Canadian Journal of Microbiology
Revue canadienne de microbiologie

Investigating the expression of F10 and G11 xylanases from *Aspergillus niger* A09 with qPCR

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2015-0394.R4
Manuscript Type:	Article
Date Submitted by the Author:	21-Apr-2016
Complete List of Authors:	Cui, Shixiu; Henan Agricultural University, College of Life Sciences Wang, Tianwen; East China University of Science and Technology, School of Biotechnology Hu, Hong; Henan Academy of Sciences, Institute of Biology Co., Ltd Liu, Liangwei; Henan Agricultural University, College of Life Sciences Song, An-Dong; Henan Agricultural University, College of Life Sciences Chen, Hongge; Henan Agricultural University, College of Life Sciences
Keyword:	<i>Aspergillus niger</i> , qPCR, F10 xylanase, G11 xylanase, Expression pattern

SCHOLARONE™
Manuscripts

1 **Investigating the expression of F10 and G11 xylanases in**
2 ***Aspergillus niger* A09 with qPCR**

3

4 Shixiu Cui^{1#}, Tianwen Wang^{2#}, Hong Hu³, Liangwei Liu¹,
5 Andong Song¹, Hongge Chen^{1*}

6 1. College of Life Sciences, Henan Agricultural University,
7 Zhengzhou, 450002, P. R. China

8 2. School of Biotechnology, State Key Laboratory of Bioreactor
9 Engineering, East China University of Science and Technology,
10 Shanghai, 200237, P. R. China

11 3. Institute of Biology Co., Ltd, Henan Academy of Sciences,
12 Zhengzhou, 450008, P. R. China

13 #Contributed equally to this work.

14 *Corresponding author, E-mail: honggeyz@163.com

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

Abstract

There exist significant differences between the two main types of xylanase, family F10 and G11. A clear understanding of the expression pattern of microbial F10 and G11 under different culture conditions would facilitate better production and industrial application of xylanase. In this study, the fungal xylanase producer *Aspergillus niger* A09 was systematically investigated in terms of induced expression of xylanase F10 and G11. Results showed that carbon and nitrogen source could influence xylanase F10 and G11 transcript abundance, with G11 more susceptible to changes in culture media composition. The most favorable carbon and nitrogen source for high G11 and low F10 production by *A. niger* A09 were xylan (2%) and $(\text{NH}_4)_2\text{C}_2\text{O}_4$ (0.3%), respectively. Following cultivation at 33°C for 60h, the highest xylanase activity (1132 IU per gram of wet mycelia) was observed. Based on differential gene expression of F10 and G11, as well as their different properties, we deduced that the F10 protein initially targeted xylan and hydrolyzed it into fragments including xylose, after which xylose acted as the inducer of F10 and G11 gene expression. These speculations also accounted for our failure to identify conditions favoring the high production of F10, but a low production of G11.

Key words: *Aspergillus niger*, qPCR, F10 xylanase, G11 xylanase, Expression pattern

Introduction

Xylan is the second most abundant polysaccharide on Earth. As an integral component of the plant cell wall, xylan accounts for roughly 1/3 of all carbohydrate in the plant, or 7-35% of the dry cell weight (Deutschmann and Dekker 2012). Xylanases are a group of enzymes that can efficiently degrade xylan to (oligo-) xylose (Ahmed et al. 2009; Adsul et al. 2009). Xylanases can be used as biocatalysts in the production of biofuels from plant biomass (Harris et al. 2014), in animal feed production (Olukosi et al. 2007), in preparation of functional food xylo-oligosaccharides (Wang et al. 2015) and in the paper and pulp industry (Buchert et al. 1994).

Due to the structural heterogeneity of xylan, the complete degradation of xylan is usually orchestrated through the function of different xylanases, including endo- β -1,4-xylanase, β -xylosidase, acetyl esterase, arabinosidase, etc. (Lee et al. 2003). However, endo- β -1,4-xylanase (EC 3.2.1.8, hereinafter referred to as xylanase) is responsible for cleaving beta-1,4-glycosidic bonds within the xylan backbone and plays a critical role in xylan degradation.

The biochemical properties of xylanase are highly dependent on their structure and origin. Based on amino acid sequence similarity and hydrophobic cluster analysis, the vast majority of xylanases are classified into glycoside hydrolase family 10 and 11, corresponding to the formerly named family F and family G, respectively (Henrissat 1991; Henrissat and Bairoch 1993). F10 xylanases have a high molecular weight (>30 kDa) and a low pI value, with a typical 8-fold α/β barrel structure. In contrast, G11 xylanases have a low molecular weight (<30 kDa) and a high pI value, with a β -jelly roll fold structure. The notable difference between the two families are their final hydrolysis products: F10

100 xylanases release smaller oligosaccharides with more xylose,
101 while G11 xylanases release larger oligosaccharides with nearly
102 no xylose. This is due to the fact that the F10 xylanase has
103 substrate binding sites smaller than those of G11 (four to five for
104 F10 while at least seven for G11, as stated by Collins et al.
105 (2005)), leading to the F10's greater catalytic versatility, or
106 lower substrate specificity than G11 (Jeffries 1996; Biely et al.
107 1997). In fact, F10 xylanases also exhibit activity on short
108 xylo-oligosaccharides, as well as on low molecular mass cellulose
109 substrate, the activity compatible with β -xylosidase and
110 cellulase, respectively (Collins et al. 2005). The property
111 differences between F10 and G11 make them a functionally
112 complementary pair for xylanase-producing microbes to digest
113 xylan efficiently.

114

115 However, as far as the application of xylanases are concerned, it
116 is conceivable that one type of xylanase may be more desirable
117 than the other. For example, the G11 xylanase should be more
118 advantageous for xylo-oligosaccharides production than an F10
119 xylanase, since the F10 xylanase releases too much xylose, a
120 side product which will increase the cost of removing
121 xylo-oligosaccharides with degree of polymerization (DP) 2-7.
122 That is probably why most work on xylo-oligosaccharide
123 production made use of G11 xylanases (Liu et al. 2008; Bragatto
124 et al. 2013; Wang et al. 2015; Qu et al. 2015). On the other hand,
125 when considering biofuel production from biomass, an F10
126 xylanase is more suitable for thorough biomass degradation
127 than a G11 xylanase. Although the production of a desirable
128 type of xylanase by heterogeneous expression in either
129 *Escherichia coli* or *Pichia pastoris* is now technically feasible,
130 xylanase production by the natural xylan-utilizing fungus
131 *Aspergillus niger* is still an attractive option. Considered a
132 generally-regarded as safe (GRAS) fungus (Schuster et al. 2002),

133 *A. niger* possesses a high protein secretion ability (de Oliveira et
134 al. 2011), the appropriate glycosylation required for enzyme
135 function (Han and Lei, 1999), and the rich enzyme complex
136 (Kniemeyer 2011). Therefore it is regarded as an ideal source for
137 exploiting xylanase as a catalyst. Currently, little has been
138 reported on gene expression patterns of *A. niger* F10 and G11
139 xylanases in response to different carbon and nitrogen sources.
140 Elucidating this may provide the possibility of selectively
141 producing either F10 or G11 simply by adjusting the culture
142 media components, making the production of a desirable
143 xylanase preparation cost-effective.

144

145 We previously noted that the high xylanase producing strain *A.*
146 *niger* A09 produced two visible xylanase bands on SDS-PAGE
147 zymogram analysis; one identified as an F10 xylanase and the
148 other a G11. This strain provides an excellent source to
149 investigate the formation pattern of F10 and G11. In this work,
150 the expression of xylanase F10 and G11 from *A. niger* A09 were
151 systematically investigated using qPCR and other related
152 methods in the hope of clarifying the expression mechanisms of
153 F10 and G11, and also to optimize culture media composition to
154 allow for the selective production of either F10 or G11 xylanases.

155

156 **Materials and Methods**

157

158 **Strain and primers**

159

160 The *A. niger* A09 strain was a lab stock copy of a previously
161 screened sample submitted to the China General
162 Microbiological Culture Collection Center. Primers used in the
163 study are listed in **Table 1**. These primers were designed based
164 on the sequences found in Genbank (18S rRNA, xylanase G11
165 (DQ147775) and F10 (XM_001389959.2)) with the aid of Beacon

166 Designer software by Premier Biosoft (Palo Alto, USA). All
167 primers were synthesized by the Sangon Company (Shanghai,
168 China).

169

170 **Chemicals**

171

172 Birchwood xylan substrate, DEPC solution, and EDTA were
173 products of Sigma (St. Louis, MO, USA). Components (peptone,
174 yeast extract) for medium preparation were from Oxiod
175 (Hampshire, UK). RNAiso™ RNA isolation kit, Real time
176 RT-PCR kit, and protein marker were bought from Takara
177 (Dalian, China). Other chemicals were of analytical grade from
178 local suppliers. Development solution 3,5-dinitrosalicylic acid
179 (DNS) was a fresh preparation in each assay.

180

181 **Culture medium**

182

183 Culture medium PDA (slants or plates) was prepared with fresh
184 potato. PDA was also used for preservation of seed culture.
185 Medium for enzyme production was prepared by supplementing
186 the selected carbon source and nitrogen source to the basic
187 medium (carbon source, 2; ammonium oxalate, 0.3;
188 $K_2HPO_4 \cdot 3H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; KCl, 0.05; $FeSO_4 \cdot 7H_2O$,
189 0.001, w%). Carbon source was selected from xylan, xylose,
190 glucose, maltose, cellobiose, arabinose, lactose; and nitrogen
191 source from yeast extract, NH_4NO_3 , $(NH_4)_2SO_4$, peptone, urea,
192 and $NaNO_3$, besides ammonium oxalate.

193

194 **Preparation of single-spore suspension**

195

196 The spores from 5-6 day's cultivation on a PDA slant were
197 aseptically transferred to autoclaved flasks with an appropriate
198 volume of distilled water and suitable amount of glass beads.

199 The flasks were then shaken on a shaker at 220 rpm for about 1
200 h (33°C) to completely disperse the spores (Ho and Ko 1997) and
201 create a spore suspension.

202

203 **Cultivation of *A. niger* and preparation of enzyme extract**

204

205 The spore suspension (1%, v/v) was inoculated into 50 mL of
206 sterilized liquid medium in 250 mL flasks in triplicate.

207 Cultivation was carried out at 33°C and 220 rpm. Following
208 growth, the filtrate of the culture broth was collected after
209 passage through 4-layered cheesecloth, and centrifuged at 4200
210 rpm for 10 min (4°C). The supernatant was used as enzyme
211 extract. Collected mycelium was subjected to biomass analysis
212 or RNA extraction after sufficient washing with distilled water.

213

214 **Enzymatic assay**

215

216 To 0.1 mL solution of Birchwood xylan (1%, in 0.2 M HAc-NaAc
217 buffer, pH4.6), 0.1 mL appropriately diluted enzyme extract was
218 added and thoroughly mixed. The reaction was kept at 50°C for
219 15 min and stopped by the addition of 0.6 mL DNS solution. The
220 mixture was then boiled for 10 min, after which distilled water
221 was added to a final volume of 5 mL, and the optical density at
222 550nm recorded to calculate the amount of reducing sugar (as
223 xylose) generated. Pre-inactivated enzyme was used as a control.
224 One unit was the amount of enzyme required for the generation
225 of 1 μ mol xylose in 1 min under the conditions specified above.
226 The calculation was done with the equation $E=1.53D \times OD_{550}$,
227 where E was the activity (U), D was the dilution, and OD_{550} was
228 the optical density from the spectrometer (Gusakov et al. 2011;
229 Rivers et al. 1984).

230

231 **Zymographic analysis**

232

233 In zymographic analysis (Leber and Balkwill 1997), proteins in
234 the sample were denatured, separated by SDS-PAGE, and
235 renatured to catalyze the substrate evenly dispersed in the gel
236 (Chen and Buller 1995). Briefly, xylan (1%) dissolved in 0.1
237 mol/L NaOH was added to a resolving gel (1/10, v/v). The pH
238 was adjusted to 8.8 with 6 mol/L HCl before the addition of
239 tetramethylethylenediamine (TEMED) and ammonium
240 persulfate (APS). The enzyme extract was mixed with loading
241 buffer and boiled for 10 min at 100°C. After centrifugation, the
242 supernatant was loaded onto the gel for SDS-PAGE. Following
243 SDS-PAGE, the gel was washed for 60 min with 2.5%
244 Triton-X100 to remove SDS, and the gel incubated in 50 mM
245 HAc-NaAc buffer for 30 min (50°C). The reaction was stopped by
246 placing the gel at 4°C. Gel staining was done by incubating the
247 gel with 1 mg/ml Congo red for 20 min with gentle shaking.
248 De-staining was performed in 1 M NaCl until colorless or light
249 yellow bands were visible in the dark-red background, after
250 which 1 M HCl was added to change the background color into
251 deep blue.

252

253 **The transcriptional analysis**

254

255 Collected mycelium were ground to a powder and used for RNA
256 isolation, following manufacturer's instructions. The
257 concentration and purity of isolated total RNA were analyzed by
258 measuring the OD₂₆₀ and OD₂₈₀ of 50-fold dilution with
259 DEPC-containing water (also as a blank). The integrity of isolated RNA
260 was checked by agarose gel electrophoresis with gel concentration of 1%.
261 For each lane, 0.8 µg of total RNA was loaded. Total cDNA was
262 obtained by reverse transcription of the isolated total RNA. The
263 mixture for cDNA synthesis can be found in Table S1.
264 Reverse-transcription was carried out at 37°C for 15 min, and

265 then the sample was incubated at 85°C for 5s to inactivate the
266 reverse transcriptase (Wacker and Godard 2005). The
267 semi-quantitative RT-PCR was carried out under the conditions
268 in Table S2 and Table S3. In the real-time PCR, the reaction
269 system specified in Table S4 was used, and the 18S rRNA gene
270 was used as the reference. For each group, three samples were
271 used: #1 was the calibration, the other two were tests. The data
272 was analyzed with $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008).

273

274 Results

275

276 The effect of different carbon and nitrogen sources on mycelium 277 growth and xylanase activity of *A. niger*

278

279 We determined xylanase activity and mycelium fresh weight at
280 two typical time points (40h and 72h) during culture of *A. niger*
281 A09 on different carbon sources (**Table 2**). At each time point,
282 the ratios of activity to mycelium fresh weight were also
283 calculated as an indicator parameter for easier comparison.

284

285 From these data, we could see that the tested carbon sources
286 could be utilized differently by *A. niger* A09. Lactose and
287 arabinose couldn't support the growth of this fungus, indicating
288 the lack of corresponding enzymes to efficiently metabolize
289 these two carbon sources. Maltose and glucose were comparable
290 in supporting growth, but were not suitable carbon sources for
291 enzyme production. Cellobiose was a good carbon source for fast
292 mycelium growth, since the cell weight reached a high value at
293 40h, comparable to that of 72h. Regarding xylanase, there was a
294 negligible change during this course. Xylan and xylose were
295 better carbon sources for the growth and enzyme production of
296 *A. niger* A09. Xylan was a better carbon source for quick and
297 sustainable accumulation of biomass, although the performance

on xylose was also acceptable. Xylan was also a better choice as carbon source for xylanase production, because a higher activity (15.3 IU/ml after 40h) could be achieved. These results suggested that, for the induced expression of xylanase, xylan was better than the monosaccharide xylose, implying that the required additional steps prior to metabolizing xylan didn't constitute a problem limiting the efficient utilization of xylan by *A. niger* A09.

Using xylan as the carbon source, the influence of different nitrogen sources on mycelium accumulation and xylanase secretion was shown in **Table 3**. Preferences for nitrogen sources were observed, and three nitrogen sources (urea, peptone, NaNO_3) couldn't be utilized by this fungus (data not shown). Of the four utilizable nitrogen sources (yeast extract, $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{C}_2\text{O}_4$, NH_4NO_3), better xylanase specific activity at extended culture time were observed with the latter three nitrogen sources. Interestingly, for most microbes, yeast extract serves as a good nutrient source for their growth. Therefore, the decrease in specific activity should be ascribed to the continued accumulation of mycelium, leading to a decreased ratio in specific activity.

Semi-quantitative analysis of the influence of carbon sources and nitrogen sources on transcription of F10 and G11

As shown in the previous experiment, *A. niger* A09 couldn't grow with lactose or arabinose as the carbon source. Therefore, xylan, xylose, glucose, cellobiose, and maltose were used as individual carbon sources to compare xylanase transcription level by semi-quantitative PCR analysis. With the 18S rRNA gene as a reference, the amounts of cDNA template for different samples were determined to get the equal quantity of reference gene

PCR products, and the appropriate cycling number was identified to ensure PCR reactions within the exponential amplification phase. Using the determined cDNA template amounts and cycling number, F10 and G11 transcripts were correspondingly amplified. As shown in **Fig. S1**, the relatively high transcription of G11 was found with xylan and xylose as the carbon source. A similar phenomenon was also observed in the case of F10, although the transcription level on these two carbon sources were slightly lower than that of G11. For G11, variation in transcription levels were more significant. However, F10 exhibited more stable and lower transcription levels with all five carbon sources tested. When the 40h and 72h samples collected from the four different nitrogen sources $[(\text{NH}_4)_2\text{C}_2\text{O}_4, (\text{NH}_4)_2\text{SO}_4, \text{yeast extract}, \text{NH}_4\text{NO}_3]$ were analyzed, G11 transcription levels were higher than those of F10, however, the semi-quantitative PCR failed to reveal meaningful differences in expression level between samples (**Fig. S2**).

Effect of carbon sources on transcriptional level revealed by real-time PCR analysis

Primers are crucial for real-time PCR reliability. A melting curve analysis of 18S rRNA, F10, and G11 gene products (**Fig. S3**) showed that the melting curve for each product had a single peak, indicating that the primer pairs used were specific for the targeted genes. The real-time PCR (**Fig.1, Table S5**) confirmed that the transcription levels of F10 and G11 were influenced, to different extents, by the carbon sources used in the medium. Using the transcription level of F10 xylanase at 40h on glucose medium as a reference, we found that, at 40h, the F10 transcription levels were relatively high with xylose and maltose as carbon source. However, when xylan and cellobiose were used, the transcription levels were rather low. When the

364 cultivation time reached 72h, the superiority of xylan as a
365 carbon source was more prominent: with an approximate
366 6.5-fold increase observed for F10. In contrast, with xylose, a
367 2.7-fold decrease in transcript abundance was observed.
368 Moreover, a similar trend was also observed with maltose and
369 cellobiose. These results together indicated that the
370 transcription of F10 xylanase was significantly dependent on
371 the induction by the available carbon source. Sugars like glucose,
372 xylose, and maltose were better carbon sources that supported
373 increased F10 transcription levels at an earlier time (40h).
374 These observations may reflect a greater ease of *A.niger* A09 in
375 metabolizing these carbon sources. A continued increase in
376 transcript abundance was only seen with xylan and cellobiose,
377 although the F10 transcription level with cellobiose was much
378 lower than that with glucose. When the G11 xylanase was
379 considered, higher transcription levels were found with xylan
380 and xylose as carbon source, which was the same result as
381 revealed by semi-quantitative PCR analysis. In the case of
382 cellobiose at 40h and that of maltose at 72h, the G11
383 transcription levels were much lower versus the reference.
384 While the G11 transcription level on xylose almost remained the
385 same from 40h to 72h, there was a sharp increase in G11
386 transcripts on xylan from 40h to 72h. Therefore, in subsequent
387 experiments, xylan was chosen as the carbon source.

388

389 **Effect of nitrogen source on transcription level revealed by** 390 **real-time PCR analysis**

391

392 Using xylan as the carbon source, seven different nitrogen
393 sources were tested (yeast extract, ammonium oxalate, NH_4NO_3 ,
394 $(\text{NH}_4)_2\text{SO}_4$, peptone, urea, and NaNO_3). Unfortunately, peptone,
395 urea and NaNO_3 could not support the fungus to accumulate
396 sufficient sample for analysis. Therefore, only the yeast extract,

ammonium oxalate, $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 were subsequently investigated. As shown in Figure 2 and Table S6, except for the yeast extract, all the other utilizable nitrogen sources exhibited an increase in the G11 transcription level with the extended cultivation time. Ammonium oxalate yielded the highest G11 transcription level. A possible reason for this phenomenon was that the fungus could utilize both the nitrogen and carbon source contained within ammonium oxalate.

Analysis of samples collected at different time points with real-time PCR

Based on the transcription analyses, we decided to use xylan and ammonium oxalate as the optimal carbon and nitrogen source, respectively. An analysis of F10 and G11 transcription levels was carried out during the cultivation process over 24h to 84h. At the same time, the weight of mycelium, xylanase activity, and specific activity were also determined (**Fig. 3**). The weight of mycelium reached a maximum at 84h, while both the xylanase activity and specific activity reached their highest value at 60h. Extended culture time (84h) was beneficial for the accumulation of mycelium; however, it didn't yield a higher xylanase activity. Since specific activity was the ratio of activity to mycelium weight, a significant decrease in specific activity was consequently observed. From **Fig. 4**, we could clearly see that the transcription level of G11 was much higher than that of F10 at each analyzed time point (**Table S7**). Zymographic analysis of samples collected at different time points showed that both F10 and G11 enzyme increased with extended time (**Fig. S4**). However, the G11 activity band was not as dominant as the transcriptional data observed, hinting that the specific activity of G11 might be lower than that of F10. It might also indicate the translation of G11 transcripts was not as efficient as that of

430 F10 transcripts.

431

432 Discussion

433

434 Nutrients are known to affect the expression of certain
435 proteins/enzymes (de Souza et al. 2013). Our initial intent was
436 to identify cultivation conditions favoring the efficient
437 accumulation of either F10 or G11 xylanase. In this study, we
438 systematically investigated the influence of carbon and nitrogen
439 sources on the transcript abundance of the two main types of
440 xylanase, F10 and G11 from *A. niger* A09. Although we failed in
441 finding a condition that favored the high production of F10 and
442 low production of G11, we successfully found that, by 60h of
443 cultivation (xylan, $(\text{NH}_4)_2\text{C}_2\text{O}_4$, 33°C) a satisfactorily high level
444 of G11 activity and low level of F10 activity could be realized.
445 The resulting xylanase preparation was more favorable for
446 xylo-oligosaccharide production than enzymes prepared under
447 other culture conditions.

448

449 Xylan was the best carbon source for the transcription of the two
450 types of xylanase. Xylose could be utilized for inducing
451 transcription of the enzymes; however, it was less efficient in
452 supporting the accumulation of mycelium and active enzymes
453 (Table 3). This result might imply that other sugars released
454 from xylan due to the hydrolysis could also be efficiently
455 metabolized by this fungus. Furthermore, the contribution from
456 other xylan components to the accumulation of mycelium and
457 xylanase activity were quite obvious, since the corresponding
458 values using xylan at each time point (40h or 72h) were higher
459 than those of using the monosaccharide xylose.

460

461 Since more significant changes in transcript abundance were
462 observed for G11 (Fig. 1, 2, 4) in each analysis, we deduced that,

the utilization of xylan was more dependent on the increased level of G11. The distinct properties of F10 and G11, in combination with the results shown in **Fig. 1, 2, 4**, enables us to propose a possible pathway by which xylan was biodegraded and utilized by fungus *A.niger* A09 (**Fig. 5**). Although transcript abundance of F10 and G11 can be significantly induced by some carbon sources (e.g. the sugars tested in this study), there is some background expression (stage 1, **Fig. 5**). This low level expression is independent of the availability of xylan (or other functionally or structurally similar compounds). The low abundance of F10 protein acts as a sensor to probe the availability of inducers. Why consider F10 to be the sensor protein? This question can be answered based on its structural features. F10 is a larger protein usually containing the carbohydrate binding module (CBM), making it a sensitive probe for inducer compounds in the surrounding environment. The strong affinity of CBM for corresponding substances makes it possible to sense the presence of inducers, even if they are at very low abundance. At the same time, from **Fig. 1**, we could also see that there was a rapid response of F10 to the availability of inducers like xylan, xylose, and other sugars.

As protein F10 probed and detected the inducer compounds (like xylan), the catalytic domain in F10 could hydrolyze the xylan into various fragments or monosaccharide (e.g. xylose, and other sugars). Although the identity of the actual inducer of increased expression of xylanase is still an open question (xylan fragment or xylose?), the results in **Fig. 1**, at least, showed that xylose could efficiently induce increased transcription levels for F10 and G11. Hence xylose is more likely the actual inducer, as suggested by de Vries et al. (1999). When xylan is present, the sensor protein F10 is capable of making more xylose quickly and efficiently because of its high reaction speed and product feature

(predominantly monosaccharide/low DP oligosaccharides) in catalysis.

Therefore, we propose the biodegradation process of xylan by *A.niger* A09 is as follows. When xylan is absent, F10 and G11 are produced due to the low level of background gene expression. The F10 protein senses the existence of xylan, and when xylan is present, F10 binds to xylan and hydrolyzes it into fragments, as well as other monosaccharides. Xylose is then transported into the cell, leading to induced transcription of F10 and G11 to produce more enzymes. At the same time, xylose and other monosaccharides are also metabolized by the fungus to support its growth. This helps explain why a better growth can be witnessed when xylan is the carbon source. This would also help explain our failure in finding the conditions favoring the high production of F10 and the low production of G11.

Acknowledgement

This study was financially supported by the Special Fund for Agro-Scientific Research in the Public Interest from the Ministry of Agriculture, China (No. 201503134), and by the Program for Innovative Research Team (in Science and Technology) in University of Henan Province (No. 15IRTSTHN014). We thank Dr. Barry Flinn for his critical modification to the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest with regard to the publication of this manuscript.

References

Adsul, M.G., Bastawde, K.B., and Gokhale, D.V. 2009.

- 529 Biochemical characterization of two xylanases from yeast
530 *Pseudozyma hubeiensis* producing only
531 xylooligosaccharides. *Bioresour Technol* 100:6488–6495.
532 doi: 10.1016/j.biortech.2009.07.064
- 533 Ahmed, S., Riaz, S., and Jamil, A. 2009. Molecular cloning of
534 fungal xylanases: an overview. *Appl Microbiol Biotechnol*
535 84:19–35. doi: 10.1007/s00253-009-2079-4
- 536 Biely, P., Vrsanska, M., Tenkanen, M., and Kluepfel, D. 1997.
537 Endo-beta-xylanases families : differences in catalytic
538 properties. *J Biotechnol* 57:151-166
- 539 Bragatto, J., Segato, F., and Squina, F.M. 2013. Production of
540 xylooligosaccharides (XOS) from delignified sugarcane
541 bagasse by peroxide-HAc process using recombinant
542 xylanase from *Bacillus subtilis*. *Ind Crops Prod*
543 51:123-129.
- 544 Buchert, J., Tenkanen, M., Kantelinen, A., and Viikari, L. 1994.
545 Application of xylanases in the pulp and paper industry.
546 *Biores Technol* 50:65–72
- 547 Chen, P., and Buller, C.S. 1995. Activity staining of xylanases in
548 polyacrylamide gels containing xylan. *Anal Biochem*

- 549 226:186–188. doi: 10.1006/abio.1995.1209
- 550 Collins, T., Gerday, C., and Feller, G. 2005. Xylanases, xylanase
551 families and extremophilic xylanases. FEMS Microbiol Rev
552 29:3–23.
- 553 de Oliveira, J.M.P.F., van Passel, M.W.J., Schaap, P.J., and de
554 Graaff, L.H. 2011. Proteomic analysis of the secretory
555 response of *Aspergillus niger* to D-maltose and D-xylose.
556 PloS One 6:e20865. doi: 10.1371/journal.pone.0020865
- 557 de Souza, W.R., Maitan-Alfenas, G.P., de Gouvêa, P.F., Brown,
558 N.A., Savoldi, M., Battaglia, E., et al. 2013. The influence
559 of *Aspergillus niger* transcription factors AraR and XlnR
560 in the gene expression during growth in D-xylose,
561 L-arabinose and steam-exploded sugarcane bagasse.
562 Fungal Genet Biol FG B 60:29–45. doi:
563 10.1016/j.fgb.2013.07.007
- 564 Deutschmann, R., and Dekker, R.F.H. 2012. From plant biomass
565 to bio-based chemicals: latest developments in xylan
566 research. Biotechnol Adv 30:1627–1640. doi:
567 10.1016/j.biotechadv.2012.07.001
- 568 de Vries, R.P., Visser, J., and de Graaff, L.H. 1999. CreA

- modulates the XlnR-induced expression on xylose of
Aspergillus niger genes involved in xylan degradation. Res
Microbiol 150:281–285
- Gusakov, A.V., Kondratyeva, E.G., and Sinitsyn, A.P. 2011.
Comparison of two methods for assaying reducing sugars
in the determination of carbohydrase activities. Int J Anal
Chem 2011:e283658. doi: 10.1155/2011/283658
- Han, Y., and Lei, X.G. 1999. Role of glycosylation in the
functional expression of an *Aspergillus niger* phytase
(phyA) in *Pichia pastoris*. Arch Biochem Biophys
364:83–90
- Harris, P.V., Xu, F., Kreel, N.E., Kang, C., and Fukuyama, S.
2014. New enzyme insights drive advances in commercial
ethanol production. Curr Opin Chem Biol 19:162–170. doi:
10.1016/j.cbpa.2014.02.015
- Henrissat, B. 1991. A classification of glycosyl hydrolases based
on amino acid sequence similarities. Biochem J 280 (Pt
2):309–316.
- Henrissat, B., and Bairoch, A. 1993. New families in the
classification of glycosyl hydrolases based on amino acid

- sequence similarities. *Biochem J* 293 (Pt 3):781–788.
- Ho, W., and Ko, W. 1997. A simple method for obtaining single-spore of fungi. *Bot Bull Acad Sin* 38:41–44.
- Jeffries, T.W. 1996. Biochemistry and genetics of microbial xylanases. *Curr Opin Biotech* 7:337-342.
- Kniemeyer, O. 2011. Proteomics of eukaryotic microorganisms: The medically and biotechnologically important fungal genus *Aspergillus*. *Proteomics* 11:3232–3243. doi: 10.1002/pmic.201100087
- Leber, T.M., and Balkwill, F.R. 1997. Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. *Anal Biochem* 249:24–28. doi: 10.1006/abio.1997.2170
- Lee, R.C., Hrmova, M., and Burton, R.A. 2003. Bifunctional family 3 glycoside hydrolases from barley with alpha-L-arabinofuranosidase and beta-D-xylosidase activity characterization, primary structure and COOH-terminal processing. *J Biol Chem* 278:5377–5388.
- Liu, M.Q., and Liu, G.F. 2008. Expression of recombinant *Bacillus licheniformis* xylanase A in *Pichia pastoris* and

- 609 xylooligosaccharides released from xylans by it. Protein
610 Expr Purif 57:101-107
- 611 Olukosi, O.A., Bedford, M.R., and Adeola, O. 2007. Xylanase in
612 diets for growing pigs and broiler chicks. Can J Anim Sci
613 87:227-235. doi: 10.4141/CJAS06005
- 614 Qu, W., Xue, Y., and Ding, Q. 2015. Display of fungi xylanase on
615 *Escherichia coli* cell surface and use of the enzyme in xylan
616 biodegradation. Curr Microbiol 70:779-785. doi:
617 10.1007/s00284-015-0781-2
- 618 Rivers, D.B., Gracheck, S.J., Woodford, L.C., and Emert, G.H.
619 1984. Limitations of the DNS assay for reducing sugars
620 from saccharified lignocellulosics. Biotechnol Bioeng
621 26:800–802. doi: 10.1002/bit.260260727
- 622 Schmittgen, T.D., and Livak, K.J. 2008. Analyzing real-time
623 PCR data by the comparative C(T) method. Nat Protoc
624 3:1101–1108. doi: 10.1038/nprot.2008.73
- 625 Schuster, E., Dunn-Coleman, N., Frisvad, J.C., and van Dijck,
626 P.W.M. 2002. On the safety of *Aspergillus niger*—a review.
627 Appl Microbiol Biotechnol 59:426–435. doi:
628 10.1007/s00253-002-1032-6

629 Wacker, M.J., and Godard, M.P. 2005. Analysis of one-step and
630 two-step real-time RT-PCR using SuperScript III. J Biomol
631 Tech JBT 16:266–271.

632 Wang, Q., Du, W., Weng, X.Y., Liu, M.Q., Wang, J.K., and Liu,
633 J.X. 2015. Recombination of thermo-alkalstable, high
634 xylooligosaccharides producing endo-xylanase from
635 *Thermobifida fusca* and expression in *Pichia pastoris*. Appl
636 Biochem Biotechnol 175:1318-1329. doi:
637 10.1007/s12010-014-1355-7

Draft

Figure captions

Fig. 1 Effect of carbon source on xylanase transcription levels revealed by real-time PCR analysis

Transcription levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Values smaller than 0 mean the transcription levels were decreased by the indicated fold-number compared with the reference (the value for F10 at 40h for samples collected from culture broth with glucose as carbon source). F10-40h stands for the level of F10 at 40h.

Fig. 2 Effect of nitrogen source on xylanase transcription levels revealed by real-time PCR analysis

Transcription levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Values smaller than 0 mean the transcription levels were decreased by the indicated fold-number compared with the reference (the value of F10 at 40h for samples collected from culture broth with yeast extract as nitrogen source). F10-40h stands for the level of F10 at 40h.

Fig. 3 The changes of mycelium weight, activity and specific activity during the culture process

Mycelium weight exhibited a continual increase, while activity and specific activity reached the maximum at 60h.

Fig. 4 Xylanase transcription levels revealed by real-time PCR analysis during the culture process

Transcription levels were calculated using the $2^{-\Delta\Delta C_t}$ method with the value of F10 at 40h for samples collected from culture broth with xylan as carbon source, and $(\text{NH}_4)_2\text{C}_2\text{O}_4$ as nitrogen source. F10-40h stands for the level of F10 at 40h.

Fig. 5 The proposed scheme of xylan biodegradation by *A.niger*

689 **A09**

690 Background level expression product F10 and G11 were secreted
691 into the medium. F10 functions as a sensor to probe for the
692 existence of the inducer xylan (or other inducers). F10 binds to
693 xylan and hydrolyzes it to produce xylose. Xylose enters the cell,
694 inducing and increased level of F10 and G11 gene expression. In
695 the regions containing the genes for F10 and G11, black
696 represent the introns, and white the exons.

Draft

Fig. 1 Effect of carbon source on xylanase transcription levels revealed by real-time PCR analysis
Transcription levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Values smaller than 0 mean the transcription levels were decreased by the indicated fold-number compared with the reference (the value for F10 at 40h for samples collected from culture broth with glucose as carbon source). F10-40h stands for the level of F10 at 40h.
83x71mm (300 x 300 DPI)

Fig. 2 Effect of nitrogen source on xylanase transcription levels revealed by real-time PCR analysis. Transcription levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Values smaller than 0 mean the transcription levels were decreased by the indicated fold-number compared with the reference (the value of F10 at 40h for samples collected from culture broth with yeast extract as nitrogen source). F10-40h stands for the level of F10 at 40h.

83x70mm (300 x 300 DPI)

Fig. 3 The changes of mycelium weight, activity and specific activity during the culture process
Mycelium weight exhibited a continual increase, while activity and specific activity reached the maximum at 60h.
83x58mm (300 x 300 DPI)

Fig. 4 Xylanase transcription levels revealed by real-time PCR analysis during the culture process. Transcription levels were calculated using the $2^{-\Delta\Delta C_t}$ method with the value of F10 at 40h for samples collected from culture broth with xylan as carbon source, and $(\text{NH}_4)_2\text{C}_2\text{O}_4$ as nitrogen source. F10-40h stands for the level of F10 at 40h.

83x65mm (300 x 300 DPI)

Fig. 5 The proposed scheme of xylan biodegradation by *A. niger* A09
Background level expression product F10 and G11 were secreted into the medium. F10 functions as a sensor to probe for the existence of the inducer xylan (or other inducers). F10 binds to xylan and hydrolyzes it to produce xylose. Xylose enters the cell, inducing and increased level of F10 and G11 gene expression. In the regions containing the genes for F10 and G11, black represent the introns, and white the exons.

Tables**Table 1. Primers used in this study.**

Primers	Sequence(5'->3')
18SrRNAF	ACTCACCAGGTCCAGACAAAATAAG
18SrRNAR	AAGCAGACAAATCACTCCACCA
F10F	CGGACAAGAATACACTGATCGAAG
F10R	TTGACAACATCCCAGGCATAAA
G11F	GCAAAACTACAACGGCAACC
G11R	AGCCCAGACCAACGACAAA

Draft

Table 2. The effect of different carbon source on xylanase production capacity and mycelium growth of *A. niger*A09

Carbon source(2%)	Activity (IU/ml)		Weight (g)		Specific activity (IU/g)	
	40h	72h	40h	72h	40h	72h
xylan	15.3±0.2	45.14±1.3	3.15±0.28	4±0.66	243±12.1	564.5±39.5
xylose	4.21±0.8	30.6±3.3	1.55±0.12	3.35±0.52	135±15.7	456.5±21.6
cellobiose	0.77±0.13	0.69±0.17	3.67±0.37	3.85±0.83	10.5±2.2	9±1.1
glucose	0.46±0.19	0.54±0.05	1.35±0.1	2.20±0.21	17±1.9	12.5±0.9
maltose	—	0.92±0.16	1.12±0.11	2.10±0.23	—	22±1.6
lactose	—	—	—	—	—	—
arabinose	—	—	—	—	—	—

Note: Activity: xylanase activity of crude enzyme; Weight: the fresh weight of mycelium after washing; Specific activity: the ratio of the total xylanase activity in crude enzyme to the total mycelium weight.

Table 3. The effect of different nitrogen source on xylanase production capacity and mycelium growth of *A. niger* A09

Nitrogen source (0.3%)	Activity (IU/ml)		Weight (g)		Specific activity (IU/g)	
	40h	72h	40h	72h	40h	72h
Yeast extract	38.3±0.86	43.5±0.21	1.8±0.2	2.2±0.1	1063±1.6	988.6±5.8
(NH ₄) ₂ C ₂ O ₄	35.8±0.24	52±0.91	1.8±0.1	2.5±0.09	993.6±1.9	1040±4.7
(NH ₄) ₂ SO ₄	32.5±0.43	45.3±0.39	1.7±0.15	2.1±0.07	955.3±5.9	1077.4±7.1
NH ₄ NO ₃	42.2±1.1	58±0.54	2.1±0.2	2.4±0.12	1004.8±6.2	1208.3±4.6

Note: Activity: xylanase activity of crude enzyme; Weight: the fresh weight of mycelium after washing; Specific activity: the ratio of the total xylanase activity in crude enzyme to the total mycelium weight.