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De novo Synthesis and Expression of a Thermostable Xylanase from *Geobacillus stearothermophilus* in *Escherichia coli**

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Abstract The endoxylanase XT6 secreted from *Geobacillus stearothermophilus* is a particularly attractive candidate for some industrial purposes and was used successfully on an industrial-scale mill trial. The gene was de novo synthesized with the codons adjusted to fit the bias of that of *Escherichia coli* and constructed into vector pET28a (+). After optimizing the expression conditions, functional xylanase XT6 was over expressed in *E. coli* with up to 65% of total protein. A maximum xylanase activity of 3,030 U/mL was obtained from cell extract against birchwood xylan. The recombinant XT6 was partly characterized and was similar with those of the native enzyme in G. stearothermophilus. This is the first report on the over expression of a de novo synthesized xylanase XT6 gene from *Geobacillus stearothermophilus*. Fig 6, Tab 1, Ref 19

Keywords *Geobacillus stearothermophilus*; xylanase; whole gene synthesis; codon optimization ; expression;
CLC Q78 : Q936

嗜热脂肪土芽孢杆菌木聚糖酶基因的合成及其在大肠杆菌中的表达*

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摘 要 来自嗜热脂肪土芽孢杆菌的木聚糖内切酶XT6在工业上有着重要的应用, 已经成功应用于工业规模的生产试验. 本文作者在合成XT6基因全序列的同时对其密码子进行了优化, 且构建重组质粒在大肠杆菌中高表达. 通过优化表达条件, 功能正常的XT6基因在大肠杆菌中成功过量表达, 蛋白表达量占细胞中总蛋白的65%. 重组表达的木聚糖内切酶XT6特性和天然酶相似, 以桦木木聚糖为底物测定细胞提取物中木聚糖酶活性, 最大活性高达3 030 U/mL. 本文首次报道了来自嗜热脂肪土芽孢杆菌中木聚糖酶基因全序列的合成和在大肠杆菌中成功过量表达. 图6 表1 参19

关键词 嗜热脂肪土芽孢杆菌; 木聚糖酶; 全基因合成; 密码子优化; 表达
CLC Q78 : Q936

Xylan is the major constituent of hemicellulose and has a high potential for degradation to useful end products. The efficient degradation of xylan requires the synergistic action of many enzymes. Among them, the most important enzyme is endoxylanase (1,4-*β*-*D*-xylanase, EC 3.2.1.8). Xylanases have wide commercial applications in industrial processes, such as feed industry, paper and pulp industry, foodstuff industry and energy industry [1]. The xylanases with different properties are suited for use in different applications, e.g., alkaline xylanases for the pulp industry. In the past few years, new xylanases with specific properties from both bacterial and fungal sources have continued to be isolated [2~5], and several techniques have been developed for engineer xylanases used in specific industrial applications [6~9].

The extracellular xylanase XT6 from *Geobacillus stearothermophilus* is stable at high temperature and pH, and has been used in the paper and pulp industry for biobleaching [10]. This enzyme was also purified, characterized and cloned in *Escherichia coli* [11~13].

In this work, the xylanase XT6 gene was codon optimized and synthesized by a PCR-based method and the enzyme was successfully overproduced in *E. coli*, which established a solid foundation for further study on its structural-functional relationship and to improve its stability by directed evolution.

1 Material & Methods

1.1 Bacterial strains and reagents

E. coli strain BL21 (DE3) (Novagen, Madison, WI) was used as the host for expression. plasmid pET-28 (+) (Novagen,

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Madison, WI)) was used to construct expression vector. QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit (Qiagen, USA) were applied in the purification of the plasmids and PCR products. Birch xylan was purchased from Sigma (Sigma Aldrich, St. Louis, USA). *Taq* polymerase and xylose were purchased from the TaKaRa Biotechnology (Dalian, China) Co. Ltd. Restriction enzymes were from New England Biolabs (NEB). All other reagents were obtained from general commercial suppliers and used without further purification.

1.2 Optimization of xylanase XT6 nucleotide sequence and oligonucleotide design for synthesis of xylanase XT6 gene

Automatic codon optimization and oligonucleotide design for polymerase chain reaction (PCR)-based gene synthesis were performed using DNAWorks 3.1 [14], developed by the Center for Information Technology, National Institutes of Health, Department of Health and Human Services (*http://helixweb.nih.gov/dnaworks/*). The total amino acid sequence of xylanase XT6 was obtained from the National Center for Biotechnology Information (Accession number: ABI49951). About 32 oligomers with overlap regions with their neighbors were synthesized (Table 1) based on the DNAWorks results.

The codons were optimized automatically to suit the codon preference of *E. coli*. The codon frequency threshold was set to be 10% in the program. Overlap melting temperatures were designed to be (62±1) °C. The P1 and P32 oligomers were designed to contain an *Nde* I restriction site and a *Bam*H I restriction site, respectively. Other than that, the DNA sequence was designed to lack the *Nde* I and *Bam*H I sites needed for subsequent manipulation.

1.3 Xylanase gene synthesis and expression vector construction

Five microliters of each of the oligonucleotide components (normalized to 10 μmol/L) were mixed and diluted to 0.08 μmol/L each. Gene synthesis was performed as follows: The PCR-based assembly was performed with 1 U of *pfu* DNA polymerase (Fermentase, Germany) in a total volume of 50 μL containing 5 μL 10×DNA polymerase Buffer, 2.5 mmol/L each of dNTPs, 1.5 mmol/L $MgCl_2$ and 5 μL oligonucleotide mixture. Thermal cycling began with a 10-min denaturing step at 94 °C and was followed by 25 cycles of 30 s at 94 °C, 30 s at 63 °C, and 90 s at 72 °C, and finally 10 min at 72 °C for extension. PCR amplification mixtures contained 1 U of *pfu* DNA polymerase, 2.5 mmol/L each of dNTPs, 1.5 mmol/L $MgCl_2$, 5.0 μL 10×DNA polymerase Buffer, 1 μL of PCR-based assembly product, and 0.8 mmol/L of the outermost oligonucleotides. The thermal cycling parameters were same as for PCR-based assembly.

The PCR amplification product was analyzed by agarose gel electrophoresis and was inserted into plasmid pET-28 (+) at restriction sites *Nde*I and *Bam*HI to construct the cloning vector pET-28-XT6 [15]. The recombinant plasmids were transformed into *E. coli* DH5α competent cells and selected on LB plates with 50 μg/mL kanamycin. The plasmids that yielded 1.2 kb fragments were sequenced by Shanghai Sangon Biological Engineering Technology And Service Co., Ltd.

Table 1 Sequences of oligomers 1~32

Oligomers	Oligomer sequence (from 5' end to 3' end)
P1	TAGGAGGTCATATGAAAAATGCGGACAG
P2	GTTCAGGGCGCTAATATGCGGTTTTTTCGCATAGCTGTCCGCATTTTTCATGATATTCC
P3	CGCATATTAGCGCCCTGAACGCGCCACAGCTGGACCAACGGTACAAAAATGAATTTACT
P4	CTGCAGCTGATAGGGTTCCACCGCCGCACCAATAGTAAATTCATTTTTGTACCGTTGGT
P5	TGGAACCCTATCAGCTGCAGAATGAGAAAGATGTGCAGATGCTGAAGCGTCATTTTAAC
P6	CTAATAGGCTTCATCACATTTTCCGCCACAATTGAGTTAAAATGACGCTTCAGCATCTG
P7	CGGAAAATGTGATGAAGCCTATTAGCATTCAGCCGGAAGAAGGGAAATTCAATTTCGAA
P8	TCGCTTTCGCAAATTTCACAATACGATCCGCCTGTTCGAAATTGAATTTCCCTTCTTCC
P9	GTATTGTGAAATTTGCGAAAGCGAATGGCATGGATATTCGTTTTCACACCCTGGTGTGG
P10	TTCTTTGTCAAGGAAGAACCACTGCGGCACTTGGCTATGCCACACCAGGGTGTGAAAAC
P11	CAGTGGTTCTTCCTTGACAAAGAAGGAAAACCGATGGTGAACGAGACCGATCCGGTGAA
P12	TTCCAAGCGTTTCAGCAGCAACTGTTTATTCTGCTCACGTTTCACCGGATCGGTCTCGT
P13	GCTGCTGAAACGCTTGGAAACCCACATCAAAACCATTGTTGAACGTTATAAGGATGATA
P14	AACTACCTCATTCACTACATCCCAGTACTTTATATCATCCTTATAACGTTCAACAATGG
P15	TGGGATGTAGTGAATGAGGTAGTTGGCGATGATGGCAAACTGCGTAATAGCCCGTGGTA
P16	TGGAACGCCACTTTAATGTAATCAATGCCCGCAATCTGATACCACGGGCTATTACGCAG
P17	TTGATTACATTAAAGTGGCGTTCCAGGCGGCACGTAAATATGGTGGCGATAACATTAAG
P18	TCTACTTCGGTATTGTAGTCATTCATGTATAACTTAATGTTATCGCCACCATATTTACG
P19	CATGAATGACTACAATACCGAAGTAGAACCGAAACGCACTGCGCTGTATAATCTGGTAA
P20	TGCCGTCAATCGGCACGCCCTCTTCTTTCAGCTGTTTTACCAGATTATACAGCGCAGTG
P21	CGTGCCGATTGACGGCATTGGGCATCAGAGCCACATTCAAATTGGCTGGCCGAGCGAAG
P22	AGGCCCAGTGCTGCAAACATATTGATGGTCTTTTCTATTTCCGCTTCGCTCGGCCAGCC
P23	GTTTGCAGCACTGGGCCTGGATAACCAAATTACCGAACTGGATGTAAGCATGTACGGAT
P24	GAATCGCATCATAGGTCGGATACGCACGCGGTGGCCATCCGTACATGCTTACATCCAGT
P25	GTATCCGACCTATGATGCGATTCCGAAACAGAAATTCCTGGATCAAGCAGCTCGGTATG
P26	TTTGTCGCTCAGCTTTTCATACAGTTTGAACAGCCGGTCATACCGAGCTGCTTGATCCA
P27	TGTATGAAAAGCTGAGCGACAAAATTAGCAATGTGACCTTTTGGGGAATTGCGGATAAT
P28	GTAATACACATCCGCACGGCTATCCAGCCAGGTGTGATTATCCGCAATTCCCCAAAAGG
P29	GCCGTGCGGATGTGTATTACGATGCCAATGGCAATGTTGTGGTCGATCCGAATGCGCCC
P30	AAACGGCGCATCTTTGCCCTTGCCTTTTTCCACTTTCGCGTAGGGCGCATTCGGATCGA
P31	GGCAAAGATGCGCCGTTTGTGTTTGGCCCGGATTACAAAGTGAAGCCGGCGTATTGGGC
P32	ATACGCGGATCCCTATTTGTGATCAATGATCGCCCAATACGCCGGCTT

1.4 Expression of xylanase XT6

Both pET-28 (+) and the recombinant pET-28-XT6 plasmids were transformed into *E. coli* BL21 competent cells and selected on LB plate containing 50 μg/mL kanamycin. Single colonies were grown in LB media containing kanamycin and shaken at 250 r/min over night at 37 °C. Then 120 μL of the culture was used to inoculate 120 mL fresh TB medium containing 50μg/mL kanamycin in a 500 mL flask and grown until an $D_{600\ nm}$ of 0.7 was reached. The expression was induced by adding 1 mmol/L isopropyl-β-D-1-thiogalactopyranoside (IPTG). The growth was continued for another 45 h, sampling (15 mL) every 5 h.

Cell pellets were recovered after centrifugation (10 min, 2 500 r/min at 4 °C), resuspended in 0.6×volume of 50 mmol/L sodium phosphate buffer (pH 7.6). Recovery of the xylanase was completed by ultrasonic (10×30 s, 10 cycles, 200 W). Cell debris was discarded after centrifugation at 4 °C, 12 000 r/min for 20 min, and the supernatant was analyzed by SDS-PAGE and enzymatic assay.

1.5 Enzyme assays

The xylanase activity was measured in a mixture consisting

of 0.2 mL of appropriately diluted enzyme solution and 1.8 mL 50 mmol/L sodium phosphate buffer (pH 7.6) containing 1% (*w*/*V*) birchwood xylan. The mixture was incubated at 50 °C for 5 min, followed by immediate chilling on ice for 5 min. The amount of reducing sugars released was determined by the dinitrosalicylic acid (DNS) method [16]. One unit of xylanase activity was defined as the amount of enzyme producing 1 μmoL of reducing equivalents per min under the assay conditions. Protein concentrations were determined by the Bradford method [17] using BSA as the standard. Same method was applied under temperatures ranging from 45 °C to 95 °C to determine the temperature effect.

2 Results & Discussion

2.1 Synthesis of codon-optimized xylanase XT6 gene

Because of the plan to express the XT6 gene in *E. coli*, the nucleotide sequence was optimized for codon usage in the oligonucleotide design process [12, 18, 19]. The synthetic gene sequence had 47.0% G+C content and only shared 24.1% similarity to the wild-type XT6 (Fig. 1), although they encoded the same amino acid sequence. Table 1 shows the total 32 oligonucleotides designed by the on-line program DNAWorks 3.1. The PCR-based de novo gene synthesis strategy is illustrated in Fig. 2-a. The optimized gene without signal peptide sequence was synthesized and Fig. 2-b shows the final amplified product with a length of 1 166 bp, which was then ligated into pET-28a (+) (Fig. 3) and subjected to sequencing. Four plasmids were sequenced with one perfect sequence and three having a single point mutation. The overall error rate was about 0.6 error/kb.

2.2 Expression of de novo synthesized XT6 in *E. coli*

The recombinant expression plasmid pET-28-XT6 was

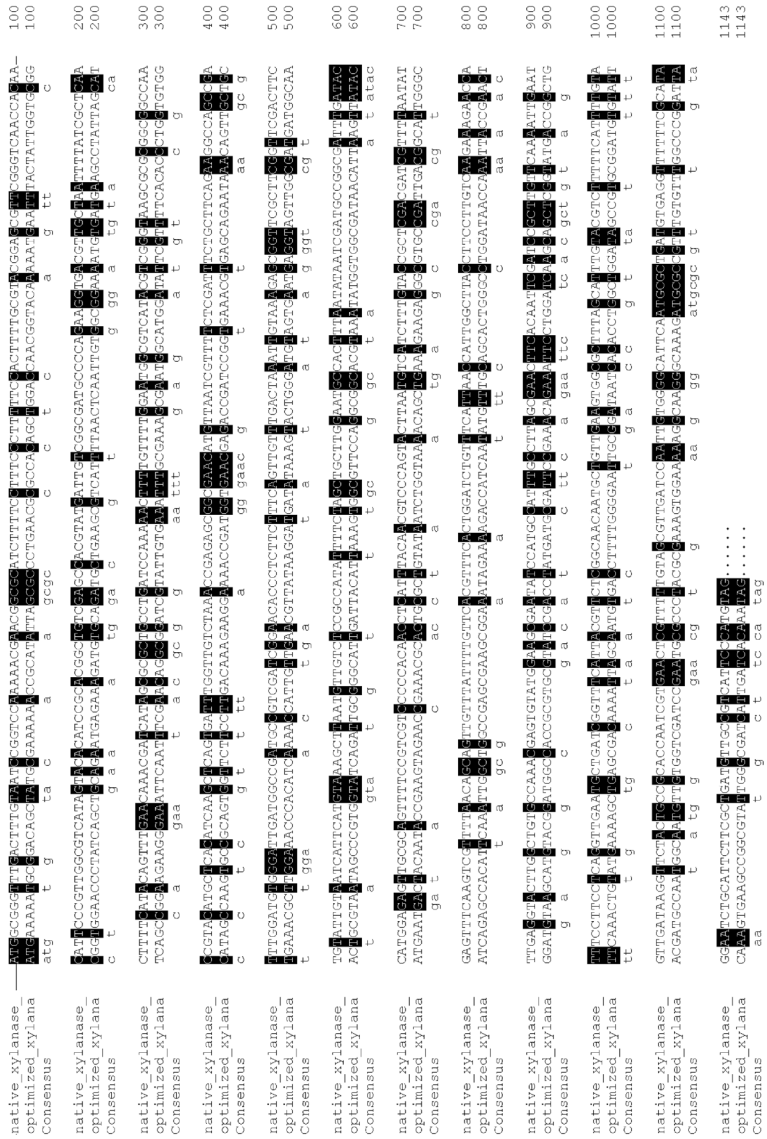

Fig. 1 Alignment of XT6 gene original DNA sequence (GenBank Accession No: Z29080) and optimized sequence generated by DNAMAN (Lynnon Biosoft, version 5.2.2)

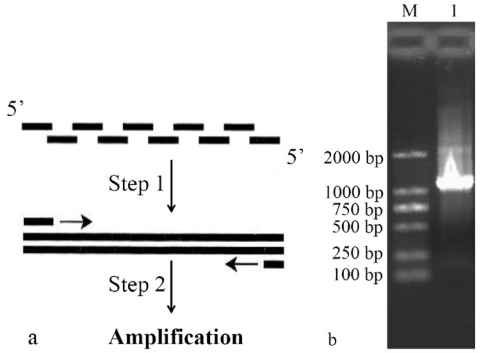

Fig. 2 Xylanae XT6 gene synthesis

a. Diagram of xylanase XT6 gene synthesis by PCR-based method. b. Agarose gel electrophoresis of gene synthesis product (Lane 1: Gene synthesis product. Lane M: Molecular weight marker

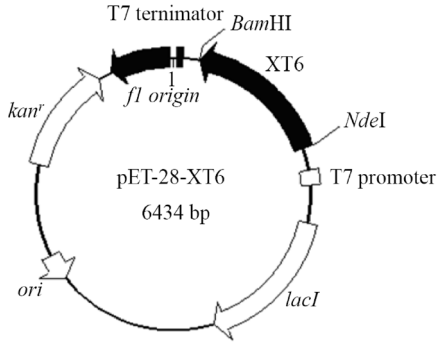

Fig. 3 Physical map of recombinant expression plasmid pET-28-XT6

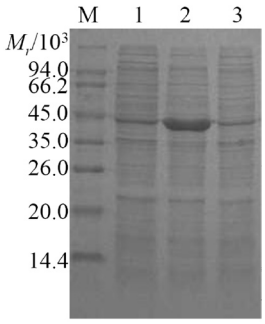

Fig. 4 SDS-PAGE analysis of recombinant XT6 in *E. coli*

Lane M: Molecular mass markers; Lane 1: pET-28-XT6 without induction; Lane 2: pET-28-XT6 induced by IPTG; Lane 3: pET-28a (+) induced by IPTG

transformed into *E. coli* BL21 (DE3) for XT6 expression. The SDS-PAGE analysis (Fig. 4) indicated that the molecular weight of expressed XT6 was about 4.3×10^3, in agreement with the calculated molecular masses [11]. The expressed codon-optimized XT6 represented about 65% of the total soluble protein of *E. coli*. The expression level was comparable to that reported for the native XT6 gene [11]. When the inducer was added at an $D_{600\ nm}$ of 0.7, enzyme activity increased continually for a period of over 30 h, reaching a level of about 1 800 U/mL culture, meanwhile, the total protein concentrations were up to approximately 2.0 mg/mL culture. Fig. 5 shows that the xylanase activity varied with total cell proteins with a specific activity of around 1 000 U/mg. The maximal xylanase activities expressed in TB were confirmed in crude cell-free extracts of recombinant *E. coli* cells and were up to 3 030 U/mL.

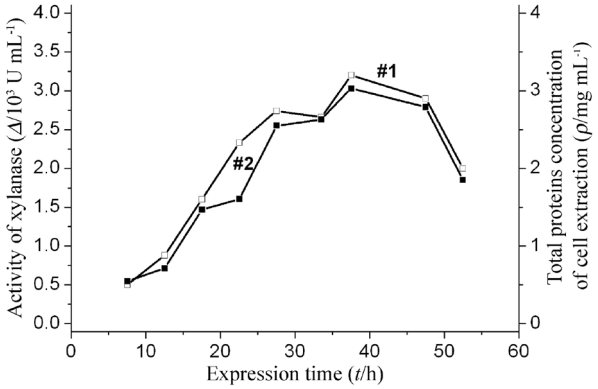

Fig. 5 XT6 expression in *E. coli* cells

E. coli BL21 (DE3) cultures were grown in TB media in shake flasks and induced (1 mmol/L IPTG).The total protein concentration of cell extracts (#1, hollow square)and activity of xylanase (#2, filled square)were changed with the induced time

2.3 Effect of temperature on xylanase XT6

The reaction rate of xylanase XT6 at different temperatures was determined (Fig. 6). The highest initial reaction rate was obtained at 80 °C. The relative activities at different temperatures were 14%, 24%, 30%, 34%, 60%, 73%, 98%, 100%, 80% and 52% at 45, 50, 55, 60, 65, 70, 75, 80, 90 and 95 °C, respectively. It was found that the enzyme had an optimal temperature at 80 °C although our experiments also showed that it was completely inactivated after treated without substrate at 80 °C for 1 h (The data were not shown). When the temperature exceeded 80 °C, the activity of the XT6 declined gradually.

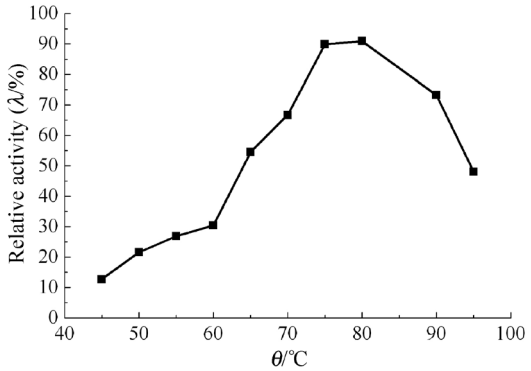

Fig. 6 Temperature profiles of xylanase XT6

Activities are shown as percentages of the maximum activity, and the values shown are the means of triplicate determinations

3 Conclusions

The bias of codon usage has been demonstrated to be related to gene expression level [19]. In this paper, xylanase XT6 from *G. stearothermophilus* that its gene was optimized for overexpression in *E. coli* and synthesized by the PCR-based method was successfully expressed in *E. coli* using pET expression system. After optimization of the expression condition, the recombinant xylanase made up 65% of total cell protein, and 3 030 U/mL xylanase activities were obtained. Its

properties were similar with those of the native enzyme in *G. stearothermophilus*. This work would pave the way for further studies on its structural-functional relationship and for improving its stability by directed molecular evolution.

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