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Engineering a high-performance, metagenomic-derived novel xylanase with improved soluble protein yield and thermostability

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ABSTRACT

The novel termite gut metagenomic-derived GH11 xylanase gene *xyl7* was expressed in *Escherichia coli* BL21, and the purified XYL7 enzyme exhibited high specific activity (6340 U/mg) and broad pH active range of 5.5–10.0. Directed evolution was employed to enhance the thermostability of XYL7; two mutants (XYL7-TC and XYL7-TS) showed a 250-fold increase in half-life at 55 °C, with a 10 °C increase in optimal temperature compared to that of wild-type XYL7. A truncated enzyme (XYL7-Tr3) acquired by protein engineering showed similar catalytic properties as the wild-type, with a tenfold increase in soluble protein yield by the mutant. The reducing sugar produced by XYL7-TC was about fourfold greater than that produced by their parents when incubated with xylan at 60 °C for 4 h. The engineered novel xylanase exhibited superior enzymatic performance and showed promise as an excellent candidate for industrial application due to its high specific activity, stability and soluble protein yield.

1. Introduction

The plant cell wall is composed mainly of hemicellulose, cellulose (1,4-β-glucan) and lignin, with hemicellulose as the second most abundant renewable polysaccharide in nature after cellulose. Xylan, the major component in hemicellulose, is a heterogeneous polysaccharide with a backbone consisting of xylopyranosyl units linked by β-1,4-glycodidic bonds [1]. Xylanase (EC 3.2.1.8), a crucial enzyme in the hydrolysis of xylan, hydrolyzes the β-1,4-glycodidic linkages in the xylan backbone resulting in xylooligosaccharide and xylose. Therefore, xylanase plays an important role in carbon circulation and shows great potential for biofuel production [2,3]. In addition, xylanase has been used widely in a variety of industrial applications ranging from food preparation to wood pulp bleaching [4].

Xylanase is produced naturally from various biological sources, such as bacteria, fungi and insects. As a primary destroyer of wood, termites harbor more than 200 types of microbial species that can produce cellulase and hemicellulase, providing an important source of enzymes for industrial applications [5]. However, the majority of microbes are unculturable. Metagenomic technology provides a powerful culture-independent strategy for obtaining new enzyme genes [6,7]. In recent years, many xylanase genes were isolated from metagenomic libraries, including *xynGR40* from goat rumen content [8], *XynH* from soil samples [9] and *XYL6419* from termite gut [10]. However, most of these wild-type enzymes showed poor thermostability, particularly for xylanases in the glycoside hydrolase family 11 (GH11) [11,12].

Most industrial processes using xylanase require a thermostable enzyme. Protein engineering based on directed evolution and rational design has made it possible to improve a number of desired enzymatic properties. Directed evolution especially is suitable for proteins without available structural information. In the early stages of directed evolution, a thermostable mutant M3 obtained from *Orpinomyces* sp. xynA showed a 401-min half-life at 60 °C, which was ~50-fold greater than that of wild-type [13]. Using XynG1-1 from *Paenibacillus campinasensis* as template, Zheng et al. screened two mutants – XynG1-1B43 and XynG1-1B43cc16 – which showed a 2-unit increase in optimal pH and 10 °C increase in optimal temperature, respectively [14].

In this study, we screened and cloned a GH11 XYL7 xylanase with higher enzyme activity, wider pH range but weaker thermostability from a fosmid metagenomic library of *Globitermes brachycerastes* gut sample. Error-prone polymerase chain reaction

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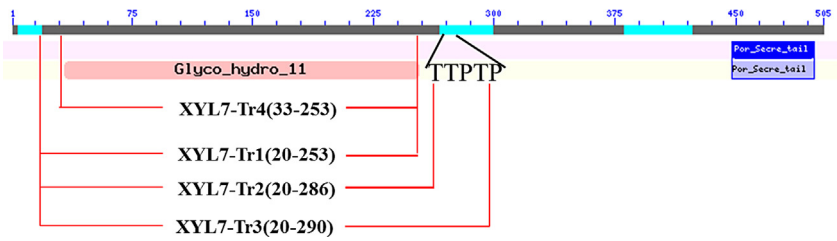

Fig. 1. Four truncated fragments of XYL7 and the name of mutants. The XYL7 gene was truncated around the predicted GH11 conserved domain.

(PCR), site-saturation and site-directed mutagenesis were applied to enhance its thermostability, and two mutants with about 250-fold greater half-life at 55 °C were isolated. These mutants showed improved xylan hydrolysis ability compared to that of the wild-type under identical conditions.

2. Materials and methods

2.1. Materials

Birchwood xylan and beechwood xylan were purchased from Sigma-Aldrich (St. Louis, MO, USA). KOD-plus DNA polymerase was obtained from Toyobo (Shanghai, China). Restriction endonucleases were obtained from Takara (Dalian, China) and used according to the manufacturers' instructions. T4 DNA ligase was purchased from New England Biolabs (Beverly, MA, USA). Site-directed mutagenesis and random mutagenesis kits were obtained from Stratagene (La Jolla, CA, USA). The AxyPrep plasmid miniprep, AxyPrep DNA gel extraction and AxyPrep PCR clean-up kits were purchased from Axygen (Axygen, Union City, CA, USA). All other chemicals and reagents were from Sangon (Shanghai, China) and were of analytical grade.

The expression vector pET-28a was obtained from Novagen (Madison, WI, USA). *Escherichia coli* TOP10, *E. coli* DH10B and *E. coli* BL21 (DE3) were used as hosts for plasmid manipulation and recombinant protein expression. The strains were cultured in Luria–Bertani (LB) medium.

2.2. Cloning and construction of wild-type and truncated *xyl7* expression vector

The wild-type xylanase gene *xyl7* was cloned from the metagenomic fosmid library using primers *xyl7*-1F and *xyl7*-1R. The signal peptides and protein family domains were predicted by Signal 4.1 [15] and Pfam [16], respectively. The signal peptide was removed from the *xyl7* gene in all constructs. Four types of fragment that included the GH11-conserved domain were cloned (Fig. 1): *xyl7*-1F and *xyl7*-2R primers were used to amplify the truncated fragment XYL7-Tr1 [amino acids (aa) 20–253], *xyl*-1F and *xyl*-3R were used to amplify XYL7-Tr2 (aa 20–286), *xyl*-1F and *xyl*-4R were used to amplify XYL7-Tr3 (aa 20–290) and *xyl*-2F and *xyl*-4R were used to amplify XYL7-Tr4 (aa 33–253) (Table 1). The forward and the reverse primers contained *Nde*I and *EcoR*I restriction sites, respectively. The PCR products were double digested and ligated into pET28a, and the resulting plasmids were used to transform *E. coli* TOP10 for plasmid amplification.

2.3. Random mutation library construction and screening

A random mutation library was constructed using error-prone PCR according to the Genemorph II Random Mutagenesis Kit manual with the plasmid pET28a-*xyl7* as template. For the second round of error-prone PCR, the plasmid corresponding to the thermostable mutant obtained from the first round was used as template. The PCR products and plasmid pET28a were double-digested and ligated with T4 DNA ligase, and the resulting plasmid was used to transform *E. coli* DH10B using the MicroPulser Electroporator 165-2100 (Bio-Rad Laboratories, Veenendaal, The Netherlands). The transformants were spread on LB plates with 50 μg/ml of kanamycin and incubated at 37 °C. The plasmids were extracted and used to transform *E. coli* BL21 to produce a mutant library.

Colonies from the mutant library were picked and cultured overnight in 96-well plates with LB medium containing 50 μg/ml kanamycin and 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cell pellets were collected by centrifugation, resuspended in 100-μl pH 7.0 Na_2HPO_4/NaH_2PO_4 buffer and split equally into two 96-well plates. One plate was heated at the designated temperature for 2 h and the other plate served as an untreated control. After heat treatment, 50 μl of 2% xylan (w/v) were added into each well. The plates were incubated at 37 °C and the enzyme reaction was stopped by heating at 100 °C for 20 min with 100-μl 3,5-dinitrosalicylic acid (DNS). Enzyme activity was measured by the absorbance value at 540 nm. Through this process, clones from the library that retained more than 50% enzyme activity relative to the control were saved as potential positive clones [17,18].

2.4. Site-directed mutagenesis and site-saturated mutagenesis

Site-direct mutagenesis and site-saturated mutagenesis were carried out according to the QuikChange site-directed mutagenesis kit protocol (Stratagene), and PCR was performed using the corresponding primers containing the mutations (Table 1). The products were digested by *Dpn* I, cloned into the vector, and the resulting plasmids were used to transform *E. coli* BL21 for screening, as described above.

Table 1
Primers used in this study.

Primer	Nucleotide sequence
xyl7-1F[a]	GAGACTCCATATGCAAGGTCCCACATGGACT
xyl7-2F	TGAGACTCCATATGAACGGCTACGACTACGAG
xyl7-1R	CGGAATTCTTACCTCACCATAACCCT
xyl7-2R	GCGGAATTCTTAGTTGCTACTCCCACTGCCGGT
xyl7-3R	GCGGAATTCTTAGCCTCCAATGGTCAAAAG
xyl7-4R	CGGAATTCTGGCGTAGGCGTGGTGCC
K51T-F	ACCGTTAGCATGACGCTCACGGGAGAT
K51T-R	ATCTCCCGTGAGCGTCATGCTAACGGT
K224E-F	CAGCACTTTAATGAATGGCATGAACTT
K224E-R	AAGTTCATGCCATTCATTAAAGTGCTG
E238D-F	GGTCCGTTATATGATGTGGCGATG
E238D-R	CATCGCCACATCATATAACGGACC
K242-F[b]	GAGGTGGCGATGNNVGTTGAATCTTAT
K242-R	ATAAGATTCAACNNBCATCGCCACCTC
K242T-F	GAGGTGGCGATGACGGTTGAATCTTAT
K242T-R	ATAAGATTCAACCGTCATCGCCACCTC
A405S-F	AGCTCTTCCGTATCGGCATCTTCTTCT
A405S-R	AGAAGAAGATGCCGATACGGAAGAGCT

[a] Underlines means the restriction sites.
[b] N = A/T/G/C; V = G/C/A; B = G/C/T.