Improvement in xylooligosaccharides production by knockout of the β -xyl1 gene in *Trichoderma orientalis* EU7-22

Chuannan Long^{1,2} · Jingjing Cui^{1,3,4} · Hailong Li^{3,5} · Jian Liu³ · Lihui Gan³ · Bin Zeng^{1,2} · Minnan Long³

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Abstract

The goal of this study was to enhance the production of xylooligosaccharides (XOs) and reduce the production of xylose. We investigated β -xylosidases, which were key enzymes in the hydrolysis of xylan into xylose, in *Trichoderma orientalis* EU7-22. The binary vector pUR5750G/*bxl*::*hph* was constructed to knock out the β -xyl1 gene (encoding β -xylosidases) in *T. orientalis* EU7-22 by homologous integration, producing the mutant strain *T. orientalis* Bxyl-1. Xylanase activity for strain Bxyl-1 was 452.42 IU/mL, which increased by only 0.07% compared to that of parental strain EU7-22, whereas β -xylosidase activity was 0.06 IU/mL, representing a 91.89% decrease. When xylanase (200 IU/g xylan), produced by *T. orientalis* EU7-22 and *T. orientalis* Bxyl-1, was used to hydrolyze beechwood xylan, in contrast to the parental strain, the XOs were enhanced by 83.27%, whereas xylose decreased by 45.80% after 36 h in *T. orientalis* Bxyl-1. Based on these results, *T. orientalis* Bxyl-1 has great potential for application in the production of XOs from lignocellulosic biomass.

Keywords *Trichoderma orientalis* · Xylanase · β -xylosidase · β -xyl1 gene · Xylooligosaccharides · Xylose

Introduction

Hemicellulose, a main element of lignocellulosic biomass, is the second most abundant polysaccharide in nature. Xylan is a major component of hemicellulose, which can be used to manufacture xylooligosaccharides (XOs) (Vazquez et al. 2000; Huang et al. 2017). However, because the xylan–lignin complex in lignocellulosic biomass resists hydrolysis, XOs

are produced in two steps: alkaline extraction of xylan from lignocellulosic biomass, followed by enzymatic hydrolysis (Vazquez et al. 2000; Akpinar et al. 2007; Chapla et al. 2012). Compared to acid hydrolysis, enzymatic hydrolysis is an attractive approach, because it does not yield undesirable by-products and does not require special equipment (Chapla et al. 2012; Bosetto et al. 2016; Huang et al. 2017). Of course, complete degradation of xylan requires xylanolytic enzymes such as endo- β -1,4-xylanase, β -xylosidases, and debranching enzymes (Collins et al. 2005; Knob et al. 2010; Bosetto et al. 2016; Huang et al. 2017). The main chain of xylan is randomly degraded by endo- β -1,4-xylanase to produce oligosaccharides. Then, the XOs are further hydrolyzed into xylose by β -xylosidases. Therefore, high endo- β -1,4-xylanase activity and the production of no β -xylosidases are desirable in XO production (Chen et al. 2009; Knob et al. 2010; Sabiha-Hanim et al. 2011; Bosetto et al. 2016).

Trichoderma spp., a well-known filamentous fungi, show good capacity to degrade lignocellulosic biomass. Hermann et al. (1997) reported that purified β -xylosidases from *Trichoderma reesei* were multifunctional β -D-xylan xylohydrolases. Chen et al. (2009) studied the activity of purified endo- β -1,4-xylanase in *Trichoderma* sp. K9301 during XO production. Choengpanya et al. (2015) reported the function of β -xylosidase in *Aspergillus niger* ASKU28. However,

✉ Minnan Long
longmn@xmu.edu.cn

¹ Jiangxi Key Laboratory of Bioprocess Engineering, Jiangxi Science and Technology Normal University, Nanchang 330013, People's Republic of China

² School of Life Science, Jiangxi Science and Technology Normal University, Nanchang 330013, People's Republic of China

³ College of Energy, Xiamen University, Xiamen 361005, People's Republic of China

⁴ National Engineering Laboratory for Green Chemical Productions of Alcohols Ethers Esters, Xiamen University, Xiamen 361005, People's Republic of China

⁵ Key Laboratory of Renewable Energy, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, People's Republic of China

there has been no report on the effect of knocking out the β -xylI gene, which encodes β -xylosidases, on XO production in filamentous fungi.

In eukaryotes, DNA double-strand break (DSBs) repair mainly occurs via homologous recombination (HR) and non-homologous end-joining (NHEJ), which act independently but competitively (Dyck et al. 1999). NHEJ is the dominant pathway in many eukaryotes such as plants, mammals, insects, and filamentous fungi (Pastink et al. 2001; Tachibana 2004). Gene targeting (knockout), which can be used to delete genes and replace alleles (Feng et al. 2012), is a useful technique to study gene function in living organisms, especially in filamentous fungi (Carvalho et al. 2010; Kück and Hoff 2010). Normally, gene replacement frequencies in filamentous fungi are less than 30%, so it is a time-consuming process to generate homologous transformants (Meyer 2008; Kück and Hoff 2010).

In this study, we investigated knockout of the β -xylI gene (GenBank no. JQ238612) from *Trichoderma orientalis* EU7-22 by homologous recombination. The differences in the activities of enzymes and production of XOs between parent strains and mutant strains were determined.

Materials and methods

Strains

The hemicellulase-producing strain *T. orientalis* EU7-22 was used as a host for knockout of the β -xylI gene (GenBank no. JQ238612). *Escherichia coli* DH5a was used for vector construction. *Agrobacterium tumefaciens* AGL1 was used to mediate transformation (Long et al. 2013).

Plasmid construction and molecular analysis of the mutant strain

The T-DNA binary vector pUR5750G/*bxl::hph* was constructed to knock out β -xylI on the backbone of pUR5750G (Fig. 1a). All 2394 bp of the β -xylI gene was amplified by PCR using primers BXL-F & R (Fig. 1b, primers F2 and R2), with the total DNA from *T. orientalis* EU7-22 used as a template. The first homologous arm (fragment I = 946 bp) of the β -xylI gene was amplified by PCR with primers BXL-QC1F (containing *KpnI*) and BXL-QC1R (containing *SacI*) (Fig. 1b, primers F1 and R1), and it was inserted into the *KpnI/SacI* site of pUR5750G. At the same time, the second homologous arm (fragment II = 925 bp) was obtained by digesting the β -xylI DNA fragment with *XbaI/HindIII* (Fig. 1b), and it was inserted into the *XbaI/HindIII* site of pUR5750G. The sequences of the primers used in this study are listed in Table 1.

Fig. 1 Schematic map of the vector and primer loci for knockout of the β -xylI gene. **a** pUR5750G; **b** F1 primer BXL-QC1F, R1 primer BXL-QC1R, F2 primer BXL-F, R2 primer BXL-R, F3 primer HtrpC-F, R3 primer BXL-YZR, F4 primer Hph-F, R4 primer Hph-R

The binary vector pUR5750G/*bxl::hph* was transferred to the parental strain *T. orientalis* EU7-22 via *A. tumefaciens*-mediated transformation (ATMT) (Long et al. 2013). The mutant strain was analyzed by PCR (Fig. 2).

Enzyme production and activity assay

Experiments were conducted in 250 mL Erlenmeyer flasks with *T. orientalis* EU7-22 and mutant strain Bxyl-1, which were incubated on a rotary shaker (37.3 °C, 180 rpm) for 3 days). The enzyme production medium (pH 5.2) consisted of the following: 1.5% (w/v) corn cobs, 1.5% (w/v) wheat bran, 0.5% (w/v) peptone, 0.4% (w/v) Tween-80, 0.04% (w/v) CaCl_2 , 0.04% (w/v) FeSO_4 , 0.08% (w/v) MgSO_4 , and 2.5 g/L KH_2PO_4 .

Table 1 Primers used in this study

Primer name	Primer sequence (5'–3')	Description
BXL-QC1F	CGGGGTACCGGCCCTGGCGCAAAACAATC	For amplification of the 946-bp fragment I
BXL-QC1R	CCGAGCTCAGCGGCAGACGACTGGTTGC	
BXL-F	ATGGTGAATAACGCAGCTCTTCTCG	For amplification of the 2394-bp <i>bxyI</i> gene
BXL-R	TTATGCGTCAGGTGTAGCATCCTTG	
Hph-F	CGACAGCGTCTCCGACCTGA	For detection of the 811-bp <i>hph</i> gene fragment
Hph-R	CGCCCAAGCTGCATCATCGAA	
HtrpC-F	AGGAATAGAGTAGATGCCGACC	For detection of the 1748-bp knockout fragment
BXL-YZR	TGTAGCATCCTTGATCTGTTGC

Fig. 2 Identification of *T. orientalis* Bxyl-1, Bxyl-2, Bxyl-3, Bxyl-4 transformants using a molecular method. **a** Primers BXL-F and BXL-R test, lanes 1–5 represent *T. orientalis* EU7-22 and transformants *T. orientalis* Bxyl-1, Bxyl-2, Bxyl-3, Bxyl-4, and total DNA as a template, respectively. **b** Primers HtrpC-F and BXL-YZR test, lanes 1–5 represent *T. orientalis* EU7-22 and transformants *H. orientalis* Bxyl-1, Bxyl-2, Bxyl-3, and Bxyl-4 total DNA as templates, respectively. **c** Primers Hph-F and Hph-R test, lanes 1–4 represent transformants *T. orientalis* Bxyl-1, Bxyl-2, Bxyl-3, and Bxyl-4 total DNA as templates, respectively. Ma 200 bp DNA ladder marker; Mb DL5000 DNA Marker

Crude enzyme was first centrifuged (6000 rpm) for 10 min to remove the cells and solid medium. Enzyme activity in the supernatant was determined. Xylanase activity was assayed according to the method of Bailey et al. (1992) by measuring the total reducing sugars released from 1% (w/v) beechwood xylan (Sigma, St. Louis, USA, X4252) (1.0 mL) in citrate buffer (50 mM, pH 4.8). One unit of

xylanase activity was defined as the amount of enzyme that produced 1 μ mol xylose per minute and was expressed in IU/mL. β -xylosidases activity was assayed with 4-nitrophenyl β -D-xylopyranoside (pNPX, 5 mM) (Sigma, St. Louis, USA, N2132) as the substrate by measuring the amount of *p*-nitrophenol released (Lachke 1988). The mixture was incubated at 50 °C for 30 min, and the reaction was stopped by adding Na_2CO_3 (0.5 M). One unit of enzyme activity was defined as the amount of enzyme needed to liberate 1 μ mol *p*-nitrophenol per min and was expressed in IU/mL. Filter paper activities (FPA) were measured as described by Ghose (1987). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μ M of reducing sugar per minute and was expressed in IU/mL.

Xylan hydrolysis

Beechwood xylan (0.5 g) was subjected to enzymatic hydrolysis of xylanase, the enzyme produced from parent strains and mutant strains. In 100 mL Erlenmeyer flasks, a fixed volume was reached by adding 25 mL buffer. The enzymatic hydrolysis experiments were performed according to the National Renewable Energy Laboratory analytical procedures, with 2% (w/v) solids loaded with 0.05 M citrate buffer (pH 4.8) into a thermostatically controlled shaker at 50 °C and the flasks placed in an incubator with a shaking speed of 150 rpm (Selig and Weiss 2008; Sabiha-Hanim et al. 2011). To prevent possible microbial growth on the sugars generated, 100 μ L of 2% sodium azide was added. Xylanase (200 IU/g xylan) was transferred to the hydrolysis broth, and samples (0.5 mL) taken after 0.5, 1, 2, 4, 6, 8, 10, 12, 24, and 36 h of hydrolysis were analyzed.

High-performance liquid chromatography (HPLC) analysis of XO and xylose

1-Phenyl-3-methyl-5-pyrazolone (PMP) (Acrose Organics, Belgium) derivatization of saccharides was carried out as described previously with proper modification (Li et al. 2013). Briefly, individual standard saccharides, mixed

standard saccharides, or hydrolysates of xylan were dissolved in 0.3 M aqueous NaOH (50 μ L), and a 0.5 M methanol solution of PMP (50 μ L) was added to each solution. Each mixture was allowed to react for 60 min at 70 °C, then cooled to room temperature and neutralized with 0.3 M HCl (50 μ L). The resulting solution was extracted with chloroform (1 mL), and the process was repeated three times; then, the aqueous layer was filtered through a 0.22 μ m membrane. Xylotetraose (X4), xylotriose (X3), and xylobiose (X2) were obtained from Megazyme (Bray, Ireland), and xylose was purchased from Merck (Germany); all were standard saccharides (mg/mL). In this study, total XOs contained xylotetraose, xylotriose and xylobiose. PMP-labeled saccharides were analyzed on an HPLC system (Thermo Scientific Ultimate 3000) using a C18 column (Dionex Bonded Silica Products) (5 μ m, 120A, 4.6 $\times$ 250 mm) and a column oven at 35 °C. The wavelength for UV detection was 245 nm. The product was eluted at a flow rate of 0.5 mL/min. Mobile phase A was phosphate buffer (40 mM, pH 6.7, 70%), and mobile phase B was acetonitrile (30%). The injection volume was 10 μ L. The statistical significance of differences between the parent strain and the mutant strain was assessed by one-way analysis of variance.

Results and discussion

Obtaining the $\Delta\beta$ -xylI mutant strain

After *A. tumefaciens* AGL1 successfully mediated pUR5750G/bxl::hph transformation into *T. orientalis* EU7-22 genome, four transformants were selected after 5 days of cultivation. These transformants were designated Bxyl-1, -2, -3, and -4, and all of them were shown to retain their mitotic stability. Genomic DNA from the four transformants was tested for the presence of the *hph* gene by PCR using specific primers Hph-F & R (Figs. 1b, primers F4 and R4, 2c).

At the same time, primers BXL-F & R (Fig. 1b, primers F2 and R2) were used to confirm the integration of the *hph* cassettes (2717 bp, *PgpdA-hph-TrpC*) into the β -xylI locus (Fig. 2a). The length of the β -xylI gene was 2394 bp, and the gap between homologous arm fragment I and fragment II was 380 bp (Fig. 1b). That means there were 2337 base pairs more in the positive transformants than in the parent strain *T. orientalis* EU7-22 (Fig. 2a, lane 1). The transformant *T. orientalis* Bxyl-1 met this length specification (Fig. 2a, lane 2).

Then, primers HtrpC-F and BXL-YZR (Fig. 1b, primers F3 and R3) were used to confirm that the *hph* cassettes were integrated into the β -xylI gene locus. The amplified fragments (1748 bp) contained the *TrpC* terminator (748 bp), the second homologous arm (fragment II = 925 bp), and sequence downstream of fragment II (75 bp). There were no bands resulting from PCR of the

parental strain *T. orientalis* EU7-22 (Fig. 2b, lane 1) or transformant *T. orientalis* Bxyl-2 (Fig. 2b, lane 3) obtained by PCR. There were, however, nonspecific amplification bands resulting from PCR of the transformants *T. orientalis* Bxyl-3 (Fig. 2b, lane 4) and *T. orientalis* Bxyl-4 (Fig. 2b, lane 5). In contrast, transformant *T. orientalis* Bxyl-1 showed the expected band after PCR amplification (Fig. 2b, lane 2). The data indicated that the *hph* cassette was successfully inserted into the β -xylI locus of transformant Bxyl-1, but that the *hph* cassette was randomly inserted into another three transformants. Therefore, the homologous recombination frequency was 25%.

Effect of β -xylI gene deletion on enzyme production

Xylanase and β -xylosidase activities and FPA for the mutant strain *T. orientalis* Bxyl-1 were 0.06, 452.42, and 0.96 IU/mL, respectively, whereas, for *T. orientalis* EU7-22s, these activities were 0.74, 422.11, and 0.95 IU/mL, respectively. These data indicated that there was no large impact of the β -xylI gene deletion on FPA activity. The xylanase activity in mutant strain Bxyl-1 only increased by 0.07%, but the β -xylosidase activity decreased by 91.89%. The β -xylosidase activity decreased significantly relative to that of the parental strain ($p < 0.01$) (Fig. 3). As expected, the $\Delta\beta$ -xylI mutation resulted in the repression of β -xylosidase expression. β -xylosidases exhibited high activity with the substrate pNPX. A previous study showed that GH10 endo- β -1,4-xylanase also exhibited detectable activity with pNPX (Li et al. 2015). This may be why minor beta-xylosidases activity was detected in mutant strain Bxyl-1.

Fig. 3 Enzyme production from *T. orientalis* EU7-22 and *T. orientalis* Bxyl-1. The significance of the difference from *T. orientalis* EU7-22 is indicated by asterisks for *T. orientalis* Bxyl-1 in each group. ** $p < 0.01$)

Hydrolysis of xylan to produce xylooligosaccharides and xylose

Figure 4 illustrates XO and xylose production from beechwood xylan by xylanase prepared from *T. orientalis* EU7-22 and *T. orientalis* Bxyl-1. As observed in the hydrolysis product curves, the concentration of xylotetraose (X4) increased rapidly to its peak value in the first 0.5 h, but then decreased and disappeared after 2 h. This pattern was similar for the two strains. The concentration of xylotriose (X3) also rapidly increased to its peak value in the first 0.5 h, then

Fig. 4 Analysis of the hydrolysis product of beechwood xylan by *T. orientalis* EU7-22 and *T. orientalis* Bxyl-1. **a** Sugar content of XOs and xylose in *T. orientalis* EU7-22 and *T. orientalis* Bxyl-1. **b** Sugar content of xylotetraose (X4), xylotriose (X3), and xylobiose (X2) in *T. orientalis* EU7-22 and *T. orientalis* Bxyl-1. The significance of the difference from *T. orientalis* EU7-22 is indicated by asterisks for *T. orientalis* Bxyl-1 in each group. * $0.05 < p < 0.01$, ** $p < 0.01$)

decreased gradually; however, xylotriose decreased faster in strain Bxyl-1 than in EU7-22. Xylotriose content decreased significantly relative to that of the parental strain at 12, 24, and 36 h ($p < 0.01$) (Fig. 4b). However, the concentration of xylobiose (X2) in the two strains sharply increased in the first 2 h; it increased gradually from 2 to 12 h, then decreased in strain EU7-22 and continued to increase through time in strain Bxyl-1. Compared to strain EU7-22, xylobiose production increased by 31.89, 31.77, 76.60, and 95.18% at 6, 12, 24, and 36 h, respectively, in strain Bxyl-1. Xylobiose content increased significantly relative to that of the parental strain at 6, 8, 10, 12, 24, and 36 h ($p < 0.01$) (Fig. 4b).

The concentration of xylose increased in the two strains at the same velocity, but there was only a slight difference between the xylose products from strain EU7-22 and Bxyl-1 in the first 6 h. Finally, the concentration of strain Bxyl-1 was lower than that of EU7-22, which decreased by 13.61, 24.12, and 45.80% at 12, 24, and 36 h, respectively. Xylose content decreased significantly relative to that of the parental strain at 24 and 36 h ($p < 0.05$) (Fig. 4a). Collectively, compared to strain EU7-22, the XOs (xylobiose, xylotriose, and xylotetraose) increased by 25.99, 24.73, 64.13, and 83.27% at 6, 12, 24, and 36 h, respectively, in strain Bxyl-1. XOs content increased significantly relative to that of the parental strain at 6, 8, 10, 12, 24, and 36 h ($p < 0.01$) (Fig. 4a).

Endo- β -1,4-xylanases hydrolyzed the β -1,4-glycosidic linkages of the xylan backbone to produce a large amount of XOs, as well as a small amount of xylose. Endo- β -1,4-xylanases from both GH10 and GH11 were not able to hydrolyze xylobiose (Li et al. 2015). β -xylosidases attack the non-reducing ends of short xylooligosaccharides to liberate xylose (Sorensen et al. 2003; Sabiha-Hanim et al. 2011; Rasmussem et al. 2006). Therefore, the hydrolysis product of xylobiose in Bxyl-1 was more than that in EU7-22, and the amount of xylose produced in Bxyl-1 was less than that in EU7-22. These results indicate that more XOs and less xylose can be produced using mutant strain *T. orientalis* Bxyl-1.

Conclusions

This study focused on β -xylosidases from *T. orientalis* EU7-22 to examine xylooligosaccharides production. The homologous integration vector pUR5750G/*bxl::hph* was constructed to knock out the β -*xyll* gene. We obtained the mutant strain *T. orientalis* Bxyl-1, which exhibited high xylanase activity and no β -xylosidase activity. XOs and xylobiose increased by 83.27 and 95.18%, respectively, for 36 h, whereas xylose decreased by 45.80% for 36 h in *T. orientalis* Bxyl-1. Based on these results, the mutant strain has great potential for application in the production of XOs from lignocellulosic biomass.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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