comprised of a
62 linear chain of D-xylopyranosyl residues linked by β-1,4 glycosidic bonds [1, 3, 4]. The
63 lateral side chains linked to xylan are formed from mannan, galactan and arabinan polymers
64 or singularly attached D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, α-L-
65 arabinofuranosyl and 4-O-methyl-glucouronic, ferulic, acetic, p-coumaric and galacturonic
66 acids [6]. However, the constituents of side chains display large variations in composition
67 among plant species. For example in softwood xylans are not acetylated and contain α-1,3-
68 glycosidic bonds at C-3 positions, whereas hardwood xylans have high levels of acetylation
69 and emerge as 0-acetyl-4-0-methylglucuronoxylan [3].

70 The ratio of each polymer within lignocellulose materials have been reported in the literature
71 to vary between the sources of lignocellulose. The majority of the carbohydrate fraction is
72 composed of cellulose and hemicellulose is the second most abundant constituent. Hydrolysis
73 of hemicellulose into sugar monomers as well as cellulose would result in the consumption of

74 more than 60 % of plant materials into valuable products and result in reduced agricultural
75 waste.

76 **3. Breakdown of Lignocellulose**

77 Xylanolytic and cellulolytic complexes have been identified and explored in saprophytic soil
78 inhabiting fungi and bacteria as they require preformed organic compounds as energy sources
79 for cellular synthesis. Unlike other eukaryotes, fungi obtain nutritional requirements by
80 secreting polymer-degrading enzymes, from their hyphal tips, to their surroundings and
81 absorb enzymatic breakdown products [7]. Moreover, competent fungal species exhibit two
82 types of extracellular catalytic systems, firstly a hydrolytic system whereby polysaccharides
83 are hydrolysed and a second oxidative ligninolytic system opens phenyl rings for the
84 degradation of lignin [5,8]. However, not all fungi are able to synthesize ligninolytic
85 enzymes. This specialized activity is reserved largely to basidiomycetous white rot fungi and
86 plant pathogenic/saprophytic fungi, including *Armillaria mellea*, *Pleurotus ostreatus*,
87 *Phanerochaete chrysosporium, Echinodontium taxodii, Aspergillus* sp., *Fusarium* sp.,
88 *Ceriporiopsis subvermispora* and *Botrytis cinerea* that have the ability to produce such
89 enzymes [8-10].

90 **3.1 Lignin Biodegradation**

91 Lignin biodegradation is an oxidative process that utilises members of the phenol oxidase
92 family of enzymes. These comprise of lignin peroxidase, manganese peroxidase and laccases,
93 that collectively catalyze the oxidation of variable phenolic moieties. Laccases are of general
94 significance because of their non-selective ability to catalyse the oxidation of a variety of
95 substrates including diphenols, polyphenols, diamines, substituted phenols and aromatic
96 amines [8]. The peroxidases of lignocellulosic fungi utilise hydrogen peroxide that is
97 generated by the enzymes glyoxal oxidase and glucose-2-oxidase [11]. These enzymes have

98 been characterised in Basidiomycota group of white rot fungi including *Strobilurus*
99 *ohshimae*, *Echinodontium toxodii, Ceriporiopsis subvermispora, Phanerochaete*
100 *chrysosporium*, *Trametes versicolor* and *Bjerkandera adusta* which oxidise phenolic lignin
101 units to diffusible products that can be acquired through hyphal tips and then utilised by
102 intracellular catabolic pathways [8-11].

103 Once dissociated from lignin cellulose and hemicellulose are available to a wider range of
104 competitive microbial species [12]. The ability to degrade plant materials efficiently arises
105 from the multiple cellulolytic and xylanolytic isoenzymes, which have diverse biochemical
106 properties that have evolved through genetic selection for substrate availability and
107 competitive efficiency within microbial communities [13].

108 **3.2 Cellulose Biodegradation**

109 Cellulose degrading microorganisms play a key role in carbon cycle by reprocessing carbon
110 fixed by photosynthesis. Many filamentous fungi have been characterized to have a complete
111 cellulolytic system, with all the enzymes required to hydrolyse cellulose efficiently via
112 synergistic actions [14]. Cellulases comprise of three classes of hydrolytic enzymes that
113 hydrolyse β-1,4- glycosidic bonds: (i) Endoglucanases, (ii) Exoglucanases and (iii) β-
114 glucosidases.

115 Endoglucanases, also known as endo-1,4-β-glucanase or endocellulase, are responsible for
116 random cleavage of internal sites within cellulose fibres. Exoglucanases (synonyms: exo-1,4-
117 β-D-glucanase, cellobiohydrolases and exocellulase) remove short oligosaccharides from the
118 ends of glucan chains, which then allow β-glucosidase to hydrolyse cellooligosaccharide
119 chains and cellobiose into glucose [15,16]. In addition to the enzymes listed above novel
120 types of cellulase enzymes have been recently identified. Swollenin (SWOI) from *T. reesei*
121 has been described to disrupt cellulosic fibres without showing any hydrolytic activity. Their
122 role is analogous to plant expansins, which are thought to promote accessibility to cellulases

123 that depolymerise cellulose fibrils [11,17].

124 3.3 Hemicellulose Biodegradation

125 Unlike cellulose, hemicellulose has a more heterogenic structure that comprises of a linear
126 main chain of β-1,4 linked D-xylose backbone and short lateral side chains of different sugar
127 residues [18,19]. Complete depolymerisation of hemicellulose fractions, into xylose and other
128 monosaccharide sugars, requires a compiled cooperative action of a diverse range of
129 enzymes. Enzymatic hydrolysis of hemicellulose commences with the removal of side chains
130 that block the sites where xylanases cleave the xylan backbone. Endo-1, 4-β-xylanase
131 enzymes do not cleave the xylan backbone randomly but cleave the glycosidic bonds in a
132 selective manner depending on the chain length and degree of branching of substrate
133 molecules and the presence of its constituents [20]. The cleavage of the xylan backbone
134 yields xylo-oligosaccharides and the final trimming is carried out by β-xylosidase that
135 hydrolyses the β-1,4 glycosidic linkages of short chain xylo-oligosaccharides and xylobiose
136 from the non-reducing termini to release xylose monomers [21,22]. Accessory side chains are
137 further removed by α-glucuronidase, acetylxylan esterase, arabinase, β-mannosidase, α-L-
138 arabinofuranosidase and other hemicellulolytic esterases [6], as illustrated in Figure 1

139 4. Sources of Xylanolytic Complexes

140 Xylanolytic and cellulolytic complexes have been identified and characterized in saprophytic
141 soil inhabiting organisms and plant pathogens. Hydrolysis of plant tissues has been reported
142 as a unifying characteristic of plant pathogens and rotting organisms since these set of
143 enzymes play a role during softening of the region of penetration and invasion of host cell
144 wall structures for obtaining nutrients [23].

145 However xylanases and β-xylosidases also originate from plants such as maize, potato tubers,
146 barley and ripening strawberry, plum and pear fruits in which they are thought to mobilize

147 polymers and remodel plant cell walls [18,23,24]. Glycoside hydrolases from plants tend to
148 be produced during ripening processes that cause fruit tissues to become softer and play a
149 crucial role in cell enlargement, germination and other physiological processes (i.e budding,
150 senescence, post-pollination processes). Sugar starvation in *Arabidopsis* has been reported to
151 induce the production of glycosyl hydrolases including β-galactosidase, β-xylosidase and β-
152 glucosidase for the mobilisation of carbon storage reserves when photosynthesis is inhibited
153 [25]. Fewer glycoside hydrolases have been characterized from plant origins, although [18]
154 have reported the purification of a β-xylosidase from cell wall of maize during senescence
155 and reported that the enzyme worked optimally at 37 °C and pH 4.5. Under optimal
156 conditions kinetic constants, K_m and k_{cat}, against the synthetic substrate p-nitrophenyl-
157 xyloside were 2.5 mM and 6.5 sec^{-1} respectively, which is relatively low compared to β-
158 xylosidases of fungal origin. The purified enzyme provided promising results through
159 synergistic activity with *Tricoderma reesei* endoxylanase to increase xylose production by 94
160 % during the hydrolysis of corn stover.

161 Yeast species such as *Sugiyamaella xylanicola* [26], *Spathaspora brasiliensis*, *Spathasopra*
162 *xylofermentans* [27] and *Candida materiae* [28] have been isolated from rotting wood and
163 demonstrated to elaborate extracellular enzymes that include xylanolytic activities in the
164 presence of xylan as a sole carbon source. The ability of these species to grow and release
165 xylose raised the idea of using these species during fermentation of five carbon substrates.
166 *Spathasopra* species are known for their ability to ferment xylose producing ethanol and have
167 the potential to be used during bioethanol production by fermentation of agricultural
168 materials [27].

169 However, the filamentous fungi remain attractive sources of robust industrial enzymes since
170 they operate in extra-cellular environments and are readily recovered from fermentates to
171 ease downstream processing. β-xylosidases produced from yeasts, for example, are generally

172 cell-associated [29], whereas filamentous fungi tend to secrete the majority into the growth
173 medium.

174 Along with saprophytic and phytopathogenic mesophilic fungi, thermophilic fungi have
175 attracted attention from researchers as sources of thermostable enzymes. It is unusual for
176 fungi to breach the upper temperature boundaries of eukaryotes and produce enzymes which
177 are stable at high temperatures to achieve faster reaction rates, however such fungi that can
178 withstand temperatures of 50 ^{o}C are of potential biotechnological use [29]. Thermophilic
179 fungal species were discovered a century ago from self-heating hay sacks. Exothermic
180 reactions associated with saprophytic/mesophilic microorganisms result in favourable
181 temperatures for thermophilic fungal spores to germinate and compete for resources at
182 temperatures above 40 °C [14]. The genome sequences of several thermophilic filamentous
183 fungi have been determined. Mining these DNA sequences has established the presence of
184 redundant xylanolytic complexes that contain enzymes with the potential to operate at
185 elevated temperatures, such as those reported from *Talaromyces thermophilus*, *Myriococcum*
186 *thermophilum*, *Scytalidium thermophilum* and *Thermomyces lanuginosus* [30-32].

187 **5. Synergy between hemicellulose hydrolyzing enzymes**

188 Due to the complexity of the hemicellulose structure, hemicellulase enzymes tend to act in
189 concert with auxiliary enzymes. Three types of synergic relationships have been observed: (i)
190 homo-synergy between main chain cleaving enzymes (i.e. Endo-1,4-β-xylanases); (ii) hetero-
191 synergy between main chain cleaving and debranching enzymes (e.g. α-L-
192 arabinofuranosidases) and (iii) anti-synergy whereby one enzyme inhibits the activity of
193 another enzyme regardless whether the primary activity is main chain cleavage or
194 debranching [3]. Interactions between selected hemicellulases have been carried out on
195 different substrates in various combinations in order to uncover the best heterosynergic
196 activity for optimum hydrolysis and for the formulation of commercial enzyme cocktails. The

197 composition of these cocktails require careful consideration because xylan degrading
198 enzymes, like other plant cell wall degrading enzymes, exhibit variation in structure and
199 substrate specificities. Xylanolytic activities can operate as domain components of
200 multifunctional enzymes that include carbohydrate binding modules of different molecular
201 sizes with varying abilities to permeate branched substrates to access the main xylan
202 backbone.

203 In several studies, incubation of endo-1,4-β-xylanases with birchwood xylan have been
204 reported to yield short chain xylooligosaccharides, with xylobiose and xylotriose
205 predominating and minor amounts of xylose [30,33-35]. Addition of β-xylosidase to
206 birchwood xylan releases negligible amounts of xylose due to the presence of a limited
207 number of non-reducing ends that are the substrate for the enzyme [36]. The presence of
208 branching hetero substituents may also be hindering exo-cutting β-xylosidase activity (Figure
209 1). However, the combination of endo-1,4-β-xylanase and β-xylosidase has been confirmed
210 to enhance the release of reducing sugar from xylans up to 25 fold that of β-xylosidase
211 treatment alone. The catalytic activity of xylanase, could be classified as a heterosynergic
212 relationship since the action of xylanase produces more unsubstituted non-reducing ends for
213 β-xylosidase to attack, hence an effective increase in substrate concentration [3,36].

214 A combined treatment of β-xylosidase and arabinofuranosidase almost doubled xylose
215 release from water extractable wheat arabinoxylan compared to β-xylosidase treatment alone.
216 The removal of 1→3 linked arabinose from singly substituted xylopyranosyls near non-
217 reducing ends provided access for β-xylosidase [37]. The ability to degrade water soluble
218 wheat arabinoxylan into reducing monomers by a combination of endo-1,4-β-xylanase and β-
219 xylosidase could be increased 2.5-fold by the addition of α-L-arabinofuranosidase [37-42].

220 **6. Influence of Carbon Source on Xylanase and β-Xylosidase Production**

221 Hydrolysis of plant polysaccharides is essential for fungal organisms to obtain energy and
222 nutrients for growth. As a consequence filamentous fungi have evolved to secrete relatively
223 large quantities of enzymes that degrade plant carbohydrates into fermentable sugars. Distinct
224 substrates, including agricultural by-products such as wheat straw, corn cobs, sugar beet pulp,
225 corn stalks and selected carbohydrates (i.e. birch wood xylan, oat spell xylan and beech wood
226 xylan) are used for the induction of cellulolytic and hemicellulolytic enzymes under solid
227 state cultivation.

228 Studies have been conducted in order to test the response of fungal organisms to different
229 carbon sources within culture media and investigate expression patterns of enzymes. Model
230 organisms like *Neurospora crassa* have been demonstrated to sense the presence of cellulose
231 in the environment and induce lignocellulolytic enzymes in response to the exposure of
232 particular carbon source available. Expression of cellulases and hemicellulases in response to
233 Avicel (crystalline cellulose) has been reported to induce 17 of 21 predicted cellulase and 11
234 of 19 predicted hemicellulase genes in *Neurospora crassa* genome on the basis of RNA
235 sequencing [43,44]. Cellulolytic enzymes are synthesized in association with hemilolytic
236 enzymes when fungal organisms are actively grown on cellulose containing media owing to
237 traces quantities of xylan that often exist in conjunction with commercial preparations of
238 cellulose that are sufficient to trigger basal transcription of hemicellulase encoding
239 components [45].

240 Expression patterns of hemilolytic enzymes in response to a range of carbon sources have
241 been studied by several research groups with similar conclusions. Xylan as the sole carbon
242 source will lead to the expression of endo-xylanase and β-xylosidase synthesis, as
243 demonstrated in *Penicillium purpurogenum* [46], *Trichoderma reesei* [47], *Scytalidium*
244 *thermophilum* [48,49]. Xylose residues released from xylobiose or xylan has been suggested

245 to induce expression of xylanolytic complexes [20] but in some microorganisms the
246 accumulation of xylose at high concentrations (45 g/L) has been observed to have a
247 repressive effect on both xylanase and β-xylosidase expression, indicating that carbon
248 catabolite repression can be triggered by co-catabolic products [47]. The presence of glucose
249 severely reduces the levels of mycelial β-D-xylosidase achievable in *Scytalidium*
250 *thermophilum* and *Penicillium sclerotiorum*, indicating that β-D-xylosidase synthesis is
251 subject to carbon catabolite repression [48,50].

252 **7. Regulation of Hemicellulase Encoding Genes**

253 Filamentous fungi are thought to respond to short chain oligosaccharides that are initially
254 released from polymeric substrates, which include derivatives such as xylitol and arabinol
255 that have been considered to induce expression of xylanolytic enzymes [11,47]. Regulatory
256 elements and binding sites of transcriptional activators have been identified in the promoter
257 regions of the genes encoding cellulolytic and xylanolytic enzymes. The transcriptional
258 activator, XlnR, regulates the xylanase-encoding genes of *Aspergillus niger* [51]. XlnR
259 contains a zinc binuclear cluster DNA binding domain that binds to the consensus sequence
260 5'-GGCTAR-3' with orthologues in other fungal species [52-55]. Transcriptome analysis of
261 wild type *A. niger* and a XlnR knockout strain revealed 25 genes to be positively regulated by
262 XlnR that included genes of β-xylosidase, endoxylanase, arabinofuranohydrolase, xylose
263 reductase, cellulases and sugar transporters [56]. Klaubauf et al. [57] have further examined
264 the secretome profiles of wild type and XlnR mutants (*ΔxlnR*) of *Fusarium graminearum*,
265 *Magnaporthe oryzae*, *Trichoderma reesei*, *Aspergillus niger* and *Aspergillus nidulans* in the
266 presence of 25 mM glucose, 25 mM xylose and 1% (w/v) beechwood xylan medium. This
267 study concluded that the regulation of core set of xylanolytic enzymes including GH11 endo-
268 xylanase, GH3 and GH43 β-xylosidases, α-glucouronidase were highly dependent on XlnR
269 transcriptional regulator but other side-chain cleaving enzymes such as α-arabinofuranosidase

270 from *Aspergillus niger* and α-galactosidase from *Trichoderma reesei* and *Aspergillus*
271 *nidulans* were unaffected by the deletion of XlnR.
272

273 Several studies have demonstrated that the expression of fungal plant cell wall degrading
274 enzymes is subject to carbon catabolite repression [34,53,58,59]. The mechanism controlling
275 the preferential utilization of substrates over alternative carbon sources is mediated by the
276 wide-domain repressor CreA which alters the transcription of XlnR [34,53,58,60]. CreA
277 binds the promotors of the genes encoding the xylanolytic enzymes and the activator *XlnR* to
278 create a double lock mechanism in which CreA represses transcription of the activator and
279 the target genes.

280 **8. Biochemical Characterisation of Xylanolytic Complexes**

281 The majority of fungal xylanases are single subunit proteins of varying molecular sizes that
282 generally fall within the range of 20-60 kDa with the exception being reports of dimeric
283 xylanases from *Talaromyces emersonii* with molecular weights of 131 and 181 kDa [14,61].
284 Several eukaryotic endoxylanases occur as glycosylated enzymes in which carbohydrate
285 groups are covalently linked to the protein or are present in dissociable complexes [29].
286 Carbohydrate content of the three xylanases, Xa, XbI and XbII encoded by *Talaromyces*
287 *byssochlamydoides* have been reported as 36.6, 31.5 and 14.2 % respectively whilst
288 carbohydrate content was not reported for the xylanases encoded by *Talaromyces emersonii*
289 [61]. It is proposed that the presence of carbohydrate moieties associated with xylanases
290 allow the proteins to tolerate higher temperatures. Xylanases of mesophilic origin such as
291 *Aspergillus* and *Penicillium* species, exhibit pH optima ranging from 3 to 7 and exhibit
292 optimal activities at temperature ranging from 40 to 60 °C [34]. It is, however, notable that a
293 few fungi of mesophilic origin produce xylanases with increased thermal stability, for
294 example the xylanase of *Ceratocystis paradoxa*, remains active at 80 °C for 1 hour [29].

295 Endoxylanases from thermophilic organisms typically exhibit pH optima ranging from 4.5 to
296 6.5 and temperature optima in the range of 55 to 65 °C.

297 Fungal β-xylosidases are often characterized as monomeric glycoproteins, however, some
298 have been reported to comprise of more than one subunit such as *Humicola insolens* β-
299 xylosidase, which is characterized as a heterodimeric protein of 68 and 17 kDa subunits that
300 could have arisen by post-translational cleavage [62]. The β-xylosidases of *Neocallimastix*
301 *frontalis* and *Aspergillus pulverulentus* have also been characterized as dimeric enzymes with
302 molecular masses 180 and 190 kDa respectively [50]. Zanoelo *et al*. [63] characterized a cell
303 wall bound β-D-xylosidase from *Scytalidium thermophilum*, which has a carbohydrate
304 content of 12 % and a molecular weight of 45 kDa. Recombinant enzymes purified from
305 yeasts or filamentous fungi tend to show variation in molecular mass from the native enzyme
306 sources due to post-translational modifications. The β-xylosidases of *Talaromyces emersonii*
307 and *Trichoderma reesei* have been expressed in *Aspergillus oryzae*, and both recombinant
308 enzymes were estimated 15 kDa larger than their theoretical molecular masses [40]. The
309 methylotrophic yeast *Pichia pastoris* is commonly used for heterologous protein expression
310 studies tends to hyperglycosylate recombinant enzymes depending on the number of N-
311 glycosylation sites within the target protein. Fungal glycosyl hydrolase family 3 β-
312 xylosidases from *Neurospora crassa* and *Aspergillus oryzae* expressed in *Pichia pastoris*
313 were observed to be hyperglycosylated with predicted molecular masses of 81.8 and 84.7
314 kDa as compared with SDS-PAGE estimates between 120–180 kDa, 153–165 kDa
315 respectively [64,65]. Similarly a 65.6 kDa *Phanerochaete chrysosporium* β-xylosidase
316 expressed in *P. pastoris* to produce a protein 83 kDa mass as a result of post-translational
317 glycosylation.

318 In contrast to endoxylanases, β-xylosidases from mesophilic and thermophilic origin exhibit
319 optimal temperature ranges between 50 and 70 °C and their thermo-stability is highly

320 variable depending on structural differences. The pH optima of fungal β-xylosidases from
321 mesophilic and thermophilic origin differ with the formers ranging from pH 2.5-4 and the
322 latter pH 5–7 [21,30,45,46,63-67].

323 β-xylosidases have been reported to exhibit bifunctional activities against the synthetic
324 substrates 4-nitrophenyl-β-D-xylopyranoside (PNPX) and 4-nitrophenyl-α-L-
325 arabinofuranoside (PNPA) [64,68]. The majority of β-xylosidases studied to date have been
326 characterised against PNPX but it should be noted that enzyme behaviour and performance in
327 the natural environment or during industrial application may differ. For example, a V_{max} of
328 1052 μmol min^{-1} mg^{-1} was recorded for recombinant *N. crassa* β-xylosidase against PNPX as
329 compared with 10.2 μmol min^{-1} mg^{-1} against xylobiose with descending values for longer
330 chain xylooligosaccharide substrates [65]. Similarly *Aspergillus oryzae* KBN616 β-
331 xylosidase exhibited greater catalytic efficiency (V_{max} and k_{cat}) against the synthetic substrate
332 PNPX rather than naturally occurring xylooligosaccharides, with a V_{max} of 250 against PNPX
333 and 25.5 μmol min^{-1} mg^{-1} against xylobiose [64]. The structural configuration of the C-O-C
334 xylosidic bond between 1,4 xylose residues is markedly different to that presented by
335 xylopyranoside 4-nitrophenyl, which is likely to affect the positioning within the active site and
336 the rate of hydrolysis.

337 **9. Xylanase classification**

338 The glycosyl hydrolases have been classified according to their amino acid sequence directed
339 structures and catalytic mechanisms [69-71]. These classifications are available in the
340 Carbohydrate Active Enzymes database (CAZy) (http://www.cazy.org/Glycoside-
341 Hydrolases.html). Fungal xylanases (endo-1,4-β-xylanase, E.C.3.2.1.8) generally fall into two
342 families of the glycosyl hydrolases (GH) that were initially named F and G, and later
343 renamed as GH10 and 11 in the consolidated scheme [72]. However, enzymes with xylanase
344 activities associated with distinct catalytic domains can also be found in GH families 5, 7, 8
345 and 43. Exoxylanase activity (exo-1, 4-β-xylanase, E.C.3.2.1.37) has also been reported from
346 the fungus *Chaetomium thermophile* [73].

347 **9.1 Glycoside Hydrolase Family 10**

348 Family GH10 xylanases have a catalytic domain molecular mass of approximately 35 kDa
349 with the possibility of translational fusions to variable accessory domains that can feature
350 alternative catalytic domains or carbohydrate binding modules. The catalytic domain
351 structure is that of an eight-fold (β/α) barrel, commonly referred to as the TIM-barrel fold
352 (Figure 2A). Structural studies of the binding of xylooligosaccharides indicate that GH10
353 xylanases contain five xylopyranose subsites A-E and that hydrolysis occurs between subsites
354 D and E [74]. Catalysis proceeds via a double displacement mechanism that retains the
355 anomeric centre [75], using two glutamate residues located on the carboxy-terminal ends of
356 core β-strands 4 and 7 [76]. Comparative studies indicate family GH10 xylanases have higher
357 affinity for shorter linear β-1,4-xylooligosaccharides than family GH11, which has been
358 proposed to be as a consequence of smaller substrate binding sites [77]. This gives the family
359 GH10 enzymes greater flexibility with respect to the degree of polymerisation but restricts
360 the enzymes ability to cleave branched chain substrates. However, GH10 xylanase of the
361 thermophillic fungus *Thermoascus aurantiacus* has been reported to show 4-fold greater

362 activity against xylotriose in which the non-reducing moiety is linked to an arabinose side-
363 chain, compared to the undecorated form of the oligosaccharide [78]. High resolution X-ray
364 crystallographic structures of GH10 xylanases from the theromophiles *Thermoascus*
365 *aurantiacus* and *Thermomyces lanuginosus* have enabled comparisons with enzymes derived
366 from mesophiles to assess the structural determinants that confer thermostability to the
367 enzymes. *Thermoascus aurantiacus* GH10 xylanases were noted to benefit from
368 improvements in hydrophobic packing, favourable interactions between charged side chains
369 with helix dipoles, the introduction of prolines at the N-termini of helices [79] and the
370 formation of salt bridges [80].

371 Structural and complementary mutational studies have highlighted that the N- and C-terminal
372 residues of GH10 xylanase from *Aspergillus niger* are disordered and could destabilise non-
373 substrate bound monomers [81]. Removal of the five disordered residues located at the N-
374 terminus of the protein, with or without the presence of C-terminal disordered leucine
375 (residue 302), resulted in a 2 to 4-fold increase in the half-life of the recombinant enzymes at
376 50°C. These data highlight a role for the protein termini in structural stability that had
377 previously been ascribed potential roles in oligomerisation and thermostability of a
378 thermophillic *Bacillus* ssp. family GH10 xylanase [82].

379 **9.1 Glycoside Hydrolase Family 11**

380 Family GH11 xylanases vary markedly in their biochemical characteristics in terms of pI,
381 thermostability, pH profiles and catalytic properties [83]. However, they have a common core
382 structure of approximately 20-25 kDa molecular mass, which is composed of two anti-
383 parallel β-sheets in the form of a β-jelly-roll. The overall jelly-roll structure is folded over to
384 create an active site cleft. From the inner β-sheet two glutamate residues are orientated into
385 the cleft, which represent the catalytic residues of a double displacement mechanism similar
386 to that used by family GH10 xylanases (Figure 2B). The topology of GH11 xylanases have

387 been described to take the shape of a right hand, with the two β-sheets and α-helix resembling
388 the palm and fingers and two loop regions forming thumb and cord [84]. Several fungal
389 GH11 xylanases have been reported to contain disulphide bridges that could confer stability
390 to extra-cellular enzymes. Molecular dynamic studies indicate that the N-terminal regions of
391 GH11 xylanases initiate protein unfolding [85], and engineering an N-terminal disulfide bond
392 has been reported to enhance enzyme thermostability [86-88]. The xylanases from
393 *Aspergillus niger* [89], *Aspergillus kawachii* [90], and *Scytalidium acidophilum* [91] contain
394 disulfide bonds that connect the cord and to strand β12, whereas the xylanases from
395 *Paecilomyces varioti* [92] and *Thermomyces lanuginosus* [93] disulfide bonds are located
396 between an α-helix and strand β11. Two disulfide bridges were observed in the family GH11
397 xylanase of the anaerobic ruminal fungus *Neocallimastix patriciarum* but the disulfide
398 located in the N-terminal region serves to tether the N-terminal located α-helix to strand β14
399 [94].

400 **10. β-Xylosidase classification**

401 The CAZy database places β-xylosidases into GH families 3, 8, 30, 39, 43, 52, 54, 116, 120
402 but β-xylosidases of fungal origin are restricted to families 3, 43 and 54. In addition, GH
403 family 31 refers to α-xylosidase activities from *Aspergillus niger* and *Aspergillus nidulans*
404 that hydrolyse α-D-xylosidic linkages

405 Glycoside families 3, 31 and 54 function by a double-displacement mechanism, whereas GH
406 43 family enzymes typically operate by inversion of the anomeric centre where aspartate and
407 glutamate are claimed to be the catalytic nucleophile base and proton donor respectively.

408 **10.1 Glycoside Hydrolase Family 3**

409 The CAZy database contains 4669 family GH3 protein sequences of which 585 are of
410 eukaryotic origin and exhibit the following catalytic activities: β-glucosidase (EC 3.2.1.21),

411 xylan 1,4-β-xylosidase (EC 3.2.1.37), N-acetylhexosaminidase (EC 3.2.1.52), glucan 1,3-β-
412 glucosidase (EC 3.2.1.58), glucan 1,4-β-glucosidase (EC 3.2.1.74), exo-1,3-1,4-glucanase
413 (EC 3.2.1.-), α-L-arabinofuranosidase (EC 3.2.1.55), β-glucosylceramidase (EC 3.2.1.45),
414 isoprimeverose producing oligoxyloglucan hydrolase (EC 3.2.1.120), coniferin β-glucosidase
415 (EC 3.2.1.126) and β-N-acetylglucosaminide phosphorylases (EC 2.4.1.-).

416 Gene sequences encoding β-xylosidases classified within family GH3 have been reported
417 from several fungal sources including *Aspergillus niger* [95], *Aspergillus oryzae* [67],
418 *Aspergillus japonicus* [96], *Aspergillus nidulans* [97], *Neurospora crassa* [65], *Humicola*
419 *insolens* [98], *Trichoderma reesei* [47] and *Talaromyces emersonii* [99]. However, structural
420 information is only available for enzyme from *Trichoderma reesei*, for which small-angle X-
421 ray scattering data has been collected and compared with the crystal structure of barley β-D-
422 glucan exohydrolase [100]. These data suggest the *Trichoderma reesei* enzyme consists of
423 three domains as opposed to two characterised for the barley enzyme. The N-terminal domain
424 consists of an $(\alpha/\beta)_8$ TIM-barrel domain and the second an αβα sandwich with the third
425 remaining unclassified. Glycoside hydrolase family 3 enzymes perform catalytic reaction by
426 a double-displacement mechanism, in which two carboxylic acid residues located in the
427 active site are involved in the formation of a covalent glycosyl-enzyme intermediate. The GH
428 3 catalytic nucleophile, aspartate, is conserved across all family members and is located in the
429 N-terminal $(\alpha/\beta)_8$ TIM barrel domain [101]. The catalytic residues of the family GH3 β-
430 xylosidases of *Trichoderma reesei* and *Talaromyces emersonii* were probed by carbodiimide-
431 nucleophile modification, which resulted in complete inactivation of the enzymes suggesting
432 that carboxyl groups are required for catalysis, which is consistent with the candidate
433 aspartate and glutamate residues identified from protein sequence comparisons [40].

434 **10.2 Glycoside Hydrolase Family 43**

435 Glycoside hydrolase family 43 contains 312 of eukaryotic origin with the following enzyme

436 activities: β-xylosidase (EC 3.2.1.37), β-1,3-xylosidase (EC 3.2.1.-), α-L-arabinofuranosidase
437 (EC 3.2.1.55), arabinanase (EC 3.2.1.99), xylanase (EC 3.2.1.8) and galactan 1,3-β-
438 galactosidase (EC 3.2.1.145), α-1,2-L-arabinofuranosidase (EC 3.2.1.-), exo-α-1,5-L-
439 arabinofuranosidase (EC 3.2.1.-).

440 Fungal sources of family GH43 β-xylosidases include *Penicillium herquei* [102],
441 *Cochliobolus carbonum* [103], *Aspergillus oryzae* [104], *Thermomyces lanuginosus* [105]
442 and *Paecilomyces thermophila* [22]. Family GH43 β-xylosidases are predicted to have
443 molecular masses between 35-62 kDa that do not contain recognisable secretion signal
444 sequences, and are therefore likely to be cell associated enzymes.

445 To date structures for members have only been determined for β-xylosidases of bacterial
446 origin. However, Ravenal *et al* [106] have modelled two GH43 enzymes from *Penicillium*
447 *purpurogenum*, a bifunctional α-l-arabinofuranosidase/xylobiohydrolase and a β-xylosidase.
448 Like other members of the family the catalytic domains have a five blade β-propeller fold that
449 contains a two subsites and a funnel shaped active site [26,107]. During catalysis, the active
450 site harbours the substrate sugar molecule at the non-reducing end and the remaining sugar
451 backbone tends to be positioned at right angles to the enzyme tertiary structure. GH43
452 enzymes perform catalytic reaction by inversion of the anomeric centre. The catalytic
453 residues are believed to be formed by aspartate as a nucleophile base and glutamate as a
454 proton donor. The catalytic reaction is executed as a single displacement reaction in which
455 one carboxylate (in this case glutamate) protonates the substrate while a second (aspartate)
456 acts as a base to activate a nucleophilic water molecule, which results in a nucleophilic
457 attack, cleavage of the glycosidic bond and inversion at the anomeric carbon.

458 **10.3 Glycoside Hydrolase Family 54 and Family 31**

459 Glycoside hydrolase family contains 37 enzymes of eukaryotic origin with α-L-
460 arabinofuranosidase (EC 3.2.1.55) and β-xylosidase (EC 3.2.1.37) activities. The majority of

461 the proteins classified within GH family 54 have α-L-arabinofuranosidase activities but two
462 from *Aspergillus awamori* and *Hypocrea koningii* are bifunctional with α-L-
463 arabinofuranosidase/β-xylosidases activities against arabinose, xylobiose and arabinose
464 linked xylobiose [101]. There are no protein structures available for β-xylosidases however
465 an α-L-arabinofuranosidase (EC 3.2.1.55) structure has been determined from *Aspergillus*
466 *kawachii* [108]. The catalytic mechanism of GH Family 54 is considered to retain the
467 anomeric carbon.

468 There are over 600 GH family 31 members of eukaryotic origin with α-glucosidase (EC
469 3.2.1.20), α-1,3-glucosidase (EC 3.2.1.84), sucrase-isomaltase (EC 3.2.1.48), α-xylosidase
470 (EC 3.2.1.177), α-glucan lyase (EC 4.2.2.13), isomaltsyltransferase (EC 2.4.1.-), α-
471 mannosidase (EC 3.2.1.24) and oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161)
472 activities. To date only one α-xylosidase has been cloned and characterized from *Asperillus*
473 *niger* which is composed of 736 amino acids and exhibit activity against p-nitrophenyl-α-D-
474 xyloside but not against p-nitrophenyl-β-D-xyloside [108]. Based on α-xylosidase structures
475 of bacterial origin, GH31 catalytic domains are $(\beta/\alpha)_8$ barrel structures that operate by
476 retaining a mechanism with aspartate as the catalytic nucleophile and proton donor.

477 **11. Industrial Applications of Endo-xylanases and β-Xylosidases**

478 Despite the general cooperative action of xylanolytic enzymes, it is the endo-xylanases and β-
479 xylosidases that are most often used to solubilize xylan in industrial processes. Industrial
480 applications include bread making, xylitol production, fruit juice clarification, processing of
481 pre-digested animal feed, bioethanol production and paper pulp processing [1,109].

482 Robust extraction methods are employed in the processing of wood to pulp and paper in order
483 to separate cellulose fibrils from lignocellulosic materials. These processes include the use of
484 concentrated acids, alkalis, hydrogen peroxide, CO_2 explosion and organic solvent treatments
485 [1,20]. Finished white pulp is bleached with acid or toxic chlorine reagents at high

486 temperature and pressure, the by-products of which represent a threat to local ecosystems. To
487 comply with environmental regulations the harsh chemical methods have been replaced with
488 alternative chlorine free bleaching such as hydrogen peroxide, ozone, sodium hypochlorite
489 and biobleaching [110]. Enzymatic treatments using endo-xylanase rich cocktails have been
490 used to enhance delignification. Hemicellulases are used in enzyme assisted bleaching to
491 soften and swell the fibre structure to enhance the efficiency of bleaching chemicals used in
492 later treatment stages [29,109]. However, it is essential for the enzyme preparation to be
493 completely free of cellulase activity, the presence of which would impair pulp quality. Most
494 fungal species co-express cellulolytic and xylanolytic to utilise plant cell wall materials.
495 However, a few strains of fungi have been reported to produce cellulase-free thermotolerant
496 xylanases that degrade the xylan of lignocellulosic components, and are suitable for
497 application in pulp bleaching bioprocesses: *Aspergillus* sp. 2M1 [111], *Talaromyces*
498 *thermophiles* stolk AX4 [112] and *Thermomyces lanuginosus* SSBP (formally known as
499 *Humicola lanuginosa*) [113].

500 Xylan rich agricultural wastes hydrolysed into its monosaccharide constituents via enzymatic
501 treatments in a form of liquors containing 30 – 40 % xylose and 20 % other sugars (i.e.
502 arabinose, galactose, and mannose) are attractive renewable feedstocks for fermentations
503 aimed to produce bio-based chemicals [114]. The most widely produced xylan derived
504 product is xylitol, which is a popular ingredient in oral care and chewing gum products.
505 Xylitol is a 5-carbon alcohol, produced by the chemical hydrogenation of xylose, that has
506 been shown to exhibit antimicrobial properties, and specifically to reduce the growth of
507 dental plaque and decrease the incidence of dental caries [115]. Xylitol is also used as an
508 artificial sweetener with less than quarter of the calories of glucose, but critically it does not
509 induce insulin release in humans, which makes it a suitable substitute for individuals with
510 diabetes [115, 116]. Xylose fermenting yeasts such as the *Candida* species *C. maltosa*, *C.*

511 *tropicalis*, *C. guilliermondii* and *C. parapsilosis* have been extensively studied for their
512 capability to ferment xylose into xylitol using xylose reductase. Microbial reduction of xylose
513 enriched broth using *Candida maltosa*, was shown to yield 213 g L^{-1} xylitol from 250 g L^{-1}
514 xylose [117]. A combined environmentally low impact process has been reported for xylitol
515 bioconversion using hydrolysates of corncob and wheat bran (1:1 ratio) first generated by
516 solid state fermentation by *Aspergillus terreus*, and then utilised as feedstock for *Candida*
517 *tropicalis* to convert 75.14 % of the xylose to xylitol [118]. Genetic engineering methods
518 have also been used to create integrated xylitol production pathways in *Saccharomyces*
519 *cerevisiae* and *Candida tropicalis* [119,120]. *Saccharomyces cerevisiae* INVSc1
520 transformants co-expressing β-1,4-xylanase and β-xylosidase from *Aspergillus terreus* and
521 xylose reductase from *Candida tropicalis* was used in a semi-aerobic fermentation to produce
522 0.71 g xylitol/g xylan [119].

523 First generation bioethanol production was based on the fermentation of the easily
524 metabolisable sugar glucose, however much research has been carried out on the conversion
525 of hemicellulose hydrolysate into second generation ethanol production [114]. Ethanol is
526 either used directly as a chemical or as an additive to gasoline up to 20 % by volume. The
527 blend of ethanol into gasoline is an improved substitute of methyl tertiary butyl ether
528 (MTBE) that may provide cleaner combustion and reduce greenhouse gas emissions [1,5].
529 Efficient production of bioethanol from lignocellulosic materials requires organisms that are
530 capable of the complete hydrolysis of agricultural waste materials and fermenting organisms
531 that convert all types of monosaccharide constituents into ethanol. Metabolic engineering
532 approaches have been employed in order to produce new strains with hyper-production
533 phenotypes for hemicellulase production and generate *Saccharomyces cerevisiae* and *Pichia*
534 *stipitis* strains capable of ethanolic pentose fermentation [120,121].

535 Xylooligosaccharides obtained by enzymatic hydrolysis of hemicellulose have been reported

536 as useful bioactive ingredients of food and health products. Similar to xylitol,
537 xylooligosaccharides are moderately sweet compounds with no off-taste and no hazardous
538 properties and can be applied to foods, juices and carbonated beverages. A few studies have
539 reported that these short chain sugars variably support the growth of probiotic *Lactobacillus*
540 and *Bifidobacterium* species promoting a number of health benefits such as the inhibition of
541 pathogenic bacteria preventing gastro-intestinal infections and improved intestinal transit
542 [122,123].

543 The use of exogenous hydrolytic enzymes in animal feed has significant potential to improve
544 nutritive values and digestibility of feed. Commercially available enzymes were initially
545 developed as silage additives in order to obtain pre-digested animal feed, however nowadays
546 such enzymes are directly added into ruminant diets. These additives are approved to increase
547 the metabolizable energy and subsequently lead to animal weight gain and improvements in
548 milk production [124, 125].

549 Enzymatic clarification of fruit juices has become a popular process in juice production. The
550 utilization of pectinases along with cellulases, xylanases and β-xylosidases for the
551 degradation of all polymeric carbohydrates found in fruit pulp can improve yields. Freshly
552 pressed juice is turbid and viscous, and without processing the carbohydrate pieces tend to
553 settle during storage. The hydrolysis of polysaccharides weakens residual cell walls that
554 results in the release of cell wall materials and a reduction in the water holding capacity, this
555 leads to efficient juice recovery with reduced turbidity and an increase in the levels of
556 reducing sugars [126]. For example, treatment of pineapple juice with *Aspergillus niger*
557 DFR-5 xylanase has been reported provide a yield of 71.3 % and a clarity value of 64.7 %
558 [127]. Endo-xylanases and β-xylosidases are also used in the brewing industry on crushed
559 barley in order to solubilize arabinoxylans that can give beers a muddy appearance with
560 lower oligosaccharides. The hydrolysis of arabinoxylans in wort reduces the beer's turbidity

thus improving its appearance and viscosity [128,129].

Endo-xylanases and β-xylosidases are employed in the baking industry due to the abundance of hemicelluloses within cereals. Wheat flour is obtained by the milling process and consists of starch and arabinoxylan. The addition of several carbohydrase enzymes such as amylases, endo-xylanases and glucanases in post-milling processes tends to modify the quality of the flour. Endo-xylanases and β-xylosidases act on the arabinoxylan fraction of the dough to liberate the water retained in the arabinoxylan that helps even distribution of water and rise in viscosity [130]. The quality of bread obtained from enzyme treated dough was improved regarding dough rise, bread shape, loaf volume and crumb structure [131]. However, it should be noted that exogenous xylanases have to overcome an endogenous wheat xylanase inhibitor protein to maximise their effects in bread making [132].

12. Concluding remarks

There are an increasing number of fungal xylanolytic enzymes either characterised or under study. These studies are prompted by the importance of hemicellulose as an abundant carbohydrate in nature. Systematic approaches are likely to yield yet further enzymes and connections as to how they interact with plants as symbionts, pathogens and saprophytes. Despite the abundance of hemicellulose, it is an underutilised resource, either as a renewable bioenergy source or a source of complex chemicals. Biotechnological applications making use of xylanolytic enzymes are increasing and will continue to do so as the concerted actions of multi-enzyme systems become better understood.

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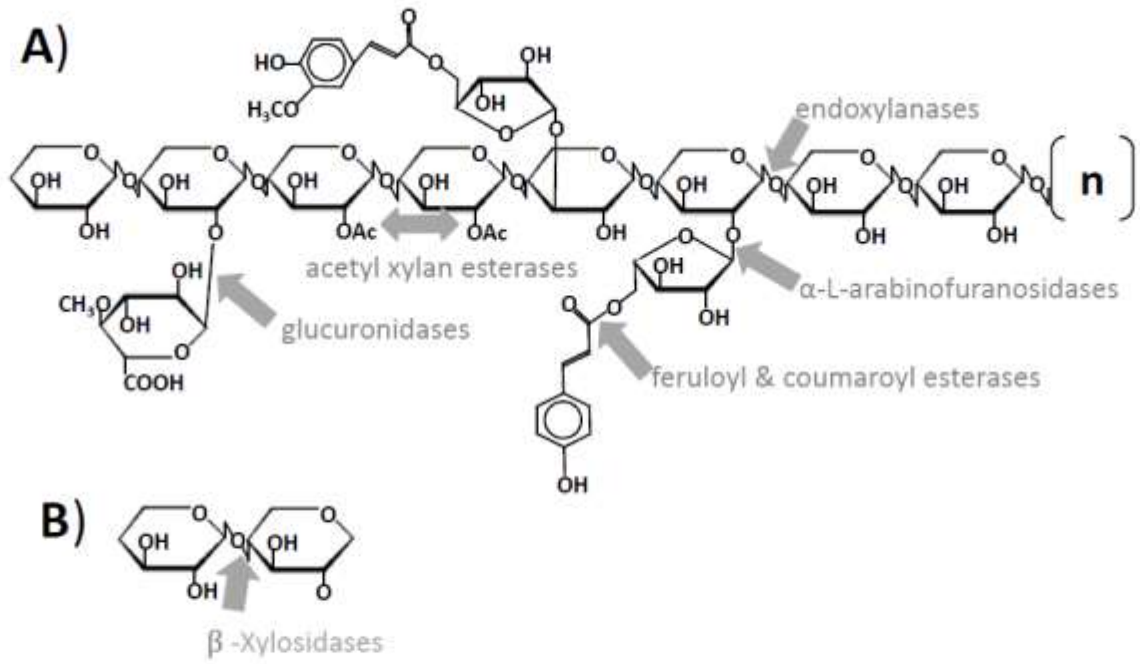
Figure Legends

Figure 1

A. Xylan polymer structure showing the actions of xylanolytic enzymes. The backbone of the substrate is composed of 1,4- β -linked xylose residues with branches of α-arabinofuranose; α -4-O-methylglucuronic acid; ferulic or p-coumaric acids. B. Hydrolysis of xylobiose and higher xylooligosaccharides by β -xylosidase.

Figure 2.

A. Structure of the family GH 10 xylanase from *Thermoascus aurantiacus* showing TIM-barrel fold with the catalytic glutamate residues projecting in to the active site cleft [80]. B. Structure of the family GH 11 xylanase from *Trichoderma reesei* showing β-jellyroll fold with the catalytic glutamate residues projecting in to the active site cleft [133].



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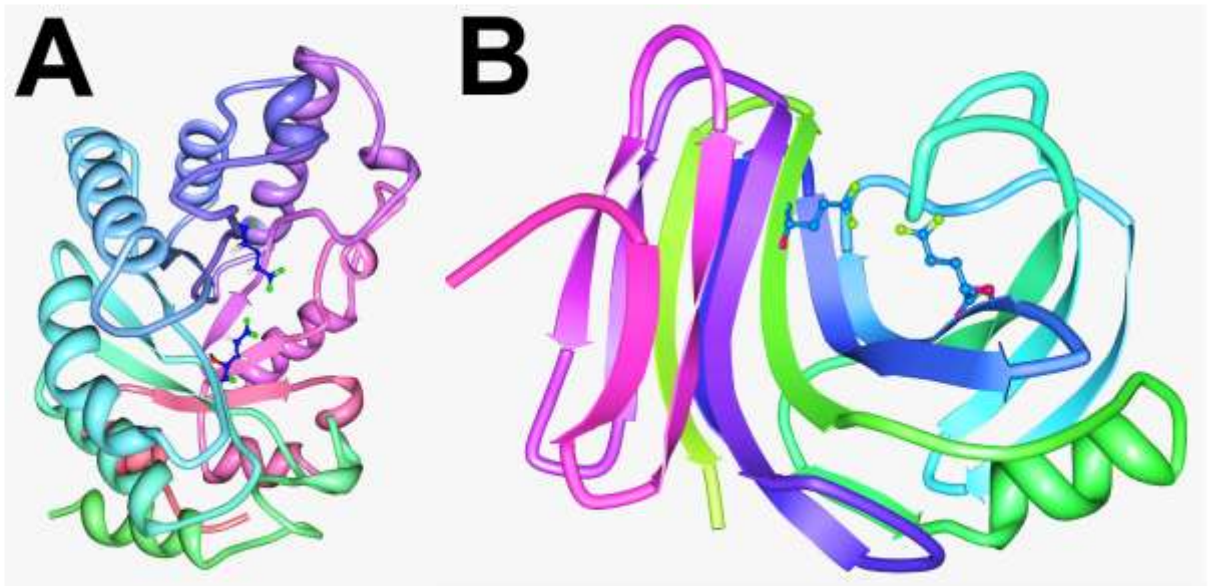

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