Research Article

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Recombinant expression of *Aspergillus niger* GH10 endo-xylanase in *Pichia pastoris* by constructing a double-plasmid co-expression system

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Abstract

BACKGROUND: Previous work has shown that GH10 endo-xylanase presented better synergistic cooperation with cellulase to achieve effective hydrolysis. However, most currently reported commercial xylanolytic enzymes from *Aspergillus niger* belong to GH11 and there have been few studies regarding GH10 xylanase. In addition, to increase overall expression of heterologous endo-xylanase in *Pichia pastoris*, a double-plasmid co-expression method, including the construction of recombinant *P. pastoris* using one expression vector followed by inserting another vector which targeted to different locus, was introduced.

RESULTS: A GH10 xylanase gene from *A. niger* BE-2 (*XynC*) was optimized and constitutively expressed in *P. pastoris* GS115. Compared with the conventional single-plasmid method, it appeared that the double-plasmid strategy could considerably increase the XynC yield by ∼33%. To further improve enzyme production, cultivation conditions were optimized at shake-flask level and then scaled up to a 5-L bioreactor. Through high cell-density fermentation, the expression level of GH10 XynC reached 1650 U mL^{-1}. The XynC showed maximum activity at 55 °C and pH 5.0, and exhibited an excellent stability over a wide range of pH from 4.5 to 7.0. The kinetic parameters K_m and V_{max} values for beechwood xylan were 3.5 mg mL^{-1} and 2327 U mg^{-1}, respectively.

CONCLUSION: The double-plasmid co-expression strategy introduced herein could greatly improve the XynC yield in *P. pastoris*. The GH10 xylanase from *A. niger* BE-2 shared similar reaction conditions with the existing enzymatic hydrolysis, which might be a good candidate to assist cellulases for industrial application.

Supporting information may be found in the online version of this article.

Keywords: codon optimization; double-plasmid co-expression system; *Pichia pastoris*; recombinant expression; xylanase

INTRODUCTION

Xylan-type polysaccharides, mainly present in hardwood and annual plants, are the second-most abundant carbohydrate in plant materials after cellulose.[1] Unlike cellulose, which has a well-defined linear structure, naturally occurring xylan often exhibits an heterogeneous branched structure with a backbone of β-(1,4)-linked xylopyranosyl and different side-chain residues, such as acetyl, L-arabinofuranosyl and 4-*O*-methylglucuronyl substituents.[2] Owing to this complex structure, various backbone cleaving and debranching enzymes are required to break down the xylan completely. Among these enzymes, endo-xylanase (EC 3.2.1.8) plays a vital role in depolymerization of the xylan by randomly hydrolyzing β-1,4-glycosidic bonds to produce xylooligosaccchrides with different lengths.[3,4]

Recently, interest in endo-xylanase production and application has gained much attention as xylanase is one of the most important accessory enzymes in the bioconversion of lignocellulosic biomass to value-added biofuels and biochemicals.[5,6] However, the existing enzymes are always limited by their high production costs and low catalytic capability, which has posed challenges to developing an economical and feasible hydrolysis process.[7] To advance the utilization of recombinant xylanase, extensive

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research on further optimization of recombinant DNA technology for large-scale expression of the enzyme in an heterologous host system and development of more efficient enzymes are necessary.[8,9]

Pichia pastoris is a well-described expression system that has been successfully utilized for the overexpression of recombinant proteins.[10] Previous work has overexpressed endo-xylanases (HoXyn11A and AnXyn10C) from *Hypocrea orientalis* and *Aspergillus niger* in *P. pastoris* with detected xylanase activities of 284 U mL^{-1} and 306 U mL^{-1}, respectively, at the shake-flask level.[11] However, even though *P. pastoris* is a good host for protein expression, there is still space for improvement at the molecular level, such as codon utilization, mRNA secondary structure and genomic copy numbers.[12] In several cases, the increase in gene copy number results in a proportional elevation in the mRNA levels of the gene, which in turn is closely related to the increase in the levels of the corresponding recombinant protein.[13] For example, around 200-fold increased productivity of tumor necrosis factor (TNF) was demonstrated in strains after the integration of >20 expression cassette copies.[14] Because multicopy events in *P. pastoris* can occur as gene insertions either at the *AOX1* or *his4* loci, to further increase copy number, a promising strategy might be using a double-plasmid method, namely construction of multicopy strains by a traditional method using one expression vector followed by additionally inserting a vector targeted to a different locus.[15] Therefore, it was hypothesized that this co-expression strategy might help to enhance the expression yield of heterologous xylanase in *P. pastoris*.

Most currently reported commercial xylanolytic preparations are derived from *A. niger*, which is a well-known filamentous fungus secreting large amounts of xylan-degrading enzymes in culture medium.[16] Based on amino acid sequence similarity and 3D structural analysis, xylanases are classified into two main glycoside hydrolase (GH) families, 10 and 11.[17] In comparison to GH11, GH10 xylanases are more capable of cleaving glycosidic linkages in the highly substituted xylan backbone within biomass and presenting better synergistic interaction with cellulase in the hydrolysis of pretreated lignocellulosic biomass.[18] However, although many GH11 xylanases from *A. niger* have been successfully expressed in *P. pastoris* or *Escherichia coli*, few studies have been made on GH10 xylanases.[19]

Therefore, in the present work, a putative GH10 endo-1,4-D-xylanase (XynC) gene from *A. niger* BE-2 was optimized, expressed and characterized in *P. pastoris*. The potential benefit of using the double-plasmid co-expression strategy to enhance the enzyme production on the transcription level was assessed. Because the main objective of this study was to actively express a high level of XynC in *P. pastoris* to match the burgeoning demand of industrial enzymes, the expression conditions of recombinant *P. pastoris* were optimized at the shake-flask level and then scaled up further in a 5-L bioreactor. The results herein indicated that XynC production yield could be greatly improved with the aid of the double-plasmid strategy, and that the GH10 XynC obtained by the present methods would be a potential candidate for industrial application.

MATERIALS AND METHODS

Strains, plasmids and growth media

Escherichia coli DH5α and *P. pastoris* GS115 were used as cloning and expression hosts, respectively. *E.coli* DH5α was grown at 37 °C in Luria–Bertani medium (LB), whereas *P. pastoris* GS115 was routinely cultured at 30 °C in yeast growth media according to the *Pichia* expression system manual from Invitrogen (Carlsbad, CA, USA). The expression vectors pPIC9K and pPICZαA (Invitrogen) were both employed as cloning and expression vectors. For the high cell-density fermentation study, bacterial standard medium (BSM) with trace element solution (PTM1) was prepared by the method described in the manual of the Multi-Copy *Pichia* Expression Kit (Invitrogen).

Sequence optimization and cloning of XynC

The cDNA of *A. niger* BE-2 GH10 xylanase (XynC, GenBank no. KJ601783) was obtained from NCBI. Multiple sequence alignments were performed using DNAMAN 5.0 (Lynnon Biosoft, USA). The codon usage was analyzed and optimized for expression in *P. pastoris* GS115 using GENE DESIGNER 2.0.[20] The DNA Star software was used to analyze the average structure, the free energy (ΔG) of mRNA folding and the sequence around the ATG distribution of G+C and to eliminate AT-rich regions. The mRNA secondary start codon was estimated and adjusted according to the work described earlier.[21] After optimization, the optimized GH10 xylanase gene (*OpXynC*) was synthesized by Sangon Biotech (Shanghai, China) and amplified by PCR using the following primers, N1-1, 5′-CCGGAATTCATGGTCCAGATTAAGGTTGCTGCTTTG-3′; N1-2, 5′-ATAAGAATGCGGCCGCTACAAAGCGTTAGCAATAGCGGTGTAAGC-3′; The PCR products were digested with *Eco*RI and *Not*I (Takara, Dalian, China) and finally inserted into pPIC9K and pPICZαA, respectively. The recombinant plasmids (pPIC9K-*OpXynC* and pPICZαA-*OpXynC*) were then transformed into *E. coli* DH5α for propagation.

Construction of single-/double-plasmid recombinant *P. pastoris* GS115

The recombinant integrating plasmid pPIC9K-*OpXynC* targeted to the *his4* locus was first linearized using *Sac*I and transformed into *P. pastoris* GS115 competent cell by a Bio-Rad Micropulser Electroporator with the conditions recommended in the manufacturer's instructions. The recombinant cells initially were screened on regeneration dextrose (RDB) medium, and then replica-plated onto yeast peptone dextrose (YPD) plates containing different G418 concentration (1.0, 2.0, 4.0 and 6.0 mg mL^{-1}) to select multicopy transformants. Selected colonies were inoculated into 5 mL buffered methanol complex (BMMY) medium containing 0.5% (v/v) methanol to induce the expression of XynC at 30 °C and 220 rpm. The transformant with the highest xylanase activity carrying single pPIC9K-*OpXynC*, namely *P. pastoris*-S, was then transformed with pPICZαA-*OpXynC* that incorporated into the *His4* locus to further increase copy number. Strains with two vectors, namely *P. pastoris*-D, likewise were screened on solid YPD plates containing 0.5 mg mL^{-1} zeocin and 0.5 mg mL^{-1} G418. A 96-well plate was used for quickly testing the xylanase activity. The general construction procedure is presented schematically in supporting information, Fig. S1.

Expression and purification of the recombinant xylanase XynC

The recombinant *P. pastoris* were cultured in BMMY at 30 °C and 220 rpm. Methanol was added to a final concentration of 0.5% (v/v) every 24 h to induce the protein expression. Cells were harvested by centrifugation (6000×*g*, 5 min) after adding the methanol for 96 h. The recombinant protein was purified by adding 75% saturated $(NH_4)_2SO_4$. After precipitation at 4 °C overnight, the precipitate was collected by centrifugation at 12000×*g* for 20 min,

and dissolved in 5 mL of 10 mmol L^{-1} Tris–HCl buffer (pH 6.0). SDS-PAGE was conducted to verify the purity of XynC, and the protein bands were analyzed using an image analysis system (Bio-Rad).

Biochemical characterization of XynC

Substrate beechwood xylan purchased from Sigma-Aldrich was used for xylanase activity analysis. The recombinant XynC was mixed with 20 mg of insoluble beechwood xylan in a final volume of 0.5 mL 50 mmol L^{-1} citric acid buffer (pH 5.0). After incubation at 50 °C for 10 min, the reaction was stopped by adding 0.5 mL 3, 5-dinitrosalicylic acid (DNS), followed by boiling for 7 min. The absorbance at 540 nm of the mixture was measured.[22] One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol min^{-1} of reducing sugars. The concentration of the purified protein was estimated by the Braford method using the bovine serum albumin (BSA) as a standard. All measurements were performed in triplicate and presented as mean values with standard deviations.

The optimal temperature of XynC was analyzed, ranging from 30 to 80 °C in increments of 5 °C. The effect of temperature on the stability of XynC was assessed by measuring residual activity after pre-incubating enzyme at 50–65 °C for 0–1 h. For determination of the effect of the pH on enzyme activity, XynC was incubated at various pH values ranging from 6.0 to 8.0. The pH stability was examined by pre-incubating XynC for 1 h at different pH values.

The kinetic constant of XynC (K_m, concentration of the substrate when the reaction velocity is equal to one half of the maximal velocity, V_{max}, for the reaction) was calculated according to the Lineweaver–Burk method. The influence of various substrate concentrations (from 1 to 20 mg mL^{-1}) on xylanase activity was assayed at 50 °C, pH 5.0 for 10 min. The values were given as the averages of three separate determinations.

In order to determine the resistance of the purified XynC to chemical reagents, organic solvents and detergents, xylanase activity was assessed by applying chemicals ($CaCl_2$, $BaCl_2$, $NiCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_2$ and EDTA) at the concentrations of 1 mmol L^{-1} and 5 mmol L^{-1}, organic solvents (ethanol, isopropanol, *n*-Butanol and glycerol) at 5% (v/v) and 10% (v/v), and detergents (SDS, Tween-20 and Tween-80) at 0.1% (v/v) and 0.5% (v/v), respectively. The enzyme was incubated with each reagent for 2 h before adding the beechwood xylan to start the reaction. All experiments were performed in triplicate.

The optimization of xylanase production in shake-flask cultures

Six major parameters, including inoculum size, initial pH, methanol concentration, reaction temperature, agitation speed and carbon (C) sources were optimized for xylanase expression at the shake-flask level, respectively. The traditional 'one-factor-at-a-time' method was used by maintaining all other parameters at constant levels except the one being studied. All enzyme activities are presented as means ± SD from three parallel experiments.

Laboratory-scale production of recombinant XynC by P. pastoris

High cell-density fermentation was carried out in a 5-L bioreactor (New Brunswick Scientifc, Edison, NJ, USA) with a working volume of 1.5 L BSM medium containing 6.6 mL PTM1. The fermentation was based on the method reported by Wang et al.[23] Temperature was initially controlled at 30 °C in the growth phase. The pH was maintained at pH 6.0 by automatic addition of 42.5% (v/v) phosphoric acid or 25% (w/v) ammonium hydroxide. Dissolved oxygen (DO) was monitored in all fermentation runs and maintained at >40% in the growth phase and 25% in themethanol induction phase by a cascaded control of agitation rate and aeration rate, respectively. When DO increased, the feeding medium, containing 50% (w/v) glycerol and 12 mL L^{-1} PTM1 was pumped into the fermenter until the OD_{600} reached above 300 (dry weight, 95 g L^{-1}). After depletion of glycerol, methanol containing 12 mL L^{-1} PTM1 was added to start the induction phase, whereas the induction temperature and pH remained at 28 °C, pH 5.5. The methanol addition rate was maintained at 6.0 mL L^{-1} h^{-1}. Samples for analysis were collected every 12 h.

RESULTS AND DISCUSSION

In-house design and synthesis of the *XynC* gene

Previous studies have shown that the codon bias often has a significant impact on the heterologous transcription and translation in the host. The existence of slightly different codes in different organisms would be a very significant barrier to the heterologous expression.[24] Thus, codons encoding the native amino acid residues of GH10 xylanase from *A. niger* BE-2 first were analyzed in order to actively express a high level of XynC in *P. pastoris* (Table S1). It was observed that the usage frequency of these codons was significantly different from that in the *P. pastoris* host. Some common codons [e.g. CGA (Arg), CGC (Arg), CCG (Pro), AGC (Ser) and CTC (Leu)] present in the native gene sequence were <10% of the usage frequency and rarely involved in the host, indicating that the natural GH10 xylanase from *A. niger* was unsuitable for its direct expression in the *P. pastoris* host.

Accordingly, the gene sequence was optimized by substituting the rare codons, reducing the GC content and regulating the secondary structure of the translation initiation region. As shown in Fig. 1, 228 bases in the *XynC* gene were modified after the optimization, and its GC content decreased from 52.0% of the wild-type (WT) to 41.8%, which is close to the level of optimal expression in *P. pastoris* (data not shown). The GC content is in agreement with that of other highly expressed genes in *P. pastoris*.[25] Comparing with the WT *XynC* gene, 18 bases of the optimized synthetic sequence formed a ring-opening structure in 5′ nucleotide section, which potentially facilitated a loose secondary structure formation at the mRNA translation initiation site and improved efficiency of the translation (Fig. S2).[23] The optimized synthetic sequence showed a 76.8% homology to the WT gene (data not shown).

Construction and screening of the single-/double-plasmid transformants

The randomly selected pPIC9K-*OpXynC* first was transformed into *P. pastoris* to express GH10 XynC. After being screened by G418 resistant with different concentration (1.0, 2.0, 4.0 and 6.0 mg mL^{-1}), four colonies (Op*XynC*-a, *OpXynC*-g, *OpXynC*-h and *OpXynC*-m) were obtained on medium containing 6.0 mg mL^{-1} G418. It was apparent that the codon-optimized XynC was successfully expressed in the recombinant *P. pastoris* as xylanase activity was detected in the crude supernatant, whereas no activity was recorded in control sample harbouring pPIC9K [Fig. 2(a)]. The *OpXynC*-h strain, namely *P. pastoris*-S, exhibited the highest xylanase activity (68 U mL^{-1}) after six days of methanol induction (0.5%) every 24 h, and was selected for the following experiments.

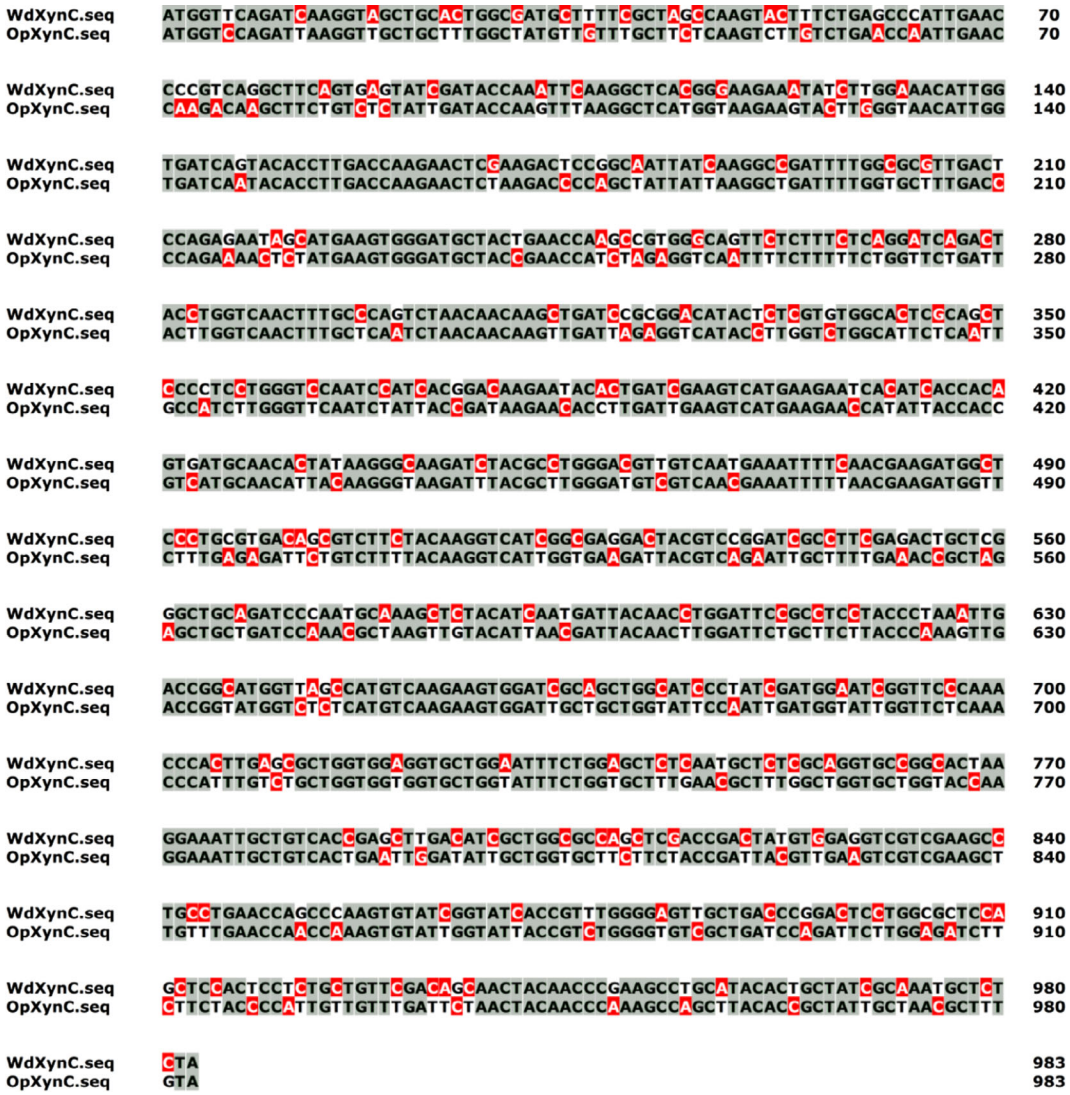

Figure 1. Sequence alignment of the genes from WT XynC (*WdXynC*) and optimized *XynC* (*OpXynC*).

Previous work has shown that the number of integrated genomic copies of the target gene often correlates with expression levels.[14] To evaluate if use of an additional expression vector targeted to different locus could further enhance XynC yield, *P. pastoris*-S incorporated into the *His4* locus was then transformed with pPICZαA-*OpXynC* targeted to the *AOX1* locus. As expected, with a shake-flask repeat screening, three transformants (*OpXynC*-h1, *OpXynC*-h2 and *OpXynC*-h3) were selected and expressed. It appeared that all three showed an obviously high xylanase activity as compared with the parental *P. pastoris*-S [Fig. 2(b)]. The *OpXynC*-h3 strain, namely *P. pastoris*-D, presented around 1.5-fold higher xylanase activity than that of *P. pastoris*-S. The comparison between single pPICZαA-*OpXynC* strain and *P. pastoris*-D on xylanase activity also was assessed. The single-plasmid transformant exhibited around two-fold lower xylanase activity than that of *P. pastoris*-D (data not shown). To simplify the experimental design, *P. pastoris*-S producing more xylanase than pPICZαA-*OpXynC* strain was used for the following analysis.

Because *P. pastoris*-D carrying two vectors showed a better expression yield after a rough assessment above, more detailed comparison between *P. pastoris*-S and *P. pastoris*-D on cell growth and xylanase production was further conducted to gain more insights into the feasibility of XynC expression by the double plasmid co-expression strategy [Fig. 2(c) and (d)]. Generally, the expression of recombinant proteins is inversely related to the rate of cell growth.[26] However, in the present study, both recombinant *P. pastoris* grew faster than the WT strain, the OD_{600} of control being 13 as compared to that of 17 in case of strains expression XynC at 56 h [Fig. 2(c)]. Although *P. pastoris*-D and *P. pastoris*-S exhibited a similar growth profile as shown in Fig. 2(c), indicating that the presence of additional integrated *XynC* genes did not induce a stress response to the growth rate of the *P. pastoris*-D after induction of the enzyme. As for xylanase yield, it was apparent that *P. pastoris*-D could significantly enhance the xylanase secretion, resulting in a 33% increase in enzyme yield of *P. pastoris*-D over that of *P. pastoris*-S at 10 days of cultivation [Fig. 3(d)]. The above results suggest that the double-plasmid co-expression method held great potential to facilitate the XynC expression in *P. pastoris*.

Biochemical properties of the xylanase XynC

In order to avoid or minimize interference in the assay by media components, XynC expressed from *P. pastoris*-D was purified

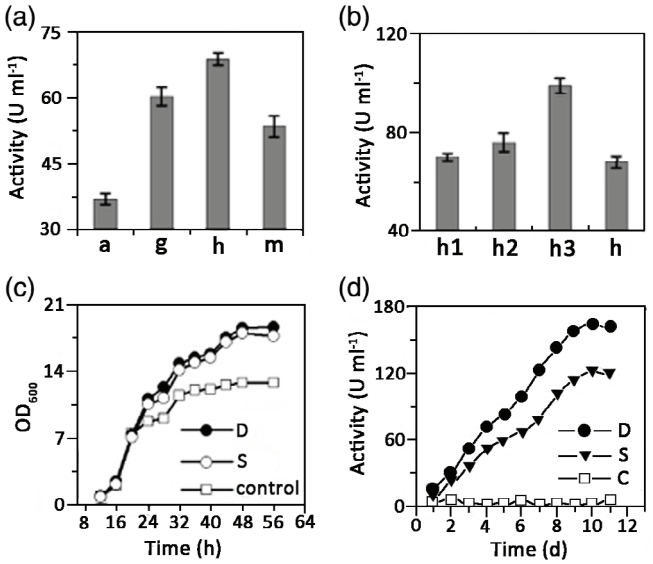

Figure 2. (a, b) Screening of recombinant *P. pastoris*; (c, d) The effects of single-/double-plasmid method on cell growth and xylanase activity, respectively. (a) Xylanase activity of single-plasmid recombinant *P. pastoris* (pPIC9K-*OpXynC*); a, g, h, m, transformants screened by selection on medium containing 6 mg mL^{-1} of G418. (b) Xylanase acitvity of double-plasmid transformants. (c) Growth profiles of WT *P. pastoris* (control) and recombinant *P. pastoris*. S, recombinant *P. pastoris* carrying pPIC9K-*OpXynC* (*P. pastoris*-S). D, double-plasmid transformant carrying two vectors (*P. pastoris*-D, pPIC9K-*OpXynC* and pPICZαA-*OpXynC*). (d) Time course of xylanase activity. S, recombinant *P. pastoris* carrying pPIC9K-*OpXynC*. D, double-plasmid transformant. C, control, *P. pastoris* carrying pPIC9K.

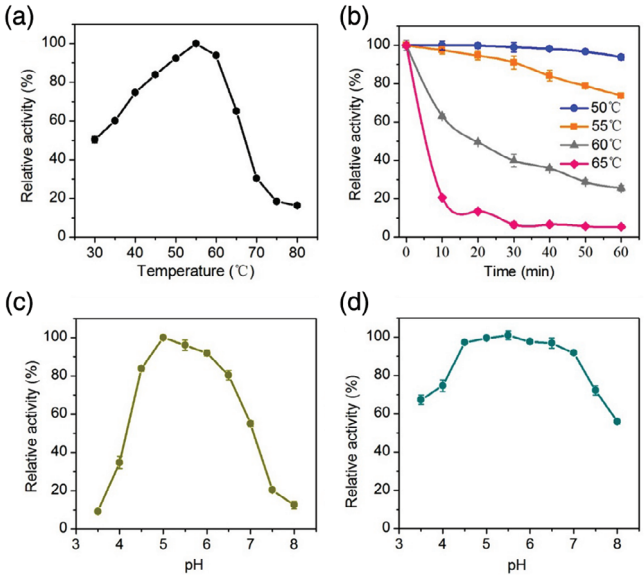

Figure 3. Biochemical properties of the recombinant XynC: (a) optimal temperature, (b) thermostability, (c) optimal pH and (d) pH stability. The maximum activity was defined as 100% (a, c). The initial activity was defined as 100% (b, d). All experiments were repeated three times.

through ammonium sulfate [$(NH_4)_2SO_4$] precipitation and showed a single band by SDS-PAGE analysis (Fig. S2). After purification, several key parameters of XynC (e.g. optimum pH, optimum temperature and kinetic parameters) were assessed systematically (Fig. 3). It appeared that the purified XynC displayed maximal activity at 55 °C and pH 5. The enzyme retained about 95% of its initial activity after 60 min incubation at 50 °C, and was stable over a wide range of pH (4.5–7.0). These properties made XynC a suitable accessory enzyme for the existing enzymatic hydrolysis, which is usually carried out at around 50 °C and pH 5 to conform to the properties of most commercial cellulases.[27] As for the kinetic parameters, the K_m and V_{max} of purified XynC against beechwood xylan were 3.5 mg mL^{-1} and 2327 U mg^{-1}, respectively. Compared with the reported xylanases from the same genus *A. niger*, such as GH11 XynA with a K_m and V_{max} of 4.8 mg mL^{-1} and 370 U mg^{-1}, and 4.4 mg mL^{-1} and 1429 U mg^{-1} for GH11 XynB, again, the GH10 XynC expressed in the present work might be a suitable alternative candidate for various industrial applications.[28]

Besides the parameters analyzed above, the effects of various additives including metal ions, organic solvents and detergents on XynC activity also were investigated (Table S2). Results revealed that most of the metal ions, such as Mn^{2+}, Fe^{2+} and Co^{2+}, had a partial inhibition on XynC activity whereas a slight stimulation was observed with the addition of Mg^{2+} (5 mmol L^{-1}). This was similar to that of the reported xylanase from *A. niger*.[27] As for the detergents, no negative effect was observed in the presence of Tween 20 and Tween 80, but the presence of SDS greatly inhibited xylanase activity. In addition, organic solvents also significantly affected XynC activity.

Optimization of xylanase production in shake-flask cultures

Optimization of induction conditions is one of the methods that can enhance the expression levels of recombinant proteins.[29] To reach reasonable production of XynC, several key factors, including inoculum size, methanol concentration and temperature, were preliminarily optimized at the shake-flask level by the one-factor-at-a-time method (Fig. 4). Because *P. pastoris*-D showed great promise to XynC expression, this recombinant strain was used for the following optimization. Briefly, inoculum size slightly affected cell growth and enzyme activity. When the inoculum size reached 6.0%, the maximum OD_{600} and XynC activity were achieved [Fig. 4(a)]. As for the initial pH, it appeared that *P. pastoris*-D was capable of growing across a wide range of pH (from pH 4.5 to pH 7). The optimal xylanase level (188 U mL^{-1}) appeared when fermentation was carried out in the medium of pH 5.5. Methanol dosage presented an obvious influence on cell growth and enzyme production [Fig. 4(c)]. The highest OD_{600} and XynC activity (275 U mL^{-1}) were observed when methanol was added to a final concentration of 1.5% (v/v). Comparatively, 0.5% (v/v) of methanol induction was required to achieve a high expression yield of GH11 xylanase from *Fusarium oxysporum* in *P. pastoris* and 1% (v/v) of methanol for *Thrichoderma reesei* xylanase were demonstrated in previous studies.[30] These differences probably were a consequence of the gene variety. It was apparent that incubation temperature was another critical variable which is strongly influencing the foreign protein expression.[31] At the optimal temperature of 28 °C, *P. pastoris*-D expressed maximum xylanase activity (295 U mL^{-1}). Oxygen aeration and agitation of the recombinant also appeared to be important parameters in obtaining optimal secretion [Fig. 4(e)]. The highest xylanase activity was measured at 250 rpm (375 U mL^{-1}), whereas <20 U mL^{-1} of activity was observed under stationary conditions. This was probably due to the methanol metabolism in the host requiring oxygen for oxidation.[30] Additionally, results showed that the agitation boosted the effect on cell growth and enzyme activity was much higher at the initial 190 rpm. When agitation speed was further increased, the growth rate and enzyme

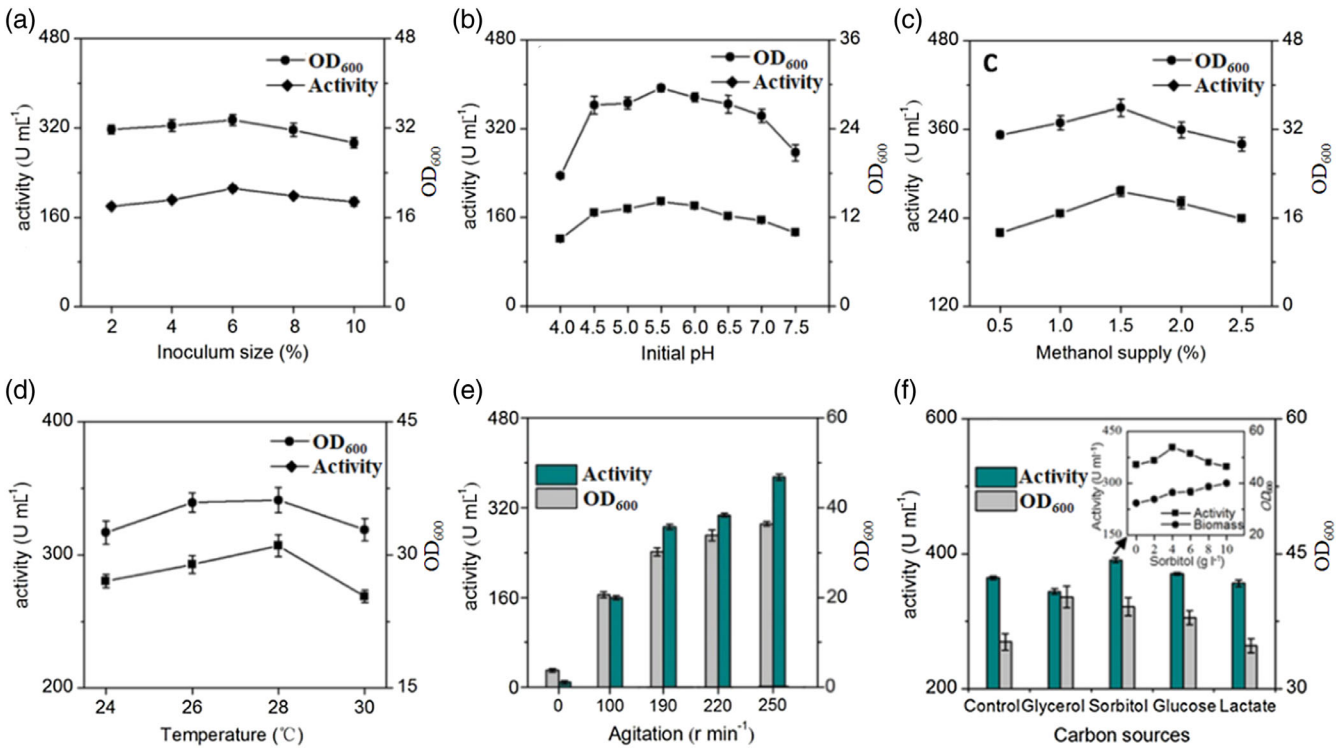

Figure 4. The effects of different cultivation conditions on xylanase activity and cell growth at the flask scale: (a) inoculum size, (b) initial pH, (c) methanol dosage, (d) reaction temperature, (e) agitation rate and (f) C sources. Control, cultivation of the double-plasmid transformant without additional C sources. Each value represents the mean of triplicate determinations.

Table 1. Comparison of XynC expressed in *P. pastoris*-S and *P. pastoris*-D under optimized conditions

	Before optimization			After optimization		
Recombinant	OD_{600}	Protein (g L^{-1})	Enzyme activity (U mL^{-1})	OD_{600}	Protein (g L^{-1})	Enzyme activity (U mL^{-1})
P. pastoris-D	29.5	0.16	166.6	40.6	0.37	403
P. pastoris-S	28.7	0.13	124.4	35.5	0.31	305

P. pastoris-D, the transformant harbouring pPIC9K-*OpXynC* and pPICZαA-*OpXynC*; *P. pastoris*-S, the transformant carrying only pPIC9K-*OpXynC*.

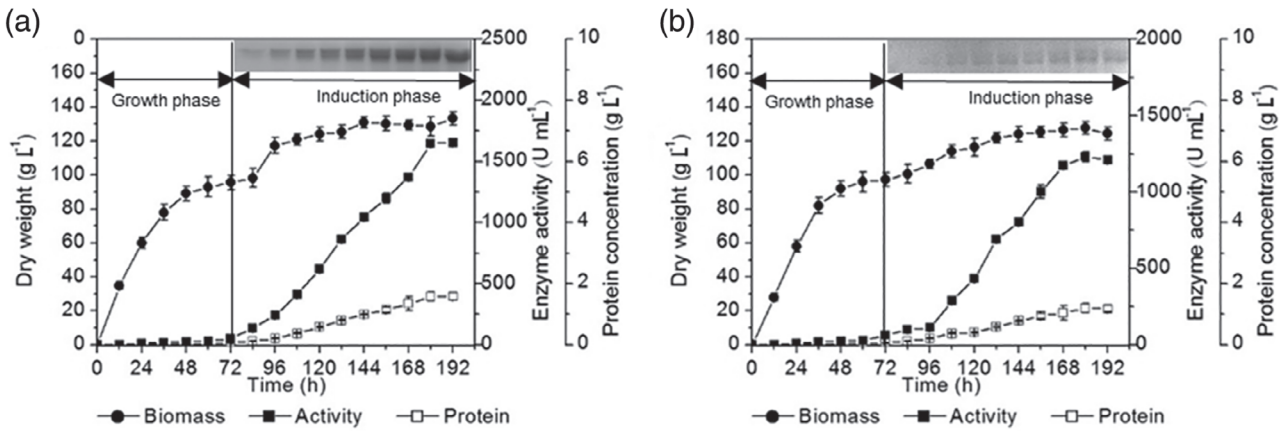

Figure 5. High cell-density fermentation of XynC from the recombinant *P. pastoris*: (a) XynC fermentation by *P. pastoris*-D and (b) XynC fermentation by *P. pastoris*-S.

production efficiency gradually reduced. Because C source has been recognized as a major factor for enzyme production, and previous work has shown that the choice of C source depends highly on the individual gene,[32] four different C sources commonly used for fermentation were next assessed [Fig. 4(f)]. Although the recombinant strain was able to utilize methanol as the sole C source (control sample), the addition of dual C sources (sorbitol and methanol) was beneficial for heterologous protein expression, resulting in maximum xylanase activity (403 U mL^{-1}) with the additional of 4 g L^{-1} sorbitol. Similar results also have been reported in the study where two C sources (sorbitol and methanol) were used for polygalacturonate lyase production in *P. pastoris*.[22]

Under optimized conditions, xylanase activity and the OD_{600} of *P. pastoris*-D were found to increase to 403 U mL^{-1} and 40.6 from 166.6 U mL^{-1} and 29.5, respectively, representing a 2.4-fold and 35% improvement (respectively) compared to the control shake flask cultivation (Table 1). Because one of the main objectives of this study was to assess if the double-plasmid co-expression strategy could further facilitate XynC production, *P. pastoris*-S was subsequently incubated at the same conditions for comparison purposes. A similar increase trend in cell growth and xylanase production after cultivation optimization was achieved. However, it should be noted that although both transformants had a similar amount of OD_{600} before and after optimization, xylanase production by *P. pastoris*-D was greatly higher than *P. pastoris*-S; again, this indicated that the double-plasmid co-expression strategy was suitable for facilitating XynC expression yield in *P. pastoris*.

Table 2. Comparison of various recombinant xylanases expressed in *P. pastoris*

Donor	Expression system			Basic enzyme property					Expression level			Source/GH family
	Promoter	*P. pastoris*	Vector	Mw (kDa)	*T* (°C)	pH	K_m (g L^{-1})	V_{max} (U mg^{-1})	DCW (g L^{-1})	Yield (g L^{-1})	Activity (U mL^{-1})	
A. niger	AOX1	GS115	pPIC9K	35.5	55	5.0	3.5	2327(BEX)	10.4	0.37	403[a]	This work/GH10
			pPIC9K/ pPICZαA						133.3	1.58	1650[b]	
A. niger	AOX1	KM71	pPICZαA	21.0	55	5.0	–	–	83.5	0.50	3676[b]	[33] /GH11
A. niger	AOX1	GS115	pPICZαA	23.0	50	5.0	18.27	0.56 mol L^{-1}(BIX)	–	–	15158[a]	[18] /GH11
A. niger	AOX1	GS115	pPICZαA	24.0	50	5.0	4.429	1429 (BEX)	–	–	10035[b]	[19] /GH11
T. reesei	AOX1	X-33	pPICZαA	21.0	60	6.0	2.1	–	66	0.35	261[b]	[9] /GH11
T. sulphureus	GAP	X-33	pPICZαA	22.0	50	5.0	11.14	588.07	–	–	105[a]	[34] /GH11

BIX, Birchwood xylan; BEX, Beechwood Xylan; Mw, molecular weight; DCW, dry cell weight.
—, not shown.
[a] Shake-flask level.
[b] Fermentor level.

High cell-density fermentation in a 5-L fermenter

From the analysis above, it was shown that the cell density was positively related to protein secretion yield in *P. pastoris*.[29] To increase the production of GH10 XynC, the recombinant xylanase production was next scaled up by high cell density fermentation in a 5-L scale bioreactor. As shown in Fig. 5, both transformants shared the same growth profile at the initial 72 h, reaching 100 g L^{-1} of dry cell weight. After induction of expression by methanol utilization, the secreted XynC was increased by time, resulting in 1650 U mL^{-1} and 1.58 g L^{-1} of xylanase activity and enzyme produced by *P. pastoris*-D and 1230 U mL^{-1} and 1.19 g L^{-1} expressed by *P. pastoris*-S, respectively. Interestingly, compared with the XynC activity (403 U mL^{-1} for *P. pastoris*-D and 305 U mL^{-1} for *P. pastoris*-S) at the shake-flask level mentioned above, both *P. pastoris*-D and *P. pastoris*-S presented around four-fold higher xylanase activity at the fermenter level. However, although *P. pastoris*-D presented much higher xylanase production than *P. pastoris*-S, the maximum biomass of both recombinant strains exhibited a similar value of approximately 127 g L^{-1} (dry cell weight). Again, it indicated that increasing the number of integrated expression cassettes could increase the yield of recombinant GH10 XynC, but would not inhibit the recombinant cell growth. Furthermore, SDS-PAGE results also confirmed that *P. pastoris*-D produced a higher amount of XynC than *P. pastoris*-S as judged by band intensities on the gels.

To date, many recombinant xylanases from different microorganisms have been successfully expressed in *P. pastoris*, but the expression yields of each protein are highly dependent on the individual enzyme (Table 2). For example, the GH11 Xyn2 from *T. reesei* Rut C-30 yielded 261 U mL^{-1} for 96 h cultivation in *P. pastoris*,[9] but 105 U mL^{-1} with XynB from *Aspergillus sulphureus*.[34] As for the GH11 XynB from *A. niger* IA-001, a maximized activity of 15 158 U mL^{-1} was achieved after 120 h of shaking.[18] Besides, the enzyme assay with different standard substrates (e.g. beechwood

xylan, birchwood xylan, oat spelts xylan) likely resulted in the difference of enzyme activity. To the best of our knowledge, this is the first time that the recombinant GH10 XynC has been expressed and characterized.

Regarding the enzyme properties, GH11 members have a relatively small size with higher pI value as compared to GH10. Given that GH11 can release a longer substituted XOS from xylan, this enzyme was initially expected to be a better candidate for biomass deconstruction.[35] Nevertheless, the GH10 xylanase is more capable of attacking highly substituted glycosidic linkage, showing a better synergistic cooperation with cellulases, and accordingly constituting a suitable enzyme for the biorefinery of lignocellulosic biomass.[36] Thus, continued GH10 xylanase discovery remains the most vital approach to ramp up the bioconversion of lignocellulosic biomass and to reduce its cost of production.

CONCLUSIONS

A double-plasmid co-expression strategy improved XynC expression level by ~33% in *P. pastoris*. This strategy can be an applied to high expression of other recombinant proteins in *P. pastoris*. After optimizing the cultivation conditions, the XynC expression level was increased 2.4-fold (403 U mL^{-1}) relative to that incubated in shake flask culture. Further improvement of expression level was achieved by high cell-density fermentation using a 5-L bioreactor, which had xylanase activity of 1650 U mL^{-1}. The recombinant GH10 xylanase exhibited a high stability in the pH 4.5–7.0 range and similar optimum reaction conditions to some commercial cellulases. These properties make the enzyme attractive for industrial purposes.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

Supporting Information

Supporting information may be found in the online version of this article.

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