

1 **The presence of trace components significantly broadens the molecular response**  
2 **of *Aspergillus niger* to guar gum**

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15

16 **ABSTRACT**

17 Guar gum consists mainly of galactomannan, and constitutes the endosperm of guar seeds that acts as a  
18 reserve polysaccharide for germination. Due to its molecular structure and physical properties, this  
19 biopolymer has been considered as one of the most important and widely used gums in industry. However,  
20 for many of these applications this (hemi-)cellulosic structure needs to be modified or (partially)  
21 depolymerized in order to customize and improve its physicochemical properties. In this study,  
22 transcriptome, exoproteome and enzyme activity analyses were employed to decipher the complete  
23 enzymatic arsenal for guar gum depolymerization by *Aspergillus niger*. This multi-omic analysis revealed a  
24 set of 46 genes encoding carbohydrate-active enzymes (CAZymes) responding to the presence of guar  
25 gum, including CAZymes not only with preferred activity towards galactomannan, but also towards (arabino-  
26 )xylan, cellulose, starch and pectin, likely due to trace components in guar gum. This demonstrates that the  
27 purity of substrates has a strong effect on the resulting enzyme mixture produced by *A. niger* and probably  
28 by other fungi as well, which has significant implications for the commercial production of fungal enzyme  
29 cocktails.

30

31 *Keywords:* Guar gum; *Aspergillus niger*; CAZymes; Plant biomass degradation; Transcriptome;  
32 Exoproteome

33

## 34 Introduction

35 Guar gum is an abundant biopolymer obtained from *Cyamopsis tetragonolobus* seed endosperm,  
36 used by the plant as nutrient reserve [1]. It mainly consists of a linear backbone chain of  $\beta$ -1,4-linked  
37 mannose units with randomly attached  $\alpha$ -1,6-linked galactose units, chemically known as galactomannan  
38 [2]. It is therefore similar in structure to galacto(gluco)mannans that constitute one of the major components  
39 of softwood (coniferous trees) hemicellulose structure of lignocellulosic biomass and agricultural wastes  
40 [3]. The intrinsic properties and structure of this low cost material make it a target in many industrial  
41 applications such as food, pharmaceutical, cosmetic, textile, paper, and mineral industries [1,4]. However,  
42 the main obstacle to a more widespread utilization of this complex polysaccharide is the need to optimize  
43 and customize its depolymerization and modify its side-chain structure to obtain desired properties.  
44 Generally, gums are depolymerized by physico-chemical treatments, which often require long processing  
45 time and have a low degree of selectivity, and sometimes also change the structural integrity of the gum  
46 [1,4,5]. These disadvantages can be overcome using enzymatic hydrolysis [6].

47 Research into the industrial applications of guar gum has expanded significantly in recent years,  
48 but there is limited knowledge relating its enzymatic hydrolysis to the design of suitable enzyme mixtures  
49 that satisfy all the requirements of specific applications [4–6]. Most enzymes required to fully deconstruct  
50 plant biomass polymers are catalogued in the carbohydrate-active enzyme databases  
51 (CAZy) (<http://www.cazy.org/>), which can be classified into families of glycoside hydrolases (GHs),  
52 glycosyltransferases (GTs), carbohydrate esterases (CEs), polysaccharide lyases (PLs), and auxiliary  
53 activities (AAs) [7].

54 The hydrolysis of galactomannan containing polysaccharides requires the action of several  
55 enzymes such as  $\beta$ -mannanases,  $\beta$ -mannosidases,  $\alpha$ -galactosidases, and acetyl-mannan esterases  
56 [8]. The  $\beta$ -mannanases cleave the mannan backbone randomly to liberate oligosaccharides of varying  
57 lengths, while  $\beta$ -mannosidases are exo-acting enzymes that release monomeric D-mannose residues from  
58 these oligosaccharides [2]. The  $\alpha$ -galactosidases and acetyl-mannan esterases catalyze the release of  
59 galactose and acetyl groups from mannan, respectively [9,10]. Based on their amino acid sequence, fungal  
60  $\beta$ -mannanases are classified to CAZy families GH5, GH26 and GH134,  $\beta$ -mannosidases to GH2,  $\alpha$ -  
61 galactosidases to GH27 and GH36, and acetyl-mannan esterases to CE16 family [8,10]. In nature, many

62 fungi secrete a broad set of these CAZymes, but the filamentous fungus *Aspergillus niger* is among the  
63 most important microorganisms capable of producing a variety of CAZymes of industrial interest at low cost  
64 [11].

65 Since the deconstruction of complex polysaccharides by fungi occurs mainly extracellularly,  
66 combining transcriptome and exoproteome analyses provides a complementary approach to identify the  
67 enzyme mixtures fungi use for plant biomass deconstruction [12]. To our knowledge, no transcriptomic or  
68 exoproteomic study of fungi grown on guar gum has so far been conducted. In the present study, a multi-  
69 omic approach and enzyme assays were used to decipher the complete enzymatic arsenal for guar gum  
70 depolymerization by the highly efficient plant biomass decomposer *A. niger*. Surprisingly, we found that  
71 trace components of guar gum (Table 1) strongly affected the set of genes/enzymes expressed/produced.

72

## 73 **Materials and methods**

### 74 *Strain and culture conditions*

75 *Aspergillus niger* N402 was propagated and grown on complete medium (CM) or minimal medium  
76 (MM) [13]. Liquid cultures were grown in a rotary shaker at 250 rpm and 30°C. Transfer experiment was  
77 performed by growing the strain for 16 h in 1 L Erlenmeyer flasks that contained 250 mL CM supplemented  
78 with 2% D-fructose as carbon source. The mycelium was harvested by filtration and washed with MM without  
79 a carbon source. One gram (wet weight) of the mycelium was transferred to 250 mL Erlenmeyer flasks  
80 containing 50 mL MM supplemented with either 25 mM D-glucose (Sigma-Aldrich), 25 mM D-mannose  
81 (Sigma-Aldrich), 25 mM D-galactose (Sigma-Aldrich), 1% mannan (1,4-β-D-mannan from carob  
82 galactomannan, Megazyme) or 1% guar gum (Sigma-Aldrich) and incubated for 2 h. Mannan and guar gum  
83 cultures were also incubated for 8 h, 24 h and 48 h. All cultures were performed as biological duplicates.  
84 Mycelium was harvested by vacuum filtration, dried between towels and frozen in liquid nitrogen. The  
85 mycelium samples were stored at -80°C prior to RNA isolation.

86

### 87 *RNA isolation and microarray preparation*

88 RNA samples for microarray analysis were prepared using TRIzol (Invitrogen) according to the  
89 instructions of the supplier. RNA was purified using a Nucleospin RNA cleanup kit (Macherey-Nagel GmBh).  
90 The RNA integrity and concentration was verified with an Agilent 2100 Bioanalyzer using a RNA6000  
91 LabChip kit (Agilent Technology). The RNA samples were amplified, labeled, hybridized, washed, and  
92 stained as described previously [14]. The microarray hybridization using the Affymetrix *A. niger* GeneChips  
93 (Affymetrix) was carried out by GenomeScan B.V. (The Netherlands, Leiden).

94

#### 95 *Analysis of transcriptome data*

96 The microarray datasets retrieved from GenomeScan were processed under the Bioconductor  
97 software package version 2.8 (<http://www.bioconductor.org/>) together with house-made scripts performed  
98 in Perl (version 5.0) and Python (version 3.0). Utilizing the R statistical environment, the probe intensities  
99 were normalized for background using the robust multiarray average method [15] with only perfect match  
100 (PM) probes. The quantiles algorithm was used to perform normalization [16]. The normalized gene  
101 expression values were then analyzed with the statistical Cyber-T tool package (<http://cybert.ics.uci.edu/>)  
102 and edgeR to detect differentially expressed genes (DEGs). A false discovery rate (FDR) cut-off value of  
103 adjusted  $p < 0.05$  was set to assess statistical significance. Principal component analysis (PCA) using R  
104 programming language was performed to verify if the biological replicates were sufficiently similar. Only  
105 DEGs with FDR values lower than 0.05 and  $\log_2$ -fold changes greater than 1.0 were considered  
106 upregulated. The gene expression values were calculated by the median polish summary method [15]. The  
107 transcriptome data generated and used in this study has been deposited at the Gene Expression Omnibus  
108 (GEO) database with accession number: GSE119310 and GSE98572.

109

#### 110 *Gene expression clustering, visualization and annotation*

111 Averaged values of all replicates were hierarchically clustered with the R package-"gplots" using  
112 the parameters of Euclidean distance and complete linkage method. Clusters were set manually based on  
113 the branch-length differences of the gene-tree. The putative functional annotations of the plant biomass  
114 targeted CAZy genes were based on previous studies with the *A. niger* strain CBS 513.88 [14,17], as well  
115 as on manual phylogenetic analysis.

116

117 *Sample preparation for exoproteomic analysis*

118           The extracellular proteins were harvested from the *A. niger* liquid cultures grown on guar gum or  
119 mannan after 24 h and 48 h of cultivation. The culture liquids were separated from the mycelia by filtering  
120 through Miracloth (Millipore), after which the collected liquid fractions were centrifuged at 4°C for 1h at 3500  
121 × *g*. Proteins were extracted from the culture liquids by precipitation with two volumes of chilled 80%  
122 acetone (Sigma-Aldrich), 20% trichloroacetic acid (Sigma-Aldrich) and 20 mM DTT (Promega). The  
123 samples were incubated on ice for 1 h and centrifuged at 3200 × *g* for 30 min. The obtained pellet was re-  
124 dissolved in chilled 20 mM DTT and 80% acetone, and incubated at -20°C overnight. The mixture was  
125 centrifuged at 3200 × *g* for 30 min and the obtained pellet was dried and resuspended in 50 µl of 0.25%  
126 (w/v) Anionic Acid Labile Surfactant I (AALSI, Progenta™, Protea Biosciences) containing 20 mM  
127 ammonium bicarbonate (pH 7.8). Five micrograms of each fraction was treated with 10 mM DTT and 55  
128 mM iodoacetamide and subjected to in-solution overnight trypsin digestion. The digestion solutions were  
129 desalted using C18 ziptips (Millipore, Billerica). The peptides were resuspended in 60 µL of 5% acetonitrile  
130 and 0.1% formic acid.

131           Five microliters of peptide digest was loaded onto 10 mm × 75 µm PicoFrit column (New Objective,  
132 Woburn, MA) packed with Jupiter 5 µm, 300 Å, C18 resin (Phenomemex, Torrance, CA) connected with a  
133 Velos LTQ-Orbitrap mass spectrometer (Thermo-Fisher). Peptide separation was done using a 90 min  
134 linear gradient generated by an Easy-LC II Nano-HPLC system (Thermo-Fisher). The acquired shotgun  
135 liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) data were searched against the  
136 *A. niger* strain NRRL3 protein databases downloaded from JGI MycoCosm  
137 ([https://genome.jgi.doe.gov/Aspni\\_NRRL3\\_1/Aspni\\_NRRL3\\_1.home.html](https://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html)) corresponding to the best hit  
138 proteins. Protein quantification analysis was based on MS precursor ion signal determined by the Proteome  
139 Discoverer Quant 1.3 (Thermo-Fisher) software using the extracted ion chromatograms for the *m/z* values  
140 of the three of the most intense peptide assigned to each protein. These values were normalized using the  
141 values calculated for the spiked pre-digested Bovine Serum Albumin (Michrom, Auburn, CA) internal  
142 standard to obtain the Ion Profile (IP) of each protein.

143

144 *Exoproteomic data analysis*

145           The normalized protein areas were used as the measurement of abundance level in the  
146 exoproteome analysis. Normalized values for all substrates are included in the Supplementary Table S1.  
147 For each protein at each time point, two biological replicates were tested and averaged. Differential protein  
148 expression data was filtered by Student *t*-test with a *p*-value cutoff 0.05. Relative protein abundance was  
149 expressed as % of total protein (% TE) values using the individually determined IP values within the same  
150 sample, and visualized with ggplot2 package in the R environment.

151

152 *Protein concentration measurements and enzyme activity assays*

153           The total extracellular protein concentrations were measured using the Pierce BCA Protein Assay  
154 Kit (Thermo Scientific) according to the manufacturer's instructions. Extracellular  $\alpha$ -galactosidase (AGL),  
155  $\beta$ -glucosidase (BGL),  $\beta$ -galactosidase (LAC),  $\beta$ -mannosidase (MND),  $\alpha$ -xylosidase (AXL),  $\beta$ -xylosidase  
156 (BXL), cellobiohydrolase (CBH), glucoamylase (GLA),  $\alpha$ -rhamnosidase (RHA), and  $\alpha$ -arabinofuranosidase  
157 (ABF) activities were assayed in a total volume of 100  $\mu$ L using 10  $\mu$ L of a 2 mM *p*-nitrophenol (pNP) linked  
158 substrate (Sigma-Aldrich) solution, 20  $\mu$ L culture liquid sample, and 25 mM sodium acetate, pH 5. The  
159 samples were incubated at 30°C for 30 min and the reactions were stopped by the addition of 100  $\mu$ L 0.25  
160 M Na<sub>2</sub>CO<sub>3</sub>, pH 5.0. These conditions are commonly used for assays in *A. niger*, while the 30 min incubation  
161 was determined based on tests with different time points. Absorbance was measured at 405 nm in a  
162 microtiter plate reader (Biorad Model 550). For the determination of specific activities, pNP concentrations  
163 of 1-10 mM were used. Endo-1,4- $\beta$ -mannanase (MAN) activity was measured using Azo-carob  
164 galactomannan (S-ACGLM) (Megazyme), according to the manufacturer's instructions. One unit of  
165 enzymatic activity was defined as the amount of protein required to release one  $\mu$ mol of the corresponding  
166 product per minute, under the assay condition used. All enzyme activity assays were calculated from two  
167 biological replicate cultures and three technical replicate measurements.

168

169 **Results and discussion**

170           In this study, we aimed to gain a comprehensive insight into the transcriptome of *A. niger* during  
171 guar gum degradation (after 2, 8, 24 and 48 h) and compare this to the transcriptome on the main guar

172 gum components D-mannose, D-galactose and mannan, as well as D-glucose that was used as a control  
173 (all after 2 h incubation). In addition to transcriptomics, the exoproteomes from guar gum and mannan, after  
174 24 and 48 h, were analyzed to facilitate the identification of extracellular CAZymes, especially those with  
175 known function in degradation of plant biomass.

176

#### 177 *Functional distribution of CAZymes in A. niger transcriptomes and exoproteomes*

178 Expression of 173 genes encoding (putative) plant biomass degrading CAZymes was detected in  
179 the microarrays, which included 143 GHs, 14 CEs, 8 PLs and 8 AAs from 41 CAZy families (Fig. 1A, Table  
180 S1-1A). Overall distribution of genes according to their CAZy families showed a large variety of functions.  
181 Of these, 57, 24, 23, 21, 17, 14, 11, and 6 CAZy genes were putatively targeted towards pectin, cellulose,  
182 (arabino)xylan, diverse activities, galactomannan, starch, xyloglucan and inulin, respectively (Fig. 1A, Table  
183 S1-1A). The majority of identified transcripts (57), as well as the higher number of CAZy family genes (GH28  
184 with 20 genes), were found within CAZy genes involved in pectin degradation. Moreover, 17 genes from 6  
185 different families were directly connected to galactomannan degradation (Fig. 1A, Table S1-1A). Diverse  
186 activities included 21 CAZy transcripts, which can have activity towards multiple substrates or unknown  
187 functions.

188 In addition, LC-MS/MS identified a total of 153 proteins in the extracellular liquid from *A. niger* guar  
189 gum and mannan cultures, of which 136 proteins possessed putative secretion signal peptides (Table S2-  
190 2A). The absence of a signal peptide from the 17 proteins could be due to the fact that possibly not all  
191 cleavage motifs for proteins have been identified, or alternatively due to technical problems during sample  
192 processing (e.g. proteins without trypsin cleavage sites) [18]. However, we do not rule out the possibility  
193 that these proteins may be subject to a non-classical secretory mechanism or may be released after cell  
194 lysis [19]. From these 153 proteins, 69 belonged to CAZymes with known functions in plant biomass  
195 degradation (Fig. 1B, Table S2-2B). Their distribution over the CAZy families was 58 GHs, 7 CEs, 1 PLs  
196 and 3 AAs, which is similar to the distribution observed based on gene expression. A large number and  
197 diversity of GHs were found in the exoproteomes, targeting a broad range of polysaccharides. In total, 22  
198 proteins were related to pectin, 13 to (arabino)xylan, eight to cellulose, seven to xyloglucan, seven to  
199 galactomannan, six to diverse activities, four to starch and two to inulin degradation (Fig. 1B, Table S2-2B).



200 Of the CAZy families predicted to encode galactomannanolytic activities, GH27 again was the largest CAZy  
201 class found within this functional classification. Guar gum, while being a galactomannan in structure,  
202 contains traces of other polymers, such as starch, cellulose, xyloglucan or pectin [1,5,20]. This diversity of  
203 compounds may lead to the release of a range of monomeric compounds (e.g. L-arabinose, D-xylose, D-  
204 galacturonic acid, L-rhamnose, D-glucose), even though present at low level, which likely explains the broad  
205 diversity of enzymatic functions that are induced during growth on plant biomass [17]. It has previously  
206 been shown that already at 1 mM concentrations, and probably even lower, sugars can act as inducers of  
207 gene expression in *A. niger* [21]. The monosaccharides released from these impurities have been  
208 demonstrated to activate several transcriptional activators involved in plant biomass degradation [22,23].

209

#### 210 *Transcriptomic analysis and identification of differentially expressed CAZy genes on guar gum*

211 To investigate substrate and time-dependent variation of the expression of the CAZyme encoding  
212 genes, four time points (2, 8, 24, and 48 hours) from *A. niger* grown on guar gum were compared to D-  
213 glucose (Glc2h), D-mannose (Man2h), D-galactose (Gal2h) and mannan (Mnn2h) cultivations at an early  
214 time point (2 h) of induction. Hierarchical clustering of the differentially expressed CAZy genes from the  
215 different C-sources after 2 h of cultivation and over a 48 h time-course of guar gum depolymerization was  
216 performed to compare gene expression (Fig. 2). These CAZymes were divided into eight groups based on  
217 their putative substrate specificities: cellulose-specific, (arabino)xylan-specific, xyloglucan-specific,  
218 galactomannan-specific, starch-specific, pectin-specific, inulin-specific and not specific substrate.

219 For the *A. niger* transcriptome data from guar gum and related carbon sources at 2 h of induction,  
220 nine clusters with distinct expression patterns were identified (Fig. 2, Table S1). When the substrate-based  
221 differential expression was compared, the D-mannose transcriptome revealed that it was highly similar to  
222 that of D-glucose and did not result in upregulation of any mannanolytic genes of *A. niger* (Fig.2, Table S1-  
223 1B). In contrast, a clear relationship between guar gum and mannan was observed (Fig. 2A, Table S1-1B).  
224 Considering that mannobiose was recently demonstrated to be the inducer of mannanolytic genes in  
225 *Aspergillus oryzae* [3], our analysis shows that the mannan fraction in guar gum was largely responsible for  
226 its expression profile.

227 The genes commonly induced by all four C-sources were present in clusters C1, C4 and C5, and  
228 encode cellulolytic, pectinolytic, amylolytic and galactomannanolytic enzymes (Fig. 2A, Table S1-1B),  
229 confirming the results from previous studies where  $\alpha$ - and  $\beta$ -glucosidases,  $\alpha$ - and  $\beta$ -galactosidases, CBHs,  
230 xylanases, arabinanases, as well as genes encoding  $\alpha$ -amylases and GLAs were detected during the  
231 utilization of D-glucose and D-galactose by *A. niger* [24,25]. In contrast, the three genes of cluster C2,  
232 encoding an  $\alpha$ -amylase (*amyA*), an exo-1,3-galactanase (EXG) and an  $\alpha$ -xylosidase (*axIA*), were highly  
233 expressed on all the tested substrates except mannan and D-mannose (Fig. 2A).

234 Clusters C3 and C6 contained 12 genes specifically induced on mannan and guar gum, including  
235 genes encoding an  $\beta$ -xylosidase (*gbgA*), three  $\alpha$ -arabinofuranosidases (*abfA*, *abfB*, *abfC*), an  $\beta$ -  
236 glucosidase (*bgI4*), an  $\alpha$ -galactosidase (*agIB*), an  $\beta$ -mannosidase (*mndA*), two  $\beta$ -galactosidases (*lacA*,  
237 *lacB*), an endoarabinanase (*abnA*), a putative acetylmannan esterase (AME) and an exo-1,3-galactanase  
238 (*exgA*) (Fig. 2A, Table S1-1B). Although there is no clear evidence of the participation of  $\beta$ -galactosidases  
239 in the hydrolysis of galactomannan, our results suggest that these enzymes play a key role during guar  
240 gum degradation. The mannan specific genes were found in cluster C7, which included two  $\beta$ -glucosidases,  
241 a cellobiohydrolase (*cbhB*), an  $\alpha$ -galactosidase, an endomannanase (*manA*), a rhamnogalacturonan acetyl  
242 esterase (*rgaeB*), a rhamnogalacturonan lyase (*rgIA*), an endoarabinanase, and a putative  $\beta$ -glycosidase  
243 (Fig. 2A, Table S1-1B). The ManR transcription factor that is activated by glucomannan has been shown  
244 to regulate expression of  $\alpha$ -galactosidase,  $\beta$ -mannosidase, acetylmannan esterase,  $\beta$ -glucosidase and  
245 cellobiohydrolase genes in *Aspergillus oryzae* [3,26]. The similarities in the transcriptome data of mannan  
246 and guar gum, as well as between the genes in these clusters and those described in the *A. oryzae* study  
247 [3,26], suggest that ManR also has a major role in controlling gene expression on these two substrates in  
248 *A. niger*.

249 The 15 genes of cluster C8 were specifically induced on guar gum and encode a broader set of  
250 enzymes than those induced on mannan, and mannan and guar gum. These include (arabino)-xylanolytic,  
251 cellulolytic, pectinolytic, inulinolytic and galactomannan active enzymes, such as  $\beta$ -xylosidases  
252 (An02g00140, *xlnD*), arabinoxylan arabinofuranohydrolase (*axhA*),  $\alpha$ -glucuronidase (*aguA*), ferulolyl  
253 esterase (*faeB*),  $\beta$ -glucosidase (*bgIM*),  $\alpha$ -galactosidase (*agIA*),  $\beta$ -galactosidase (*lacC*), invertases (*sucA*,  
254 *sucB*), exo-1,6-galactanase (An03g01050),  $\alpha$ -amylase (*amyC*), and  $\alpha$ -fucosidase (*afcC*) (Fig. 2A, Table S1-

255 1B). Expression of this broader set of functions on guar gum is likely the result of the impurities in guar gum  
256 (Table 1) that trigger the activation of several transcription factors. The expression of *xlnD* and *aguA* was  
257 shown to be controlled by the hemicellulolytic regulator XlnR [27], which responds to the presence of xylose  
258 [28], while *axhA* is under control of both XlnR [27] and AraR, which responds to the presence of L-arabinose  
259 [29]. This indicates the role of monosaccharides not originating from galactomannan in gene expression  
260 during growth on guar gum. Apparently, these inducing compounds persist over time, suggesting that they  
261 are either not preferential carbon sources, or that they are only slowly released from the oligomeric or  
262 polymeric structures they are compounds of. Finally, cluster C9 contained 99 genes that did not have a  
263 significant difference of expression in all the C-sources tested (Fig. 2A, Table S1-1B).

264 The time-dependent clusters during growth of *A. niger* on guar gum were well separated according  
265 to the studied time points. As shown in Fig. 2B and Table S1-1C in Supplementary data, expression of  
266 many plant biomass degradation related CAZy genes increased strongly from 2 h to 8 h and then reduced  
267 again at the later time points. This expression profile can be explained by the release of monosaccharides  
268 that act as inducers for several transcriptional activators as mentioned above. As these other inducing  
269 compounds are only present in very small amounts, it is not surprising that their influence disappears again  
270 at later time points, resulting in a narrower set of expressed genes.

271 Fifty-four CAZy genes from clusters C1 and C9, including activities against (arabino)xylan,  
272 cellulose, galactomannan, pectin and starch, were medium to highly expressed independent of the time  
273 point (Fig. 2B, Table S1-1C), with several of them being the most abundant CAZy genes expressed. In  
274 contrast, 72 genes grouped in C5 and C6 did not have a differential expression pattern during 48 h of guar  
275 gum induction (Fig. 2B, Table S1-C).

276 Eight 2 h specific genes were found in clusters C2 and C3, including an  $\alpha$ -arabinofuranosidase  
277 (*abfA*), a  $\beta$ -xylosidase, a feruloyl esterase (*faeB*), a  $\beta$ -galactosidase (*lacB*), two  $\alpha$ -galactosidases (*aglC*), a  
278  $\beta$ -fructofuranosidase/invertase (*sucA*) and an  $\alpha$ -amylase (*amyA*) (Fig. 2B, Table S1-1C). Several of these  
279 genes (*amyA*, *aglC*, *sucA*) have been shown to be expressed in the presence of low levels of D-glucose  
280 [24] or sucrose [30], suggesting that free sugars present in guar gum are likely responsible for this  
281 expression profile. As these would be quickly consumed by *A. niger*, this likely explains the reduction in  
282 their expression at later time points.

283           The 8 h time specific genes were found in clusters C4 and C8. A high number of those genes  
284 encoded pectinolytic enzymes, such as *pmeB*, *pelA*, *xghA*, *pgaC*, *pgaE*, *pgalI*, *pgaX*, *pgxB*, *lacA*, *rhgA*,  
285 *rgIA*, *rgaeA* and *rgxC* (Fig. 2B, Table S1-1C). A similar gene expression pattern corresponding to enzymes  
286 acting on the pectin backbone has previously been observed for *A. niger* in presence of D-galacturonic acid  
287 and L-rhamnose during the initial degradation of pectin [31]. However, a low expression of exo-pectinolytic  
288 genes after 8 h was observed, indicating that possibly the main pectin chains are metabolized first, which  
289 would make the main chains of these polymers more accessible to endo-enzymes at later stages of fungal  
290 growth. Apparently, guar gum contains small traces of pectin polysaccharides or polymer decorations that  
291 induced the expression of exo-acting pectinolytic GHs mostly during early degradation. The (temporal)  
292 diversity of expression of pectinolytic genes has previously been reported for a selected set of genes [31],  
293 and has recently been confirmed in a global CAZy expression study [17] and two studies addressing the  
294 molecular mechanism of pectinolytic regulation [32].

295           Cluster C7 mainly contained genes that were not or lowly expressed at 2h, (highly) induced at 8h  
296 and remained expressed at 24 h and 48 h (Fig. 2B). The majority of these genes corresponded to (arabino)-  
297 xylanolytic, cellulolytic, xyloglucanolytic and not specific functions, such as *axeA*, *xlnC*, *faeA*, *eglC*, *cbhA*,  
298 *eglC* and lytic polysaccharide monooxygenases (LPMOs) (Fig 2B, Table S1-1C). The strong induction of  
299 those genes was an unexpected result, since the trace contaminants would be expected to be metabolized  
300 by the fungus in the first hours of degradation, and guar gum does not contain cellulose or high  
301 concentrations of xylose. This observation could suggest that the late induction of those genes is  
302 triggered even when no inducing sugar is present. Interestingly, all of these genes have been shown to be  
303 regulated by the zinc finger transcription factor XlnR in *A. niger* [17,28,33]. A detailed analysis of an *A.*  
304 *niger xlnR* deletion mutant revealed low levels of expression of a subset of genes coding for degradative  
305 CAZymes induced by carbon starvation, which can scout for available carbon source [25]. This finding may  
306 explain why the induction of xylanolytic and cellulolytic genes occurs without the presence of inducer sugars  
307 in later stages of cultivation on guar gum. In contrast, the regulation of galactomannanolytic genes showed  
308 time-dependent variation, reaching a maximum expression level at 8 h (Fig 2B, Table S1-1C). The *A. niger*  
309 genome contains 12 candidate CAZymes related to the degradation of the main chain and side-chains of  
310 galactomannan [34,35]. However, the presence of a single  $\alpha$ -galactosidase,  $\beta$ -mannosidase and endo-

311 mannanase has been shown to be sufficient for efficient degradation of galactomannan from different  
312 carbon sources [2,8,9]. The early upregulation of the exo-acting galactomannolytic genes could be  
313 related to the presence of oligosaccharides in guar gum that would allow a quick release of D-galactose  
314 and D-mannose from the substrate.

315

#### 316 *Comparative analysis of the upregulated CAZy transcripts from substrate and time-dependent variation*

317 We generated Venn diagrams to compare upregulated genes from different substrates after 2 h of  
318 incubation on guar gum (GG2h) vs. 2 h of incubation on D-glucose (Glc2h), D-mannose (Man2h), D-  
319 galactose (Gal2h) and mannan (Mnn2h). Overall, six and two genes were specifically upregulated after 2 h  
320 on guar gum compared to D-glucose, and D-mannose, respectively (Fig. 3A, Table S1-1D). In addition,  
321 seven genes were upregulated on guar gum compared to D-glucose and D-mannose, seven compared to  
322 D-glucose, D-mannose and D-galactose, four compared to D-glucose, D-mannose and mannan, and one  
323 gene compared to D-glucose and mannan. A core set of 10 genes was upregulated on guar gum compared  
324 to all four other substrates, including three xylanolytic (*axhA*, *axIA* and *aguA*), two cellulolytic (*An07g09760*  
325 and *bgIM*), an inulinolytic (*sucA*), an amylolytic (*amyA*), and two pectinolytic (*rha* and *lacA*) genes. The  
326 absence of galactomannan specific genes in any of these comparisons is likely in part due to them being  
327 mainly expressed at later time points during growth on guar gum (see below). Also, most of the genes  
328 differentially expressed between guar gum and the other substrates after 2 h have been demonstrated to  
329 be under control of XlnR, AraR, AmyR and/or InuR [17,30].

330 A similar comparison was performed to identify the time dependent upregulated genes on guar  
331 gum, by comparing the 2 h data (GG2h) to that of 8 h (GG8h), 24 h (GG24h) and 48 h (GG48h), resulting  
332 in a total of 106 significantly differentially expressed genes (Fig. 3B, Table S1-1E). As expected from the  
333 analysis described above, the largest number of specifically differently expressed genes was observed for  
334 the comparison between 2 h and 8 h (34), while the number was much lower for the comparison with 24 h  
335 (4) and 48 h (5). Most of these genes have no known role in galactomannan degradation, suggesting that  
336 they are likely expressed in response to monosaccharides present in the substrate or released from the  
337 impurities at later time points. The exceptions were two AGLs and one AME (8 h) and one MND (24 h). In  
338 contrast, only 13 genes were upregulated at all later time points compared to 2 h, consisting of four

339 cellulolytic genes (*cbhA*, *cbhB*, *eglA*, *eglC*), two amylolytic genes (*aamA*, *amyC*), one xyloglucanolytic gene  
340 (*xeg*), one arabino-xylanolytic gene (*axeA*), two mannanolytic genes (*manA*, AME), and three genes with a  
341 not yet defined substrate (one multicopper oxidase encoding gene, *mcoB*, and two LPMO encoding genes).  
342 The expression pattern of *manA* suggests that significant degradation of galactomannan does not occur  
343 until 8 h of cultivation and supports the preferential hydrolysis of smaller oligomer by the exo-acting  
344 enzymes before the polymeric fraction of guar gum is hydrolyzed.

345

#### 346 *Exoproteome analysis of guar gum and mannan degradation by A. niger*

347 The exoproteomes of *A. niger* grown on guar gum and mannan were analyzed to examine the  
348 extracellular enzymatic responses during the degradation of guar gum in the later stages of the fungal  
349 cultivations. A larger number of proteins, 59 and 27, was detected at 48 h compared with 48 and 18 proteins  
350 at 24 h of guar gum and mannan induction, respectively, suggesting accumulation of secreted enzymes in  
351 the culture medium over time (Fig. 4, Table S2-2B). However, it was evident that there was a notable  
352 difference between the abundance of transcripts and the extracellular proteins detected. These differences  
353 seem to be more related to different factors such as biological process of gene regulation during and after  
354 the transcription, mechanisms of induction and repression of sugar catabolism, changes in the  
355 environmental conditions (pH, temperature, and ionic strength), or even technical artifacts during sample  
356 processing [12,18,34]. Nevertheless, a good correlation between expression levels and LC-MS/MS  
357 identification of secreted proteins were noted.

358 The number of extracellular (arabino)xylanolytic proteins increased from 7 to 13 and 1 to 3 in guar  
359 gum and mannan, respectively (Fig. 4). Cellulolytic proteins increased from 6 to 8 and 3 to 4 in guar gum  
360 and mannan, respectively. The number of galactomannanolytic proteins was more similar between both  
361 substrates. In contrast, inulinolytic proteins were not detected in mannan cultures. The number  
362 of pectinolytic proteins remained constant (16) at 24 and 48 h of induction on guar gum, whereas a small  
363 number of proteins was found at both time points for mannan (from 4 to 6). Similarly, the number of  
364 xylanolytic proteins increased in guar gum, but only two proteins were detected in mannan. The amount of  
365 amylolytic proteins during later stages of degradation of guar gum and mannan was very similar.

366 It was noticed that despite the relatively low total abundance of pectin degrading proteins on both  
367 substrates, a highly secreted endoarabinanase (*abnC*) was detected only in the later stages of the mannan  
368 cultivations (Fig. 4). As expected, the relative abundances of the proteins within galactomannan functional  
369 category were similar during the cultivation in guar gum and mannan (Fig. 4). The most abundant proteins  
370 were found within this category, including an An02g11150 GH27 AGL, an An05g01320 GH5 MAN, an  
371 An11g06540 GH2 MND, and an An07g08940 CE16 AME (Fig. 4, Table S2-2C). Taken together, our results  
372 confirm the previous observations of their orthologs (*aglB*/AO090003001305, *manG*/AO090010000122,  
373 *ameA*/AO090005001552 and NCU08412) in the distantly related species *A. oryzae* [3] and *N. crassa* [36]  
374 as critical enzymes for mannan utilization. The combined results of the exoproteome and the transcriptome  
375 analysis confirmed that, in this experimental setting, mannan and guar gum induced a similar pattern of  
376 expression of genes encoding galactomannan specific enzymes and these enzymes were secreted.

377 The presence of a large variety of (arabino)xylan, inulin, pectin, xyloglucan degrading enzymes led  
378 us to hypothesize that only some of these enzymes may be important for guar gum (galactomannan)  
379 hydrolysis, while the others were induced by the presence of trace components present in the guar gum  
380 used in this study.

381 Although guar gum is extracted from the same plant species, its molecular composition can be  
382 influenced by the conditions and method of extraction used [4]. Previous physico-chemical analysis have  
383 shown that pure guar gum can contain up to 13% of impurities such as soluble sugars and traces of  
384 proteinaceous matter [4,20], which may originate from the remaining seed coat in the extracted guar gum.  
385 Our results suggest that this low amount of impurities acts as a potent inducer for the production of many  
386 hydrolases (even at low concentrations), suggesting that the purity of the substrate strongly affects the  
387 response of the fungus. Therefore, the difference in gene expression compared to the monosaccharides  
388 could be accounted to the activation of the hydrolytic enzymes targeted for the substrate impurities of guar  
389 gum.

390 Additionally, the transcriptome and exoproteome of *A. niger* were compared, resulting in a common  
391 set of 46 significantly upregulated CAZymes shared between them. This set contained 10 (arabino)-  
392 xylanolytic, 10 pectinolytic, seven galactomannanolytic, six cellulolytic, five xyloglucanolytic, four amylolytic  
393 and two inulinolytic genes/enzymes (Table 2). We also detected LPMOs and MCO-related enzymes after

394 8 h of induction on guar gum in the exoproteomic and transcriptomic samples, even though their  
395 corresponding genes were not highly expressed (Fig. 2, Table S1 and S2). Recently, Nekiunaite et al. [37]  
396 identified several oxidoreductases co-expressed with amylolytic hydrolases during the deconstruction of  
397 starch-containing substrates by *Aspergillus nidulans*. The role of these oxidoreductases could possibly be  
398 in the oxidative cleavage of linkages in guar gum to boost its hydrolysis by the hydrolytic depolymerizing  
399 enzymes [38,39].

400

#### 401 *Validation of the activities of the guar gum related CAZymes produced by A. niger*

402 To estimate the potential of the upregulated CAZymes identified by transcriptomic and  
403 exoproteomic analyses, several enzyme activities were determined in samples from the cultures after 8 h,  
404 24 h and 48 h:  $\alpha$ -galactosidase (AGL),  $\beta$ -galactosidase (LAC), endomannanase (MAN),  $\beta$ -mannosidase  
405 (MND),  $\alpha$ -xylosidase (AXL),  $\beta$ -xylosidase (BXL),  $\beta$ -glucosidase (BGL), cellobiohydrolase (CBH),  
406 glucoamylase (GLA), rhamnosidase (RHA) and arabinofuranosidase (ABF).

407 The results show that *A. niger* produced all the 11 tested activities during growth on guar gum and  
408 mannan (Fig. 5). At 8 h, most of the enzymes showed a reduced activity in comparison with 24 h. After 24  
409 h, AGL, MAN, MND, BGL, CBH, GLA, and ABF activities increased substantially on both guar gum and  
410 mannan. Likewise, at 48 h cultivation on mannan, AGL and MND activities increased strongly compared  
411 with those detected on guar gum. However, at the same time point of analysis, the BGL, CBH, and GLA  
412 activities from guar gum treatments were higher than that of mannan. Interestingly, only the MAN activities  
413 of guar gum and mannan cultures showed similar levels of activity. The maximal galactomannanolytic  
414 activities were reached for AGL, MAN and MND after 48 h of incubation, which was also the case for  
415 cellulolytic and several xylanolytic activities (Fig. 5). In contrast, AXL, BXL and RHA activity were low  
416 compared to the other enzyme activities. This difference correlates with the weak expression of RHA in *A.*  
417 *niger* and the fact that this enzyme has a low activity [17,40]. The measured enzyme activities were  
418 generally in good agreement with the putative key CAZymes found in the *A. niger* transcriptomes as well  
419 as in the exoproteomes during cultivation on guar gum.

420

#### 421 **Conclusion**



422 This is the first report with an integral approach to identify the key enzymes produced by *A. niger*  
423 during growth on guar gum. Interestingly, guar gum, consisting mainly of galactomannan, triggered the  
424 production of a rich and complex set of hydrolytic CAZymes with most pronounced activities towards  
425 (arabino)-xylan, cellulose, galactomannan, and pectin. This indicates that the small amounts of other mono-  
426 , oligo- and polysaccharides than galactomannan in guar gum have a major influence on gene expression  
427 and protein production and can strongly alter the composition of the enzyme mixture produced by this  
428 fungus. The implications of this for commercial production of enzyme cocktails are very high, as this  
429 indicates that variations in the impurities of feed stocks used for enzyme productions may significantly alter  
430 set of enzymes in the resulting cocktail and therefore its effectivity.

431

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553

554

555 **Table 1.** Composition analysis of guar gum.

<b>Component</b>	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Total sugar
<b>Mol%</b>	2.2	0.4	56.9	35.4	3.2	1.7	83.6

556

557

558 **Table 2.** CAZymes related to biomass deconstruction that were significantly ( $p < 0.05$ ) upregulated in the *A.*  
559 *niger* N402 transcriptomes and exoproteomes from guar gum compared to mannan.

ID_CBS513.88	CAZy family	Enzyme code/gene	Function	Putative substrate	log2 fold change
An18g04810	GH28	RGX_ <i>rgxC</i>	exo-rhamnogalacturonase	pectin	8.639498
An16g00540	GH95	AFC	$\alpha$ -fucosidase	xyloglucan	6.932792
An15g05370	GH28	PGA_ <i>pgall</i>	endopolygalacturonase	pectin	6.842149
An05g01320	GH5	MAN_ <i>manA</i>	endomannanase	galactomannan	6.365687
An01g12150	GH35	LAC_ <i>lacA</i>	$\beta$ -galactosidase	galactomannan, pectin	5.866629
An14g02760	GH12	XEG_ <i>eglA</i>	xyloglucan-active endoglucanase	xyloglucan	5.466683
An07g08940	CE16	AME	putative acetylmannan esterase	galactomannan, xylan	5.377879
An08g11070	GH32	SUC_ <i>sucA/sucI</i>	invertase/ $\beta$ -fructofuranosidase	inulin	5.356922
An14g01790	GH27	AGL	$\alpha$ -galactosidase	galactomannan	4.930603
An15g02300	GH54	ABF_ <i>abfB</i>	$\alpha$ -arabinofuranosidase	(arabino)-xylan, pectin	4.360588
An02g11150	GH27	AGL_ <i>aglB</i>	$\alpha$ -galactosidase	galactomannan	4.291878
An09g01190	GH43	ABN_ <i>abnA</i>	endoarabinanase	pectin	4.154161
An11g06540	GH2	MND_ <i>mndA</i>	$\beta$ -mannosidase	galactomannan	4.142109
An08g10780	GH43	BXL_ <i>gbgA</i>	$\beta$ -xylosidase	(arabino)-xylan	3.956593
An01g00330	GH51	ABF_ <i>abfA</i>	$\alpha$ -arabinofuranosidase	(arabino)-xylan, pectin	3.838550
An03g06740	GH28	PGX_ <i>pgxB</i>	exopolygalacturonase	pectin	3.367877
An07g08950	GH5	EGL_ <i>eglC</i>	endoglucanase	cellulose	3.062056
An04g09700	GH28	XGH_ <i>xghA</i>	xylogalacturonase	pectin	3.046303
An08g01900	GH43	BXL	$\beta$ -xylosidase	(arabino)-xylan	3.023174
An09g03300	GH31	AXL_ <i>axlA/xylS</i>	$\alpha$ -xylosidase	xyloglucan	2.992657
An11g00200	GH3	BGL_ <i>bglM</i>	$\beta$ -glucosidase	cellulose	2.979334
An01g10350	GH35	LAC_ <i>lacB</i>	$\beta$ -galactosidase	galactomannan, pectin	2.939864
An07g09330	GH7	CBH_ <i>cbhA</i>	cellobiohydrolase	cellulose	2.922187
An08g01710	GH51	ABF_ <i>abfC</i>	$\alpha$ -arabinofuranosidase	(arabino)-xylan, pectin	2.915692
An16g02760	GH95	AFC_ <i>afcC</i>	$\alpha$ -fucosidase	xyloglucan	2.753815
An06g00170	GH27	AGL_ <i>aglA</i>	$\alpha$ -galactosidase	galactomannan	2.679696
An12g08280	GH32	INX_ <i>inuE/inul</i>	exo-inulinase	inulin	2.617590
An02g02540	CE16	AME	putative acetylmannan esterase	galactomannan, xylan	2.554184
An04g09690	CE8	PME_ <i>pmeB</i>	pectin methylesterase	pectin	2.494095
An03g00940	GH10	XLN_ <i>xlnC/xynA</i>	$\beta$ -1,4-endoxylanase	(arabino)-xylan	2.436745
An03g01050	GH5	GLN	exo-1,6-galactanase	pectin	2.184780
An02g10550	GH43	ABN_ <i>abnC</i>	endoarabinanase	pectin	2.120337
An04g06920	GH31	AGD_ <i>agdA</i>	$\alpha$ -glucosidase	starch	2.095693
An11g03340	GH13	AMY_ <i>aamA</i>	$\alpha$ -amylase	starch	2.014945
An12g10390	CE1	FAE_ <i>faeB</i>	feruloyl esterase	(arabino)-xylan, pectin	1.895975
An03g06550	GH15	GLA_ <i>glaA</i>	glucoamylase	starch	1.854618
An11g02100	GH1	BGL	$\beta$ -glucosidase	cellulose	1.692845
An01g13660	AA1	MCO_ <i>mcoB</i>	multicopper oxidase	not specific	1.599671

An03g00960	GH62	AXH_ <i>axhA</i>	arabinoxylan arabinofuranohydrolase	(arabino)-xylan	1.565415
An01g09960	GH3	BXL_ <i>xlnD/xynD</i>	$\beta$ -xylosidase	(arabino)-xylan	1.534312
An01g11660	GH7	CBH_ <i>cbhB</i>	cellobiohydrolase	cellulose	1.510050
An08g04630	GH131	EGL	endoglucanase	cellulose	1.469976
An01g01870	GH74	XEG_ <i>egjC</i>	xyloglucan-active endoglucanase	xyloglucan	1.461051
An14g02670	AA9	LPMO	lytic polysaccharide monooxygenase	not specific	1.394944
An01g10930	GH31	AGD_ <i>agdB</i>	$\alpha$ -glucosidase	starch	1.344306
An01g00780	GH11	XLN_ <i>xlnB</i>	$\beta$ -1,4-endoxylanase	(arabino)-xylan	1.278865

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560



561 **Figure captions**

562

563 **Fig. 1.** Comparison of functional distribution of plant biomass degrading CAZy genes/enzymes  
564 expressed/produced by *A. niger* during cultivation on guar gum and related carbon sources. (A) Functional  
565 distribution of CAZyme encoding genes identified in the fungal transcriptome from guar gum cultures. (B)  
566 Distribution of identified proteins in *A. niger* exoproteomes on guar gum and mannan. The corresponding  
567 CAZy transcripts and proteins related to plant biomass degradation are divided by their predicted  
568 substrates. The pie chart represents the total number of genes or proteins identified according to its putative  
569 substrate. GH (glycoside hydrolase), AA (auxiliary activity), CE (carbohydrate esterase) and PL  
570 (polysaccharide lyase).

571

572 **Fig. 2.** Hierarchical clustering comparing the expression pattern of *A. niger* CAZyme encoding genes (A)  
573 after 2 h growth on D-glucose, D-mannose, D-galactose, mannan and guar gum, and (B) over 48 h of growth  
574 on guar gum. The transcriptional expression profiles from D-glucose, D-mannose, D-galactose, mannan and  
575 guar gum were normalized and clustered. The resulting heat maps were divided into clusters. The top color  
576 bar represent the gene expression value from downregulated (blue) to upregulated genes (yellow). Further  
577 details of the clusters are presented in Table S1, as are the description of the enzyme functions represented  
578 by the 3-letter codes.

579

580 **Fig. 3.** Venn diagrams showing the shared upregulated *A. niger* CAZy genes in (A) 2 h on guar gum (GG2h)  
581 vs. 2 h on D-glucose (Glc2h), D-mannose (Man2h), D-galactose (Gal2h), and mannan (Mnn2h), respectively,  
582 and (B) genes upregulated over 48 h on guar gum. A list containing the IDs and the respective annotation  
583 of the significantly upregulated ( $p < 0.01$ ) genes grouped in the Venn diagram is provided in Table S1.

584

585 **Fig. 4.** Relative abundance of CAZymes identified in *A. niger* exoproteomes after 24 h and 48 h of growth  
586 on guar gum (GG) and mannan (Mnn). The extracellular CAZymes were grouped by their predicted function  
587 towards (arabino)xylan, cellulose, galactomannan, inulin, pectin, xyloglucan, starch and not specific  
588 substrate. Difference in the percentage of relative abundances is represented by five sizes of circles from

589 0.01-0.09%, 0.1-0.9%, 1.0-3.9%, 4.0-7.8% and 12-15.7%. The different colors of the circles represent the  
590 treatments of the samples: red = GG24h, green = GG48h, blue = Mnn24h and purple = Mnn48h. Details of  
591 the enzymes and values are given in Table S2.

592

593 **Fig. 5.** Extracellular CAZyme activities detected from the culture supernatants of *A. niger* at 8 h, 24 h and  
594 48 h of cultivation on guar gum and mannan. The activities of  $\alpha$ -galactosidase (AGL),  $\beta$ -galactosidase  
595 (LAC), endomannanase (MAN),  $\beta$ -mannosidase (MND),  $\alpha$ -xylosidase (AXL),  $\beta$ -xylosidase (BXL),  $\beta$ -  
596 glucosidase (BGL), cellobiohydrolase (CBH), glucoamylase (GLA), rhamnosidase (RHA), and  
597 arabinofuranosidase (ABF) were evaluated. The vertical bars show the standard deviation of two biological  
598 replicates and three technical replicate reactions.

599