J. Microbiol. Biotechnol. (2010), **20**(3), 467–473
doi: 10.4014/jmb.0908.08028
First published online 20 January 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

High-Level Expression of an *Aspergillus niger* Endo-β-1,4-Glucanase in *Pichia pastoris* Through Gene Codon Optimization and Synthesis

Zhao, Shumiao[1†], Jun Huang[1†], Changyi Zhang[1], Ling Deng[1], Nan Hu[2], and Yunxiang Liang[1]*

[1]*State Key Laboratory of Agricultural Microbiology and College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, P.R. China*
[2]*College of Life Science and Pharmacy, Nanjing University of Technology, Nanjing 210009, P. R. China*

Received: August 25, 2009 / Revised: October 20, 2009 / Accepted: October 21, 2009

To improve the expression efficiency of recombinant endo-β-1,4-glucanase in *P. pastoris*, the endo-β-1,4-glucanase (*egI*) gene from *Aspergillus niger* was synthesized using optimized codons. Fourteen pairs of oligonucleotides with 15 bp overlap were designed and the full-length *syn-egI* gene was generated by two-step PCR-based DNA synthesis. In the synthesized endo-β-1,4-glucanase gene *syn-egI*, 193 nucleotides were changed, and the G+C content was decreased from 54% to 44.2%. The *syn-egI* gene was inserted into pPIC9K and transformed into *P. pastoris* GS115 by electroporation. The enzyme activity of recombinant *P. pastoris* stain 2-7# reached 20.3 U/ml with 1% barley β-glucan and 3.3 U/ml with 1% carboxymethylcellulose (CMC) as substrates in shake flasks versus 1,270.3 U/ml and 220.7 U/ml for the same substrates in 50-l fermentors. The molecular mass of the recombinant protein was approximately 40 kDa as determined by SDS–PAGE analysis, the optimal temperature for recombinant enzyme activity was 70°C, and the optimal pH was 5.0 when CMC was used as the substrate.

Keywords: *Aspergillus niger*, codon optimization, endo-β-1, 4-glucanase, expression, *Pichia pastoris*

Cellulose, which is one of the most abundant biopolymers in the world, is the major component of plant cell walls [18]. Biological degradation of cellulose is catalyzed by a cellulase complex consisting of three types of enzymes, endo-β-1,4-glucanase (E.C. 3.2.1.4), cellobiohydrolase (exo-β-1,4-glucanase, E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21); the endo-β-1,4-glucanases have strict cleavage specificity since only they hydrolyze β-1,4-glycosidic bonds to decrease the length of the cellulose chains [3, 19]. This enzyme has many useful applications, such as biopolishing cotton fabric, improving the processing of paper pulp, de-inking paper, enhancing the efficiency of laundry detergents, and increasing the utilization efficiency of plant materials in animal feed [8, 9].

Endo-β-1,4-glucanase can be produced by a broad range of organisms, including microbes, plants, and animals [10, 16]. However, production of this enzyme is mostly from strains of *Trichoderma* and *Aspergillus* [4]. Recently, genes encoding endo-β-1,4-glucanases of several different fungal species have been studied, such as *A. aculeatus* [24], *A. kawachii* [28], *A. niger* [13], *Fusarium oxysporum* [30, 31], and *T. reesei* [29]. For production at the industrial level, heterologous expression of endo-β-1,4-glucanase has also been carried out in a number of host organisms, including *Escherichia coli* [5, 10, 16], *Saccharomyces cerevisiae* [13, 28, 29], *A. niger* [27], and *Pichia pastoris* [25]. Among these host organisms, the *P. pastoris* expression system is an excellent system for expressing secreted heterologous proteins [20]. However, like most living organisms, yeasts display general bias towards a subset of the 61 possible sense codons [22]. This difference in codon usage between the original and heterogeneous host has a significant impact on the expression level of recombinant proteins [6]. Codon optimization is a promising technique for increasing protein expression level [34]. The optimization of codon regions encoding a target protein according to the codon bias of the host cell can usually result in an average 10- to 50-fold increase in target protein production [23, 32].

In this study, we designed and constructed a full-length synthetic gene by changing the codons to those preferred by *P. pastoris* in order to achieve high-level expression of heterologous endo-β-1,4-glucanase.

*Corresponding author
Phone: +86-27-87281040; Fax: +86-27-87280670;
E-mail: fa-lyx@163.com

†These authors contributed equally to this work.

Materials and Methods

Strains, Plasmids, and Medium

E. coli DH5α was stored by our laboratory. *P. pastoris* expression vector pPIC9K and *P. pastoris* host strain GS115 (his$^-$ mut$^+$) were purchased from Invitrogen Co., Ltd. Oligonucleotides, Pyrobest DNA polymerase, DNA marker, T4 DNA ligase, pMD18-T simple vector, and restriction enzymes *EcoR*I, *Sac*I, and *Not*I were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China).

Minimal dextrose (MD) medium, minimal methanol (MM) medium, buffered glycerol complex (BMGY) medium, buffered methanol complex (BMMY) medium, fermentation basal salts medium (BSM), and PTM1 trace salts solution were prepared according to the instructions for the *Pichia* Expression Kit [17].

Synthesis of the *syn-egI* Gene

syn-egI was synthesized according to the amino acid sequence of *A. niger* glucanase (GenBank Accession No. AF331518). Using DNAWorks [7], 28 oligonucleotides were designed based on the codon usage of *P. pastoris*, each having about 53 nucleotides and 15 bp overlaps within the nucleotide sequence, which gave a theoretical melting temperature of 55–60°C (Table 1).

The 28 oligonucleotides were assembled by two-step PCR as shown in Fig. 1 [26]. First, fragment S1 was obtained using primers P1–P14 by a two-step method: the first step was performed with a mixture of oligonucleotides P1–P14 under the following conditions: 94°C for 2 min; 30 cycles at 94°C, 60°C, and 72°C, each for 30 s; 72°C for 10 min. Then, to amplify the target fragment S1, 1 μl of the product resulting from the first-step PCR was used for the second-step PCR with forward primer P1 and reverse primer P14. The fragment S2 was obtained by a similar two-step PCR approach using primers P15–P28. Then, the two products S1 and S2 were gel-purified and mixed equally as template for the following PCR, in which oligonucleotides P1 and P28 were used to amplify the full-length gene.

Table 1. Primers used in synthesis of codon-optimized β-1,4-glucanase.

	Primer sequence (5'–3')
PD1	ggagaattcgttttcgaatggtttggatctaatgaatccggagctgag
PD2	tagtctgtaccccaaacacctgggatgttagtaccgaactcagctccggattc
PD3	gtgtttggggtacagactacatcttcccagacccatccgctatttctacattg
PD4	tgaactctgaagaagttcataccctatcaatcaatgtagaaatagcggatgg
PD5	gtatgaacttcttcagagttcaattcatgatggaacgtttgttgccagattct
PD6	gccaagtactcttcatcgtaagaaccagtcatagaatctggcaacaaacgttc
PD7	acgatgaagagtacttggctaacttaactactgttatcaaggctgttactgacgg
PD8	tagttgtgtggatcaaccaaagcatgagcaccaccgtcagtaacagccttgat
PD9	ttggttgatccacacaactacggtagatacaatggtgagatcatctcttct
PD10	ctaagttttcccagaaagtctggaaatcagaagtagaagtagaagagatgatctcaccat
PD11	cagactttctgggaaaacttagctggtcagtacaaagataacgacttggtcat
PD12	ccatgtcatggtattcgttgttggtatcaaacatgaccaagtcgttatctttg
PD13	caacgaataccatgacatggaccaagatttggtcttgaacttgaatcaagctg
PD14	agttgcacctgcagctctgataccgttgatggcagcttgattcaagttcaaga
PD15	agctgcaggtgcaacttctcagtacattttcgtcgagggtaactcctggaccg
PD16	tcatgttatcgttaacatcaacccaggtccaagcaccggtccaggagttaccc
PD17	ggttgatgttaacgataacatgaagaaccttactgatccagaagataagattg
PD18	cagagtctaagtattgatgcatttcgtaaacaatcttatcttctggatcagt
PD19	aaatgcatcaatacttagactctgatggttccggtacctccgaaacttgtgtt
PD20	cttcggtaacacgctccttaccaatagtttcagaaacacaagtttcggaggta
PD21	aaggagcgtgttaccgaagctactcaatggttgaaggataacaagaaggttgg
PD22	atcgttggatcctcctgcgtactcaccaatgaaaccaaccttcttgttatcct
PD23	gcaggaggatccaacgatgtttgtagatctgctgtctccggaatgttagagt
PD24	gcacccttccaaacgtcagtgttgtttgccatgtactctaacattccggagac
PD25	acgtttggaagggtgcctcctggtgggcagctggaccttggtggggtgacta
PD26	taggcagtaccatctggtggttccaaggagaagatgtagtcaccccaccaagg
PD27	accagatggtactgcctacactggtatgttggatatcttggaagcctatctgt
PD28	tacgctgaagcttgagtgcggccgcttacagataggcttccaagatat

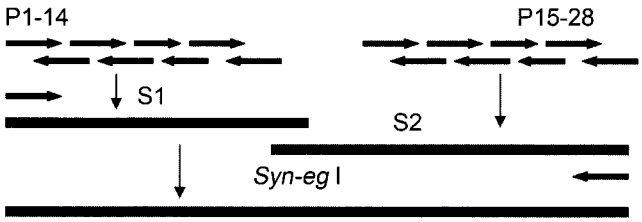

Fig. 1. The strategy for the synthesis and assembly of *syn-egI* using a two-step DNA synthesis method.

Construction of Expression Vectors

The above *syn-egI* gene was cloned into the pMD18-T simple vector, generating plasmid pMD18T-*syn-egI*. The sequence of the gene was verified by sequencing, and the target fragment was separated from pMD18T-*syn-egI* *via* *Not*I and *EcoR*I digestion followed by gel extraction and then insertion into pPIC9K to generate recombinant vector pPIC9K-*syn-egI*.

Transformation and Screening for Transformants

pPIC9K-*syn-egI* DNA (5–10 μg) was linearized with *Sac*I and electrotransformed into *P. pastoris* strain GS115 (1.5 kV, 200 Ω, 25 μF; Bio-Rad Gene Pulser System). Recombinant clones, grown on MD medium, were transferred to MM plates to identify the His$^+$Mut^S phenotype. To observe the transparent zone generated by secreted β-1,4-glucanase, all transformants were spot-inoculated onto a BMMY plate with 1% CMC and incubated for 48 h at 30°C according to the method of Teather *et al.* [33]. Then, clones displaying enzyme activity were picked from the plates, inoculated into 50 ml of BMGY, and cultured at 30°C in a gyratory shaker at 250 rpm for 48 h. The cells were harvested by centrifugation at 3,000 rpm for 5 min at 4°C and resuspended in 20 ml of BMMY. Subsequently, the cultures were incubated at 30°C for 48 h; 1% methanol was added every 24 h to maintain β-1,4-glucanase induction. The clone with the highest β-1,4-glucanase activity was chosen for testing enzyme production by fermentation in a fermentor.

High Cell Density Fermentation at Laboratory Scale

A 50-l fermenter (Biof-6000; Chinabeauty, China) equipped with two six-bladed disc impellers for aeration and electrodes for measuring pH and dissolved oxygen was used. The positive strain (No. 2-7#) with the highest glucanase activity was grown in 20 l of BSM with PTM1 trace salts solution at 30°C and pH 5.0 with the pH adjusted automatically by addition of ammonium hydroxide.

The initial cultivation terminated when all glycerol was consumed (about 20 h). Continuous glycerol feeding was carried out for about

6 h in the subsequent glycerol-fed phase, and the yield of cellular wet weight reached about 100 g/l at the end of this phase. After that, 100% methanol was added at 5 ml/h·l for 100 h. During fermentation, the condensed air flow was kept above 20% with an agitation rate of 1,000 rpm. Samples were collected from the culture every 4 h and analyzed for cellular wet weight, enzyme activity, and production of extracellular peptides by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as described previously.

Glucanase Assay

The β-1,4-glucanase activity was determined with 1% (w/v) barley β-glucan (Sigma) and 1% (w/v) CMC (Sigma) as substrates, and the reducing sugar content was measured using the 3,5-dinitrosalicylic acid method [21] with D-glucose as the standard. The assay was carried out in a 2-ml volume containing an appropriate amount of enzyme solution and 1% substrate (w/v) dissolved in 0.1 mol/l acetic buffer (pH 5.0) and incubated at 40°C for 30 min. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 μmol reducing sugar from the substrate per minute.

Characterization of the Recombinant Enzyme

The optimal pH for enzyme activity was determined at 40°C with CMC. A Na_2HPO_4/citric acid buffer was used for pH 4.0–8.0 and a NaOH/glycine buffer was used for pH 8.0–10.0. The optimal temperature was determined by performing the standard assay at various temperatures from 40°C to 90°C. Thermal stability of the enzyme was estimated by determining residual enzyme activity after incubation at 40–90°C for 30 min.

The EMBL accession number of the sequence reported in this paper is FN434112.

RESULTS

Synthesis of the *syn-egI* Gene and Construction of the Expression Vector

The *syn-egI* gene was synthesized using a two-step PCR method (Fig. 1). To reduce errors potentially introduced during PCR amplification, the designed gene was subdivided into two fragments: the S1 fragment (477 bp), assembled with oligonucleotides P1–P14, and the S2 fragment (482 bp), with oligonucleotides P15–P28. The two PCR products were then assembled into the full-length *syn-egI* gene (948 bp) including restriction sites by overlap-extension PCR (Fig. 2). The *syn-egI* nucleotide sequence showed 78.8% identity to the wild-type. Compared with the original *egI* gene, 193 nucleotides were changed, which led to the optimization of 121 amino acid codons (Fig. 3), and a reduction in G+C content from 54% to 44.2%, closer to the average G+C content of other highly expressed genes in *P. pastoris* [15].

The *syn-egI* gene was first cloned into the pMD18-T simple vector, generating plasmid pMD18T-*syn-egI*, and the gene sequence was confirmed by sequencing. The target fragment from pMD18T-*syn-egI* was then digested with *NotI* and *EcoRI* and the resulting fragment was inserted into pPIC9K as an in-frame fusion with the α-factor signal sequence, yielding pPIC9K-*syn-egI*.

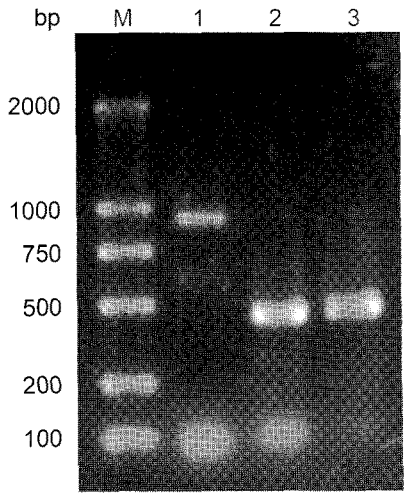

Fig. 2. PCR product of the synthetic fragment of *syn-engI* detected by 1% agarose gel electrophoresis.
Lane M, DNA marker; lane 1, PCR product of the synthetic full-length *syn-engI* with restriction sites; lane 2, PCR product of the synthetic fragment S1 of *syn-engI*; lane 3, PCR product of the synthetic fragment S2 of *syn-engI*.

Screening of Transformants by Shake-Flask Fermentation

The recombinant vector was electroporated into *P. pastoris* GS115 competent cells. A set of 46 transformants was tested by Congo red staining on CMC agar plates, in which a clear halo can be seen around positive colonies (Fig. 4); 45 transformants showed endo-β-1,4-glucanase activity. These transformants were all used for a shaker flask test. After 96 h of induction in a shaker flask, one recombinant (No. 2-7#) exhibited the highest enzyme activity (20.3 U/ml with 1% barley β-glucan and 3.3 U/ml with 1% CMC as substrates). Therefore, this recombinant was chosen to study enzyme production in high cell density fermentation.

High Cell Density Fermentation

To obtain a large amount of active protein, fed-batch high cell density fermentation was performed at a laboratory fermentor scale with a 20-l culture. During the initial glycerol-batch and glycerol-fed-batch phases, the biomass increased exponentially to 101 g/l and the glycerol was depleted by 26 h. The induction phase was then initiated by feeding methanol as the sole carbon source, which lasted about 100 h longer. The β-1,4-glucanase activity of the culture was determined every 4 h during induction. As shown in Fig. 5, the highest level of β-1,4-glucanase production was reached at 96 h after induction (enzyme activity was 220.7 U/ml with 1% CMC substrate, and 1,270.3 U/ml with 1% barley β-glucan substrate), whereas the highest biomass was obtained at 100 h after induction (393 g/l).

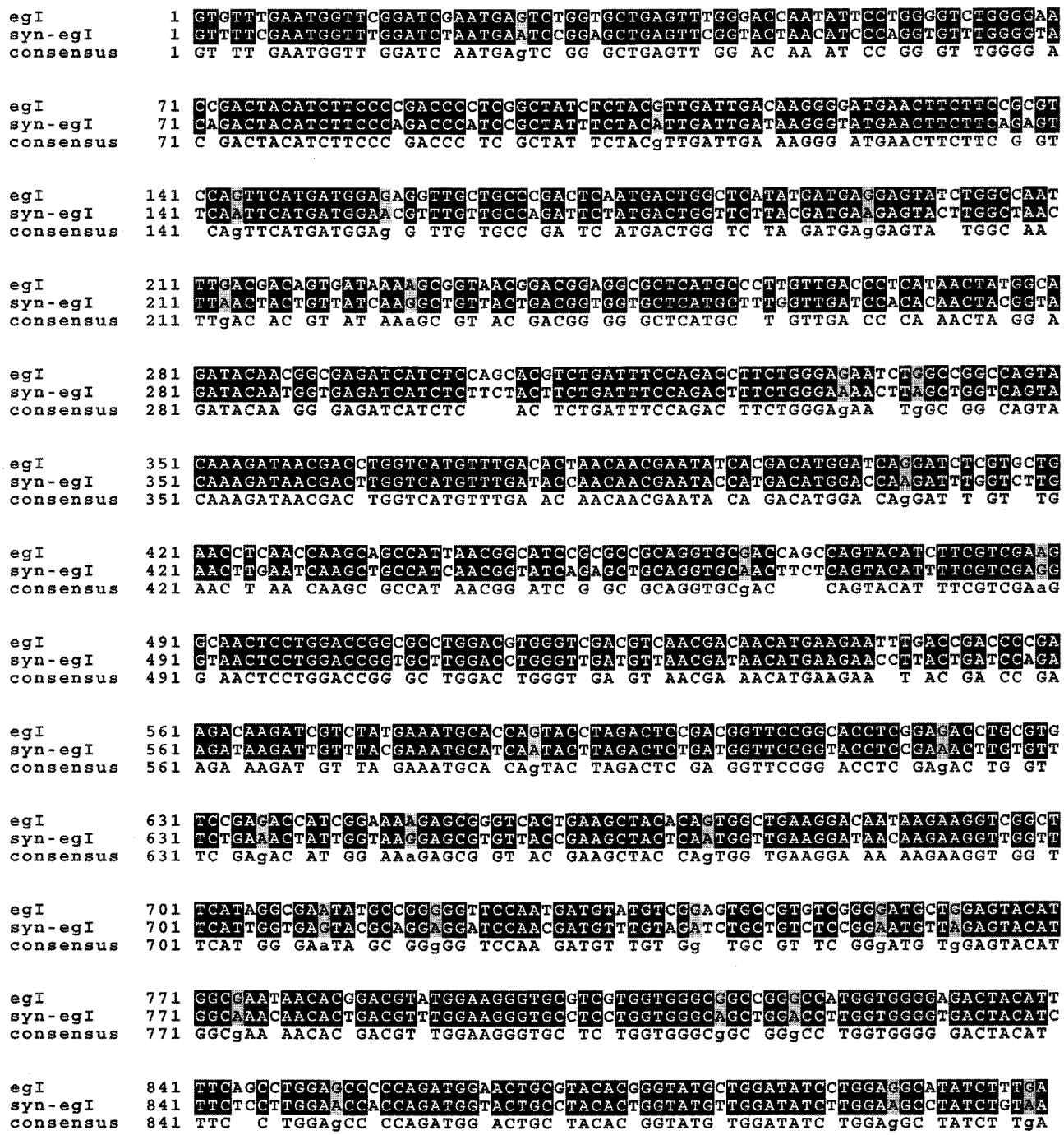

Fig. 3. Alignment of *A. niger* wild-type *eg*I nucleotide sequence (*eg*I) with optimized *eg*I (*syn-eg*I).
The synthetic sequence was generated by Vector NTI Suite (Invitrogen Co., Ltd.).

SDS–PAGE Analysis

The supernatant (5 μl) was used for SDS–PAGE analysis. After staining with Coomassie brilliant blue, the target band exhibited an apparent molecular mass of approximately 40 kDa, which was the mass predicted from the sequence (Fig. 6). Similar results were reported for expression of the *A. niger* endo-1,4-glucanase in *S. cerevisiae* [13].

Characterization of Recombinant Enzyme

The characteristics of the recombinant enzyme were further investigated using CMC as the substrate. The optimal pH for enzyme activity was 5.0 (Fig. 7). The effect of pH on enzyme stability was studied by incubating the enzyme in buffers of different pH at 40°C for 30 min. The enzyme was stable over a wide pH range. More than 90% of the

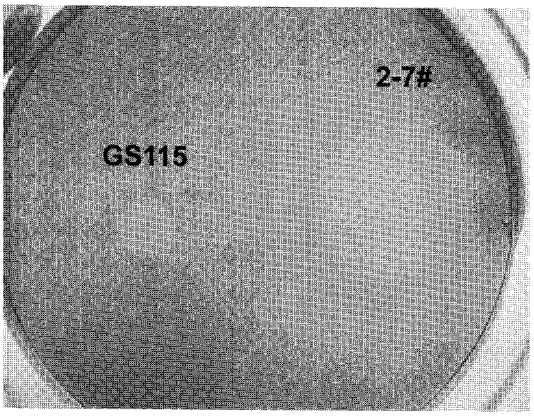

Fig. 4. Activity staining of secreted synthetic endo-β-1,4-glucanase on CMC plate observed after induction.
The transformants were spot-inoculated onto a 1% CMC plate, grown for 48 h at 30°C and stained by 0.1% Congo red for 30 min, and then washed with 1 mol/l NaCl to remove unbound dye and then immersed into 0.5% acetic acid for fixation.

total activity was retained after incubation at pH 3–9 (Fig. 7). The optimal temperature was about 70°C (Fig. 8). The enzyme was stable at high temperatures. More than 95% of enzyme activity was retained after treatment at 60°C for 30 min, and more than 90% was retained after treatment at 70°C. However, only 20% of the activity was retained at 80°C for 30 min (Fig. 8). These results indicate that the recombinant enzyme produced in yeast is relatively stable at high temperature, in accordance with Hong *et al.* [13], who expressed *A. niger egI* in the yeast *S. cerevisiae* and found that the optimal temperature for the recombinant enzyme activity was 70°C and the optimal pH was 6.0.

DISCUSSION

P. pastoris has become an important host organism for the production of recombinant proteins [20]. It is easy to

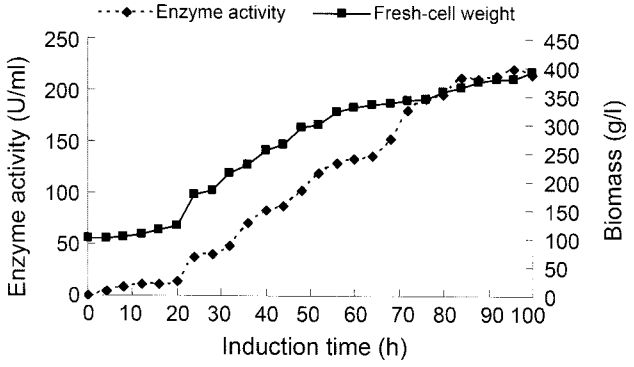

Fig. 5. Secretion of recombinant endo-β-1,4-glucanase in high-density *P. pastoris* fermentation cultures.
The fed-batch fermentation was performed in a laboratory 50-l fermentor. The β-1,4-glucanase activity was determined by incubating at 40°C for 30 min in pH 5.0 with 1% (w/v) CMC (Sigma) as the substrate.

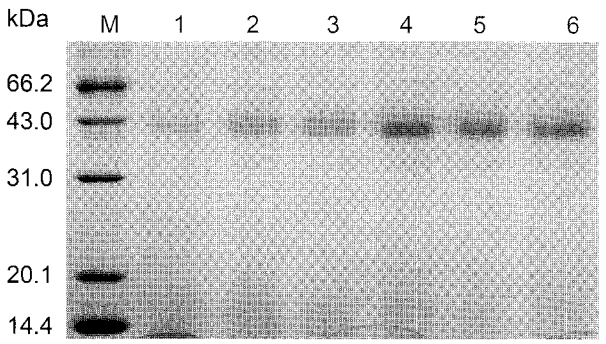

Fig. 6. Analysis of recombinant endo-β-1,4-glucanase by SDS–PAGE.
A 5-μl aliquot of culture supernatant was loaded at different induction times. Lane M, protein molecular marker; supernatants in lanes 1, 2, 3, 4, 5, and 6 were induced for 16, 32, 48, 64, 80, and 96 h, respectively.

cultivate, and its mechanism of posttranslational modification and disulfide bond formation enables proper folding of molecules [34]. In this study, we have successfully expressed an *A. niger* endo-β-1,4-glucanase in *P. pastoris* to a high level *via* codon optimization and batch fermentation.

In recent years, the codon optimization technique has been widely used to increase the expression of foreign proteins in *P. pastoris* [14, 15, 20, 34]. Generally, this is accomplished by replacing all rare codons with preferred codons, eliminating AT-rich stretches, and adjusting the G+C content to match that of other highly expressed genes in *P. pastoris* [15]. Various experimental procedures for PCR-based codon optimization have been developed – from the modification of a few codons about 5' codon adaptation [36], to the extensive rewriting of up to 1,000 bp of DNA by overlap PCR [11]. More recently, Xiong *et al.* [36] reported a simpler, rapid, high-fidelity, and cost-effective two-step PCR-based DNA synthesis method for long gene sequences. With this strategy, we have designed and synthesized the full-length *syn-egI* gene (909 bp) and then expressed it in *P. pastoris*.

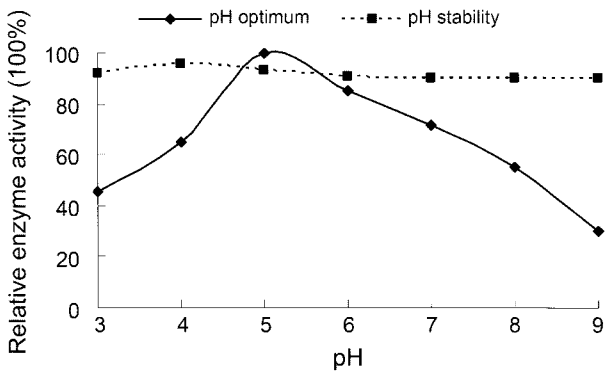

Fig. 7. Effect of pH on recombinant endo-β-1,4-glucanase activity.
The enzyme stability was determined at 40°C for 30 min with CMC.

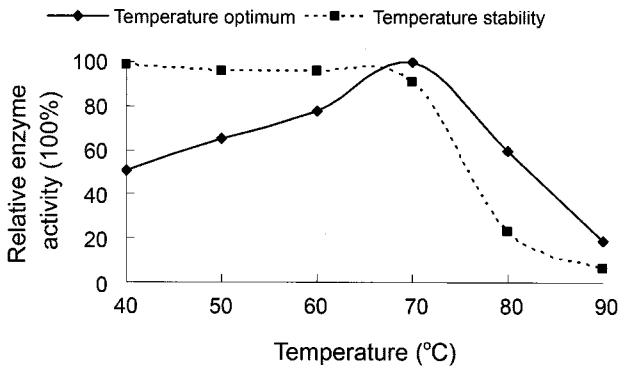

Fig. 8. Effect of temperature on recombinant endo-β-1,4-glucanase activity.
The enzyme stability was determined in pH 5.0 for 30 min with CMC.

The endo-β-glucanase family includes several types, such as endo-β-1,4-glucanase (E.C. 3.2.1.4), endo-β-1,3(4)-glucanase (E.C. 3.2.1.6), endo-β-1,3-glucanase (E.C. 3.2.1.39), and endo-β-1,3-1,4-glucanase (E.C. 3.2.1.73) [12]. Endo-β-1,4-glucanase catalyzes the endohydrolysis of β-1,4-D-glucosidic linkages in cellulose. It also hydrolyzes 1,4-linkages in β-D-glucans that also contain 1,3-linkages [2]. The recombinant enzyme of *A. niger* in this study has higher specific activity on soluble β-1,3-1,4-glucans (1,270.3 U/ml) than the endo-β-1,3-1,4-glucanase from *B. licheniformis* (333.7 U/ml) [34], but endo-β-1,3-1,4-glucanase activity was lower than that from *Paenibacillus* sp. (4,554 U/ml) [26]. This is in agreement with the reports by Bauer *et al.* [2] and Ajithkumar *et al.* [1], in which endoglucanases from the hyperthermophilic archaeon *Pyrococcus furiosus* hydrolyze β-1,4 bonds in mixed-linkage (1→3),(1→4)-β-D-glucans, and endoglucanases from the mesophilic fungus *T. reesei* hydrolyze cellulose, respectively [1, 2]. To our knowledge, the level of endo-β-1,4-glucanase production achieved in this study is the highest ever reported, and the properties of the recombinant enzyme also show great potential for industrial production, especially for utilization in animal feed additives and beer brewing. Further studies will focus on pilot scale-up fermentation of *P. pastoris* expressing the recombinant glucanase.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (No. 30800028) and National Basic Research Program of China (No. 2009CB724700).

References

1. Ajithkumar, A., R. Andersson, M. Siika-aho, M. Tenkanen, and P. Aman. 2006. Isolation of cellotriosyl blocks from barley β-glucan with endo-1,4-β-glucanase from *Trichoderma reesei*. *Carbohyd. Polym.* **64:** 233–238.
2. Bauer, M. W., L. E. Driskill, W. Callen, M. A. Snead, E. J. Mathur, and R. M. Kelly. 1999. An endoglucanase, EglA, from the hyperthermophilic archaeon *Pyrococcus furiosus* hydrolyzes β-1,4 bonds in mixed-linkage (1→3),(1→4)-β-D-glucans and cellulose. *J. Bacteriol.* **181:** 284–290.
3. Beguin, P. and J. P. Aubert. 1994. The biological degradation of cellulose. *FEMS Microbiol. Rev.* **13:** 25–58.
4. Bhat, M. K. 2004. Cellulases and related enzymes in biotechnology. *Biotechnol. Adv.* **18:** 355–383.
5. Cazemier, A. E., J. C. Verdoes, H. J. M. Op den Camp, J. H. P. Hackstein, and A. J. J. van Ooyen. 1999. A β-1,4-endoglucanase-encoding gene from *Cellulomonas pachnodae*. *Appl. Microbiol. Biotechnol.* **52:** 232–239.
6. Chang, S. W., G. C. Lee, and J. F. Shaw. 2006. Codon optimization of *Candida rugosa lip1* gene for improving expression in *Pichia pastoris* and biochemical characterization of the purified recombinant LIP1 lipase. *J. Agric. Food Chem.* **3:** 815–822.
7. David, M. H. and L. Jacek. 2002. DNAWorks: An automated method for designing oligonucleotides for PCR-based gene synthesis. *Nucleic Acids Res.* **30:** e43
8. Dienes, D., A. Egyhazi, and K. Reczey. 2004. Treatment of recycled fiber with *Trichoderma* cellulases. *Ind. Crop. Prod.* **20:** 11–21.
9. Duan, X. Y., S. Y. Liu, W. C. Zhang, Q. X. Zhang, and P. J. Gao. 2004. Volumetric productivity improvement for endoglucanase of *Trichoderma pseudokoingii* S-38. *J. Appl. Microbiol.* **96:** 772–776.
10. Feng, Y., C. J. Duan, H. Pang, X. C. Mo, C. F. Wu, Y. Yu, *et al.* 2007. Cloning and identification of novel cellulase genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases. *Appl. Microbiol. Biotechnol.* **75:** 319–328.
11. Hale, R. S. and G. Thompson. 1998. Codon optimization of the gene encoding a domain from human type 1 neurofibromin protein results in a threefold improvement in expression level in *Escherichia coli*. *Protein Expr. Purif.* **12:** 185–188.
12. Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280:** 309–316.
13. Hong, J., T. Hisanori, A. Shunichi, K. Yamamtot, and H. Kumagai. 2001. Cloning of a gene encoding a highly stable endo-β-1,4-glucanase from *Aspergillus niger* and its expression in yeast. *J. Biosci. Bioeng.* **92:** 434–439.
14. Hu, S., L. Li, J. Qiao, Y. Guo, L. Cheng, and J. Liu. 2006. Codon optimization, expression, and characterization of an internalizing anti-ErbB2 single-chain antibody in *Pichia pastoris*. *Protein Expr. Purif.* **47:** 249–257.
15. Huang, H., P. Yang, H. Luo, H. Tang, N. Shao, T. Yuan, Y. Wang, Y. Bai, and B. Yao. 2008. High-level expression of a truncated 1,3-1,4-β-D-glucanase from *Fibrobacter succinogenes* in *Pichia pastoris* by optimization of codons and fermentation. *Appl. Microbiol. Biotechnol.* **78:** 95–103.
16. Huang, Y., G. Krauss, S. Cottaz, H. Driguez, and G. Lipps. 2005. A highly acid-stable and thermostable endo-β-glucanase from the thermoacidophilic archaeon *Sulfolobus solfataricus*. *Biochem. J.* **385:** 581–588.

17. Invitrogen: *Pichia* Expression Kit: A manual of methods for expression of recombinant proteins in *Pichia pastoris*. Version M. Invitrogen, San Diego, CA, 2002
18. Kitamoto, N., M. Go, T. Shibayama, T. Kimura, Y. Kito, K. Ohmiya, and N. Tsukagoshi. 1996. Molecular cloning, purification and characterization of two endo-1,4-β-glucanases from *Aspergillus oryzae* KBN616. *Appl. Microbiol. Biotechnol.* **46:** 538–544.
19. Lynd, L. R., J. Paul, P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **66:** 506–577.
20. Macauley-Patrick, S., M. L. Fazenda, B. McNeil, and L.M. Harvey. 2005. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* **22:** 249–270.
21. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426–428.
22. Nakamura, Y., T. Gojobori, and T. Ikemura. 1999. Codon usage tabulated from the international DNA sequence database; its status 1999. *Nucleic Acids Res.* **27:** 292.
23. Nikolay, S. O., J. S. Willem, and A. J. Maarten. 2002. Optimization of the expression of equistatin in *Pichia pastoris*. *Protein Expr. Purif.* **24:** 18–24.
24. Ooi, T., A. Shinmyo, H. Okada, S. Murao, T. Kawaguchi, and M. Arai. 1990. Complete nucleotide sequence of a gene coding for *Aspergillus aculeatus* cellulase (F1-CMCase). *Nucleic Acids Res.* **18:** 5884.
25. Palomer, X., E. Dominguez-Puigjaner, M. Vendrell, and I. Llop-Tous. 2004. Study of the strawberry Cell endo-(1,4)-glucanase protein accumulation and characterization of its *in vitro* activity by heterologous expression in *Pichia pastoris*. *Plant Sci.* **167:** 509–518.
26. Peilong, Y., P. Shi, Y. Wang, Y. Bai, K. Meng, H. Luo, T. Yuan, and B. Yao. 2007. Cloning and overexpression of a *Paenibacillus* β-glucanase in *Pichia pastoris*: Purification and characterization of the recombinant enzyme. *J. Microbiol. Biotechnol.* **17:** 58–66.
27. Rose, S. H. and W. H. van Zyl. 2002. Constitutive expression of the *Trichoderma reesei* β-1,4-xylanase gene (*xyn2*) and the β-1,4-endoglucanase gene (*egI*) in *Aspergillus niger* in molasses and defined glucose media. *Appl. Microbiol. Biotechnol.* **58:** 461–468.
28. Sakamoto, S., G. Tamura, K. Ito, T. Ishikawa, K. Iwano, and N. Nishiya. 1995. Cloning and sequencing of cellulase cDNA from *Aspergillus kawachii* and its expression in *Saccharomyces cerevisiae*. *Curr. Genet.* **27:** 435–439.
29. Saloheimo, A., B. Henrissat, A. M. Hoffren, O. Teleman, and M. Penttila. 1994. A novel small endoglucanase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast. *Mol. Microbiol.* **13:** 219–228.
30. Sheppard, P. O., F. J. Grant, P. J. Oort, C. A. Sprecher, D. C. Foster, F. S. Hagen, A. U. Upshall, G. L. McKnight, and P. J. OHara. 1994. The use of conserved cellulase family-specific sequences to clone cellulase homologue cDNAs from *Fusarium oxysporum*. *Gene* **150:** 163–167.
31. Shuyan, L., D. Xinyuan, L. Xuemei, and G. Peiji. 2006. A novel thermophilic endoglucanase from a mesophilic fungus *Fusarium oxysporum*. *Chinese Sci. Bull.* **51:** 191–197.
32. Sinclair, G. and F. Y. M. Choy. 2002. Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.* **26:** 96–105.
33. Teather, R. M. and P. J. Wood. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **8:** 777–780.
34. Teng, D., Y. Fan, Y. L.Yang, Z. G. Tian, L. Luo, and J. H. Wang. 2007. Codon optimization of *Bacillus licheniformis* β-1,3-1,4-glucanase gene and its expression in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **74:** 1074–1083.
35. Vervoort, E. B., A. Van Ravestein, N. N. van Peij, J. C. Heikoop, P. J. van Hasstert, G. F. Verheijden, and M. H. Linskens. 2000. Optimizing heterologous expression in *Dictyostelium*: Importance of 5' codon adaptation. *Nucleic Acids Res.* **28:** 2069–2074.
36. Xiong, A. S., Q. H. Yao, and R. H. Peng. 2004. A simple, rapid, high-fidelity and cost-effective PCR-based two-step DNA synthesis method for long gene sequences. *Nucleic Acids Res.* **32:** e98.