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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

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Abstract 1,3-1,4-β-D-glucanase is an important endoglycosidase in the brewing and animal feed industries. To achieve high-level expression of recombinant glucanase in *Pichia pastoris*, we designed sequences encoding the α-factor signal peptide from *Saccharomyces cerevisiae* and the truncated 1,3-1,4-β-D-glucanase from *Fibrobacter succinogenes* as a whole. The codons encoding the 52 amino acids of the signal peptide and 106 residues of the glucanase protein were optimized for expression in *P. pastoris*; 189 nucleotides were changed. The G+C content was adjusted to 48–49%, and AT-rich stretches were eliminated to avoid premature termination. The messenger ribonucleic acid secondary structure near the AUG start codon was also adjusted to ensure efficient translation; the resulting glucanase production was twofold higher compared with that achieved with gene structure optimization alone. We also propose a new fermentation strategy for the induction phase, in which 5/95% glycerol/methanol mixed feed was used in days 1–3 and 100% methanol was used on days 4–6. By comparison with methanol feed and glycerol/methanol-mixed feed alone, the yield of recombinant glucanase increased by 38.5 and 16.5%, respectively. The expressed optimized recombinant 1,3-1,4-β-D-glucanase constituted ~90% of the total secreted protein, reaching up to 3 g l^{-1} in the medium.

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Introduction

1,3-1,4-β-Glucan, the major component of cereal cell walls (Buliga et al. 1986), is composed of β-D-glucosyl residues linked through β-1,3 and β-1,4 glycosidic bonds. This glucan type may cause severe problems in brewing and feed industries because of high viscosity (Bielecki et al. 1991). The enzyme, 1,3-1,4-β-D-glucanase (lichenase, EC 3.2.1.73), specifically cleaves the 1,4-β-D-glucosidic bonds adjacent to 1,3-β-linkages (Heinemann et al. 1996) and therefore has received much attention in basic and applied research (Li et al. 1996; Mathlouthi et al. 2002; Planas 2000). For example, supplementation of this enzyme in animal feed improves feed conversion efficiency and reduces sanitary problems such as sticky droppings (Mathlouthi et al. 2002).

A number of glucanase genes have been isolated and characterized from bacteria, fungi, and higher plants, including *Bacillus amyloliquefaciens* (Olsen et al. 1991), *B. licheniformis* (Planas et al. 1992; Viladot et al. 1998), *B. subtilis* (Hinchliff and Wendy 1984), *Clostridium thermocellum* (Schimming and Schwarz 1991), *Fibrobacter succinogenes* (Teather and Erfle 1990), *Paenibacillus* sp. (Yang et al. 2007), *Rhizopus arrhizus* (Clark et al. 1978), *Streptococcus bovis* (Ekinci et al. 1997), strawberry (Shi et al. 2005), and winter rye (Yaish et al. 2006). Among these, *F. succinogenes* plays a key role in plant fiber degradation in the rumen (Teather and Erfle 1990), and thus this organism's glucanase gene has received much attention. Several strategies have been attempted to produce this enzyme; these include heterologous expression in host organisms (Chen et al.

2001; Liu et al. 2007; Wen et al. 2005) and gene truncation at the C terminus (Tsai et al. 2005; Wen et al. 2005).

The methylotrophic yeast, *Pichia pastoris*, is an excellent host in which to express heterologous proteins using the methanol oxidase promoter (Sreekrishna et al. 1997) and recover them as secreted forms (Sue et al. 2005). To further improve protein expression, many factors that influence protein production have been optimized in this system, including heterologous gene copy number (Clare et al. 1991), A+T composition (Romanos et al. 1992), translation start codon (AUG) context (Sreekrishna et al. 1993), messenger ribonucleic acid (mRNA) 5′ and 3′ untranslated regions (UTRs; Sreekrishna et al. 1993), manipulating heterologous gene codons for optimal usage in the host (Teng et al. 2007), altering the secretion signal (Xiong et al. 2006), and fermentation conditions (Plantz et al. 2006). However, these optimization methods neither are considered in the mRNA level nor have been integrated as a whole to optimize gene expression.

A truncated 1,3–1,4-β-D-glucanase from *F. succinogenes* having increased specific activity (10,800 U mg^{-1}) and high thermal stability (retaining 80% activity at 90°C for 10 min) has been expressed in *P. pastoris*; however, the secreted glucanase was only 0.11 mg ml^{-1} in the supernatant, thereby limiting applications in industry (Wen et al. 2005). The goal of our present study was to achieve high-level expression of *F. succinogenes* glucanase in *P. pastoris* by optimization of the parameters mentioned above, as well as mRNA secondary structure. To our knowledge, we have achieved the highest reported expression (3 g l^{-1}) of the 1,3-1,4-β-D-glucanase in *P. pastoris*.

Materials and methods

Chemicals, plasmids, strains, and media

Lichenan was purchased from Sigma (St. Louis, MO), and all the other chemicals were of analytical grade. All oligonucletides were synthesized by Sangon (Shanghai, China). T4 deoxyribonucleic acid (DNA) ligase and restriction endonucleases were purchased from Promega (Madison, WI). Endo-β-*N*-Acetylglucosaminidase H (Endo H) was purchased from New England Biolabs (Beverly, MA). *Escherichia coli* JM109 and vector pUC19 were obtained from TaKaRa (Dalian, China). *P. pastoris* GS115 and vector pPIC9 were purchased from Invitrogen (San Diego, CA).

Regeneration dextrose (RDB) medium, minimal dextrose (MD) medium, minimal methanol (MM) medium, buffered glycerol complex (BMGY) medium, buffered methanol complex (BMMY) medium, and fermentation Basal Salts medium (BSM) were prepared according to the manual of *Pichia* Expression kit (Invitrogen 2002).

Design of glucanase gene and α-factor prepro-leader sequence

To better express the truncated glucanase from *F. succinogenes*, we designed three complete mRNAs (*α-factor+bgl*; *α-factor+bgl-m*, and *α-factor-m+bgl-m*; Fig. 1) that included the 5′ and 3′ UTRs, which were defined in the manufacturer's instruction of '*Pichia* Expression kit' (Invitrogen 2002). *α-factor* is the signal peptide of the mating pheromone from *Saccharomyces cerevisiae*. A novel strategy was used to optimize the codons of the enzyme according to the following three steps. First, the gene, *bgl-m*, was designed by changing the codons for the amino acid sequence of the truncated 1,3-1,4-β-D-glucanase from *F. succinogenes* (Wen et al. 2005) to an appropriate codon ratio according to the codon usage frequency of highly expressed genes in *P. pastoris* (Zhao et al. 2000). The codons were further adjusted based on those commonly found in the more highly expressed genes, like *AOX1* (GenBank accession no. U96967), *GAP* (GenBank accession no. U62648), and the *actin* gene (GenBank accession no. AF216956) in *P. pastoris*. The G+C content was adjusted to an appropriate range based on the content of highly expressed genes in *P. pastoris*. Second, the DNAStar program (Burland 2000)

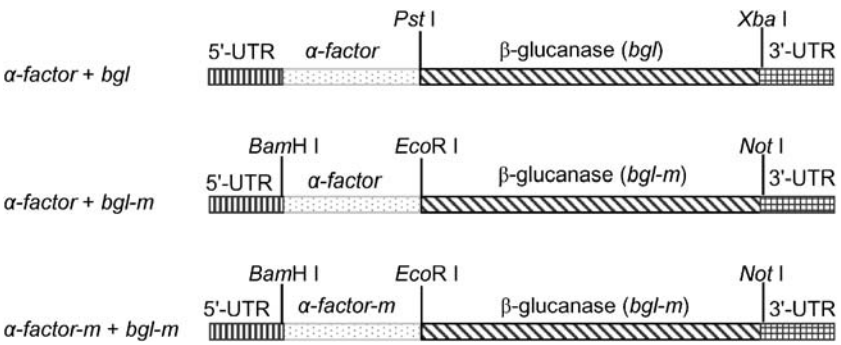

Fig. 1 Schematic representation of the complete glucanase mRNAs used in the study. *α-factor+bgl* included the wild-type α-factor signal peptide and native β-glucanase; *α-factor+bgl-m* included the wild-type signal peptide and optimized β-glucanase; *α-factor-m+bgl-m* included the optimized signal peptide and optimized β-glucanase. The *AOX1* promoter was used for each of the mRNAs

was used to analyze and optimize both the average distribution of G+C and the same codons and to eliminate AT-rich regions. Using the same strategy, the signal peptide, *α-factor-m*, was designed based on the sequence of the α-factor prepro-leader without altering the amino acid sequence. Third, the mRNA secondary structure, the free energy (ΔG) of mRNA folding, and the sequence around the AUG start codon were predicted, analyzed, and adjusted by RNA-folding analysis (the RNAstructure 4.4 program; Mathews et al. 2004) and Rensselaer bioinformatics system (http://www.bioinfo.rpi.edu) to adjust some of the parameters of the complete mRNA, including the 5′ and 3′ UTRs.

Construction of glucanase gene and α-factor prepro-leader sequence

A total of 28 oligonucleotide fragments, each about 58 bp in length, were designed and synthesized by Sangon. Fragments A1–A8 and B1–B6 were located in the sense DNA strand (5′–3′), and a1–a8 and b1–b6 in the antisense DNA strand (3′–5′). The corresponding sense and antisense fragments overlapped by about 29 nucleotides. The gene *bgl-m* was constructed with these oligonucleotide fragments by phosphorylation, annealing, and ligation into pUC19 (Fig. 2). The complete *bgl-m* and the recombinant signal peptide *α-factor-m* were sequenced.

Construction of the expression vector

To construct the expression vector, the modified signal peptide, *α-factor-m*, was inserted into the *P. pastoris* vector pPIC9 between the unique *Bam*HI and *Eco*RI sites to generate the plasmid, pPIC9m. Then, *bgl-m* was inserted into pPIC9 and pPIC9m between the *Eco*RI and *Not*I sites, producing the constructs pPIC9-*bgl-m* and pPIC9m-*bgl-m*, respectively. The *bgl-m* was in frame with the signal peptide in the recombinant expression vector. The presence of the *α-factor-m* and *bgl-m* were confirmed by restriction enzyme digestion, electrophoresis, and sequencing.

Expression of *bgl-m* with the original or modified signal peptide in *P. pastoris*

The plasmid pPIC9-*bgl-m* and pPIC9m-*bgl-m* were linearized by *Bgl*II and transformed into *P. pastoris* GS115 at the *AOX1* locus (*HIS*$^+$, *MUT*^{s}) by electroporation using Gene Pulser (BioRad, Hercules, CA). The recombinant clones grown on RDB medium were cultured on MM and MD plates at 30°C for 2 days. Transformants that grew normally on MD but showed little or no growth on MM were selected following the '*Pichia* Expression kit' manufacturer's instructions (Invitrogen 2002). The selected clones were further incubated in 3 ml BMGY medium and

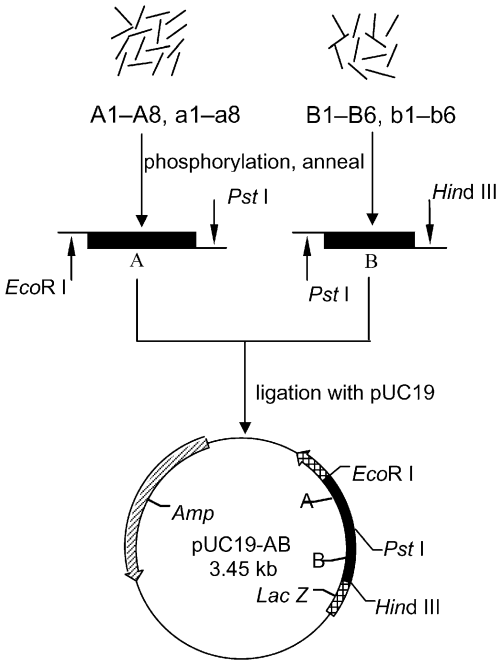

Fig. 2 Strategy for the construction of the optimized β-glucanase (*bgl-m*) gene using oligonucleotide phosphorylation and annealing. Fragments A1–A8 and B1–B6 located on the sense DNA strand (5′–3′) and a1–a8 and b1–b6 located on the antisense DNA strand (3′–5′). Corresponding sense and antisense oligonucleotides overlapped by ~29 nucleotides

incubated at 30°C in a gyratory shaker at 250 rpm for 2 days. Cells were harvested by centrifugation at 3,250×*g*, 4°C for 10 min and incubated in 1 ml BMMY medium for 2 more days. The positive clones were further determined by glucanase activity assay.

Expression of *bgl-m* at the flask level

To determine glucanase expression, the positive clones with high enzyme activity were further incubated in 45 ml BMGY and then transferred to 15 ml BMMY in a 100-ml flask for 4 days and induced with methanol (1%, v/v) every 24 h. Glucanase activity and production were analyzed every 24 h by activity assay and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), respectively.

Expression of *bgl-m* at the fermentation level

The positive strain with the highest glucanase activity was grown in a 3.7-l fermenter (Bioengineering KLF 2000, Switzerland) to scale up fermentation. The basic operation of *Pichia* fermentation was performed according to Invitrogen guidelines. All fermentations began at batch growth phase in 2.0-l BSM at 30°C and pH 5.0, and the pH was maintained automatically with ammonium hydroxide. The initial cultivation terminated when all glycerol was consumed (about 18 h), and the yield of cellular dry weight

reached 27 g l^{-1} at batch phase. Continuous glycerol feeding was carried out for about 6 h in the subsequent glycerol-fed phase, and the yield of cellular dry weight reached 45 g l^{-1}. Three induction strategies, denoted A, B, and C, were used to induce glucanase expression. In strategy A, 100% methanol was fed at 3 ml h^{-1} l^{-1} for 6 days; in strategy B, 5% glycerol +95% methanol were mixed and fed at 3 ml h^{-1} l^{-1} for 6 days; in strategy C, 5% glycerol+95% methanol were mixed and fed at 3 ml h^{-1} l^{-1} for 3 days, and then 100% methanol was added at 3 ml h^{-1} l^{-1} for the subsequent 3 days. Culture samples were collected every 12 h and analyzed for cellular dry weight, enzyme activity, and production of extracellular peptides (by SDS-PAGE).

Purification of recombinant glucanase

The cell-free supernatant from fermentation was recovered after centrifugation at 12,000×*g* for 10 min at 4°C. Proteins were precipitated from the cell-free supernatant by saturation with ammonium sulfate at 4°C and recentrifugation. The precipitate was then resuspended in 100 mM sodium citrate, pH 8.0 (buffer A), and dialyzed against the same buffer. Dialysate (10 ml) was applied to a HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia Biotech, Sweden) equilibrated with buffer A. Proteins were eluted from the column with a 0–1 M linear gradient of NaCl in buffer A. Glucanase activity eluted mainly as a single peak, and those fractions were pooled and analyzed by SDS-PAGE. The enzyme activity was determined as described below, and the concentration of the purified enzyme was determined by the Bradford assay, using bovine serum albumin as a standard (Asryants et al. 1985).

Deglycosylation analysis

Purified recombinant 1,3-1,4-β-D-glucanase (r-Bgl, ~5 μg) was deglycosylated using 250 U of Endo H for 2 h at 37°C according to the manufacturer's instructions (New England Biolabs). The deglycosylated and untreated r-Bgl were analyzed by SDS-PAGE.

Glucanase activity assays

The enzymatic activity of 1,3-1,4-β-D-glucanase was measured using lichenan as a substrate, and the reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959; Wen et al. 2005) with glucose as the standard. The standard enzyme activity assay was followed: 50 μl of enzyme solution was incubated with 450 μl substrate solution (1% lichenan (w/v) in 0.1 M phosphate citric acid buffer (0.1 M citric acid and 0.2 M Na_2HPO_4, pH 6.5)) at 50°C for 10 min; the reaction was terminated by boiling in water for 5 min and then adding 1.5 ml of DNS solution, and the absorption was measured at 540°nm after addition of 1 ml deionized water. All glucanase activity determinations were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute from 1% lichenan (w/v) at 50°C.

Properties and kinetics of r-Bgl

The pH vs glucanase activity profile was determined by incubating the purified r-Bgl with lichenan in phosphate citric acid buffer (pH 2.0–8.0) or NaOH–glycine buffer (0.1 M glycine, pH 8.0–10.0), respectively. The temperature vs glucanase activity profile was determined by measuring glucanase activity from 25 to 70°C at 5°C intervals in buffers with optimal pH. Thermal stability was measured by assessing the residual enzyme activity after incubation of the enzyme at 90°C for 5, 10, 15, 20, and 30 min. The remaining activity was measured using the standard assay. The K_m, V_{max}, and k_{cat} values for the r-Bg1 were calculated from Lineweaver–Burk plots derived from the activity assay at 55°C in phosphate citric acid buffer (pH 6.5) with 1–15 mg ml^{-1} lichenan as the substrate.

Table 1 Codon optimization process

Processing method	Signal peptide (α-factor)			Glucanase gene		
	Codons changed	Nucleotides changed	G+C content (%)	Codons changed	Nucleotides changed	G+C content (%)
Optimization of codons[a]	42	46	49.1	98	116	50.9
Adjustment of codons[b]	5	5	49.5	11	13	50.1
Adjustment of G+C[c]	6	6	48.3	15	19	48.8
Final result[d]	52	56	48.3	106	133	48.8

[a] The change of codons to an appropriate ratio according to the codon usage frequency of highly expressed genes in *P. pastoris*
[b] Further adjust the codons in accordance with codons used in *P. pastoris* genes that are most highly expressed
[c] Adjust the G+C content to an appropriate level
[d] The final change in the codon/base number is not a simple equivalent to the sum of the above three processing method because some bases changed back to the original ones in the later stages of design.

Results

Design and synthesis of the *bgl-m* gene

The process by which codons were optimized for usage in *P. pastoris* is shown in Table 1. The *α-factor-m* was synthesized according to the amino acid sequence of α-factor prepro-leader. The codons in *α-factor-m* encoding the 52 amino acids of the α-factor prepro-leader were optimized, resulting in 56 nucleotide changes, and the G+C content was increased from 41.2 to 48.3% (Fig. 3a). The synthesized 758-bp *bgl-m* (GenBank accession no. EU169241) showed 82.3% identity with the wild-type *bgl* after averaging the distribution of both the G+C content and the optimized codons and eliminating the AT-rich region (Fig. 3b). Compared with the original *bgl* gene, the codons encoding 106 residues in *bgl-m* were optimized, in which a total of 133 nucleotides were changed, and the G+C content was reduced from 53.4 to 48.8%, closer to the G+C content of other high-expression genes in *P. pastoris*. Moreover, to avoid premature termination, AT-rich stretches were eliminated by changing AT-rich codons to AT-deficient ones.

The putative secondary structures of the mRNAs *α-factor+bgl*, *α-factor+bgl-m*, and *α-factor-m+bgl-m* were analyzed and compared. The ΔG of mRNA folding were −399.7 kcal mol^{-1} for *α-factor+bgl*, −417.7 kcal mol^{-1} for *α-factor+bgl-m*, and −427.5 kcal mol^{-1} for *α-factor-m+bgl-m*. By comparison with *α-factor+bgl* and *α-factor+bgl-m*, no obvious complex secondary structures existed in *α-factor-*

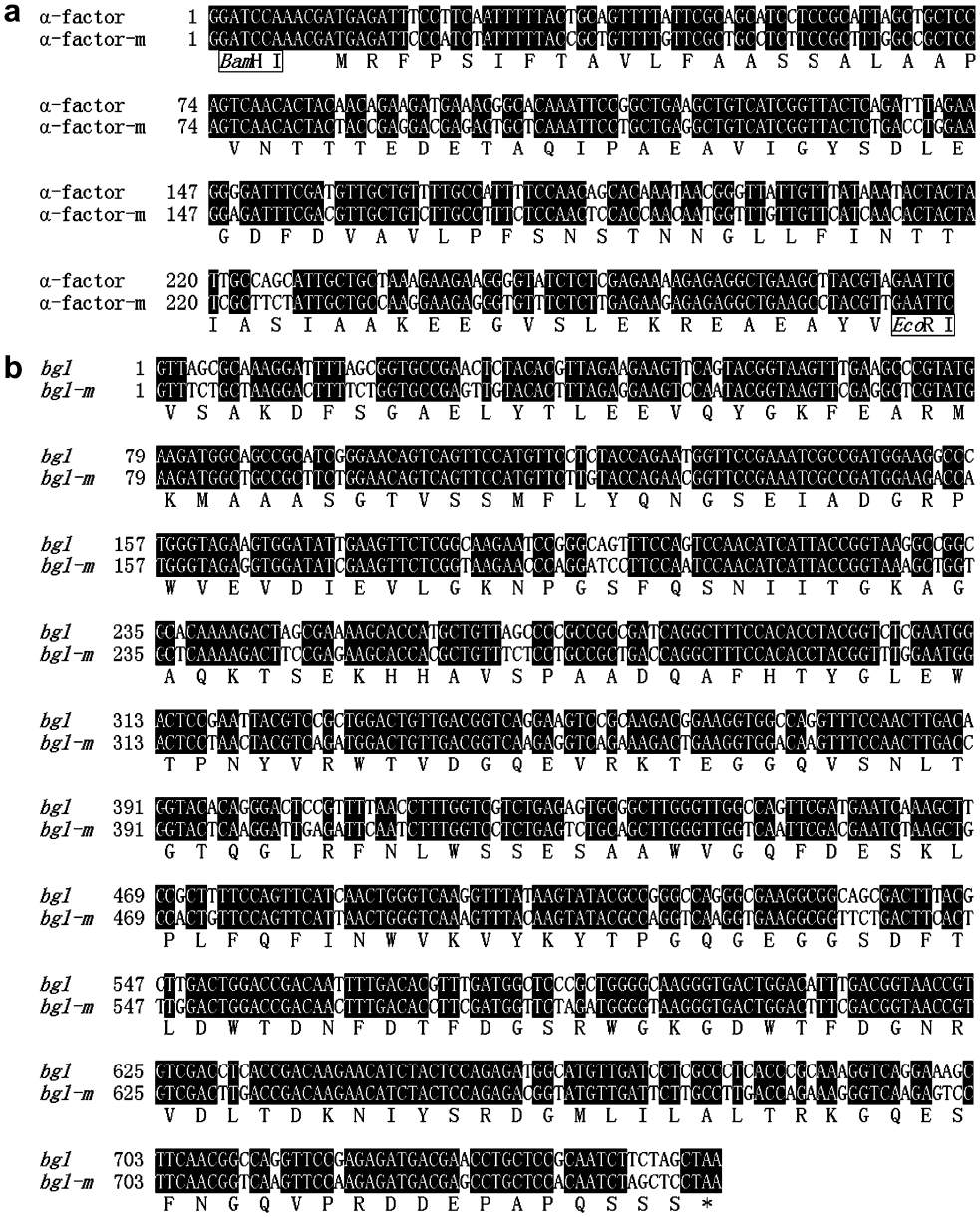

Fig. 3 Alignment of nucleotide sequences between the native and optimized gene using the box-shade program (www.ch.embnet.org/software/BOX_form.html). **a** The wild-type *α-factor* and optimized *α-factor-m*. **b** The native 1,3–1,4-β-D-glucanase (*bgl*) and optimized 1,3–1,4-β-D-glucanase (*bgl-m*). The *asterisk* indicates the stop codon

m+bgl-m. Simultaneously, the mRNA secondary structure near the AUG start codon was also adjusted by substituting synonymous codons at the relevant codon sites, and the modified AUG region was relatively free of secondary structure to ensure efficient translation of the mRNA as predicated by RNA-folding software analysis (Fig. 4).

Expression of *bgl-m* containing the original or modified signal peptide

Approximately 300 transformants of *P. pastoris* containing the constructs pPIC9-*bgl-m* and pPIC9m-*bgl-m* were replica plated onto MD and MM, respectively. About 130 transformants of each construct were positive strains having glucanase activity from 67 to 432 U ml^{-1}. Among these positive transformants, five transformants with the highest enzyme activity were selected for each construct for further analysis by the shaker flask test. After 4 days of induction, the average activity of the five pPIC9m-*bgl-m* constructs with the modified signal peptide was 1,337±72 U ml^{-1}, which was approximately twofold higher than that of the original signal peptide construct (721±47 U ml^{-1}). The SDS-PAGE analysis yielded similar results. Glucanase yield and induction days for the three constructs in shaker flasks are presented in Table 2.

High-cell-density fermentation

Within 24–26 h after inoculation, the cells had consumed the glycerol carbon source and reached about 48 g l^{-1} cellular dry weight, corresponding to a value at optical density at 600 nm of 140–150. After batch and fed-batch phases, the induction phase was initiated by feeding methanol and/or glycerol. In strategy A, methanol was the sole carbon source and inducer. After 6 days induction, the cellular dry weight reached 79.8 g l^{-1} (Fig. 5a), and the glucanase activity of the culture medium was 11,013±411 U ml^{-1} (Fig. 5b). In strategy B, 5% glycerol was supplemented. The cellular dry weight reached 110 g l^{-1}, and the glucanase activity was 13,104± 510 U ml^{-1}. In strategy C, the glucanase activity increased from 6,859 to 10,084 U ml^{-1} at 84 h induction, after the mixed feed was changed to methanol alone at 72 h. The cellular dry weight was 96 g l^{-1}, and the glucanase activity of the culture medium was 15,249±441 U ml^{-1} after 6 days (Fig. 5b). The maximal secreted concentration of r-Bgl was about 3 g l^{-1}, accounting for 90% of the total protein in the medium (Fig. 6a).

Purification, deglycosylation, and SDS-PAGE analysis of r-Bgl

The r-Bgl was purified to electrophoretic homogeneity by ammonium sulfate precipitation and anion-exchange chro-

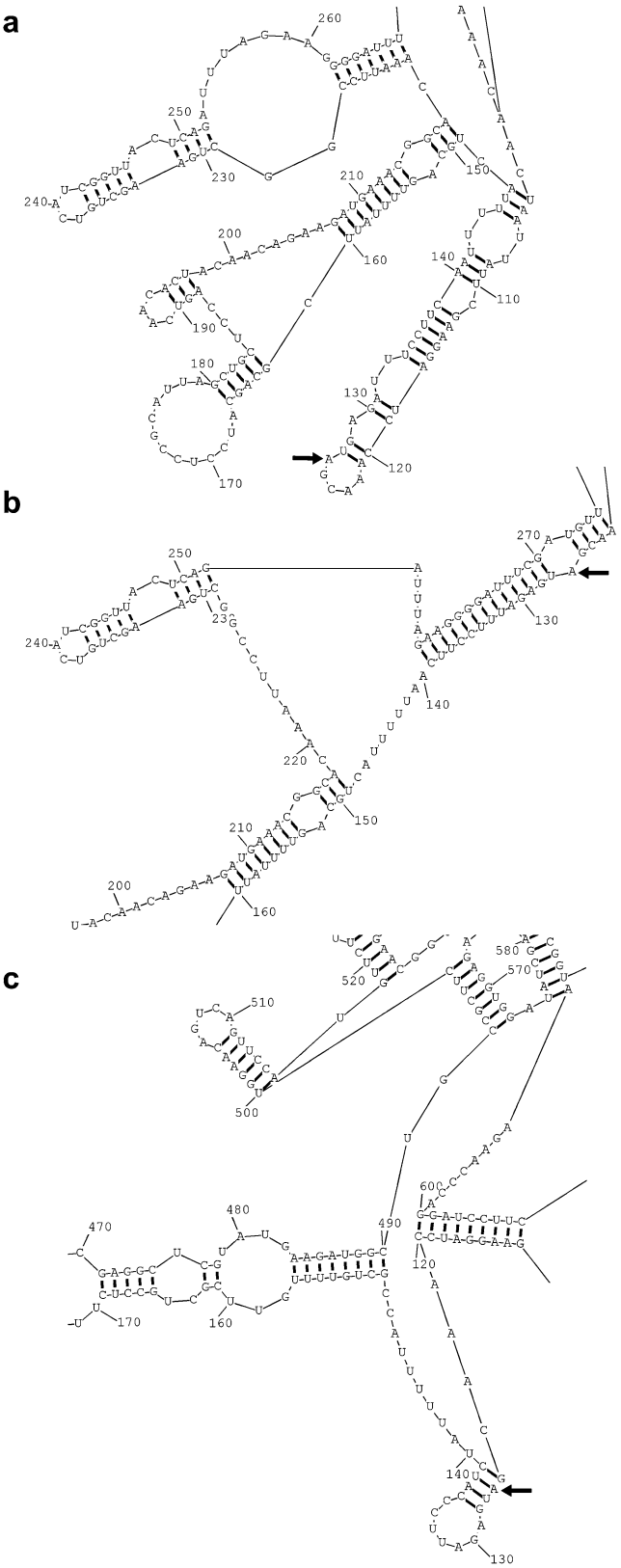

Fig. 4 Potential mRNA secondary structures around the AUG (*arrow*) start codon of the genes α-*factor+bgl* (**a**), α-*factor+bgl-m* (**b**), and α-*factor-m+bgl-m* (**c**), as predicted by RNA Structure 4.4. To open the secondary structure around AUG, **c** unties fewer hydrogen bonds and thus costs less energy by comparison with **a** and **b**

Table 2 Comparison of three constructs expressed at the shaker flask level

Construct	Description	Induction time (days)	Glucanase yield (mg l^{-1})	Reference
pPICZ-*TFGlu*	*α-factor+bgl*	15	110	Wen et al. 2005
pPIC9-*bgl-m*	*α-factor+bgl-m*	4	139±8.7	This study
pPIC9m-*bgl-m*	*α-factor-m+bgl-m*	4	258±12.6	This study

matography (see "Materials and methods"). The purified enzyme was visualized as two bands of ~33 and ~35 kDa on SDS-PAGE (Fig. 6b), respectively. Endo H treatment yielded a single band of 31 kDa, suggesting that the 33 and 35 kDa bands contained different degrees of glycosylation.

Characterization and kinetic studies of r-Bgl

The enzyme characteristics of r-Bg1 were determined using lichenan as substrate (the analyses are not shown). The r-Bgl activity was high over a pH range of 5.0–8.0, with an optimum pH of 6.5. The optimal temperature was about 55°C. The enzyme was stable at high temperatures, as greater than 78% of enzyme activity was retained after incubation at 90°C for 30 min. Kinetic parameters were determined for the overall hydrolytic reaction. The reactions were carried out at the optimal pH 6.5 at 55°C. K_m, V_{max}, and k_{cat} values for r-Bgl were 2.55 mg ml^{-1}, 4,776±138 μmol min^{-1} mg^{-1}, and 2,218±116 s^{-1}, respectively.

Discussion

P. pastoris has been developed as an excellent host for heterologous protein expression. It can express proteins at high levels intracellularly and extracellularly. The codon optimization technique has been widely used to increase the expression of foreign proteins in *P. pastoris* (Sue et al. 2005; Teng et al. 2007; Xiong et al. 2006). Generally, this is accomplished by replacing all codons with preferred codons, eliminating AT-rich stretches, and adjusting of the G+C content to match that of other highly expressed genes in *P. pastoris*. Based on these ideas, the expression of the

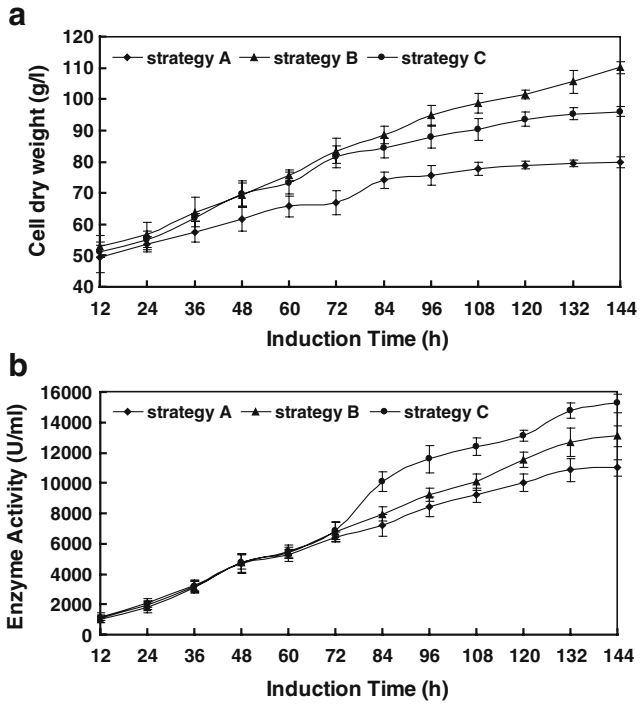

Fig. 5 Fermentation process for *P. pastoris* carrying *α-factor-m+bgl-m* construct using three different fermentation strategies: *A*, 100% methanol feed for 6 days; *B*, 5% glycerol+95% methanol-mixed feed for 6 days; and *C*, 5% glycerol+95% methanol-mixed feed for the first 3 days and 100% methanol feed for the subsequent 3 days. **a** The induction time vs cellular dry weight. **b** Glucanase activity vs induction time. The values in both panels are given as the mean±SD of three replicates

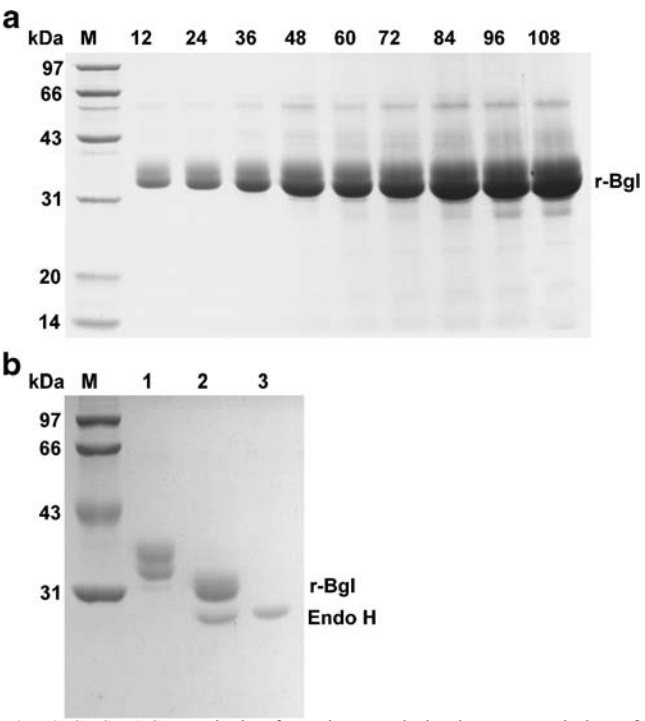

Fig. 6 SDS-PAGE analysis of r-Bgl. **a** Postinduction accumulation of r-Bgl. *M* Protein molecular weight standards. The lane headings *12*, *24*, *36*, *48*, *60*, *72*, *84*, *96*, and *108* indicate the total induction time in hours. **b** Analysis of purified and *N*-deglycosylated r-Bgl by Endo H. *M* Protein molecular weight standard. *Lane 1*, purified r-Bgl; *lane 2*, the N-deglycosylated r-Bgl and Endo H (29 kDa); *lane 3*, Endo H

β-1,3-1,4-glucanase gene from *B. licheniformis* was improved tenfold in *P. pastoris* (Teng et al. 2007), but the protein concentration was only 0.25 g l^{-1} in the medium after high-density fermentation. In our present study, the codons were optimized using a novel strategy. First, we optimized codons by considering the signal peptide and the structure gene as a whole. Second, we optimized codons to an appropriate ratio according to the codon usage frequency of highly expressed genes in *P. pastoris* (Zhao et al. 2000), and we further adjusted them according to the more highly expressed genes. Third, we analyzed the prospective mRNA, including potential mRNA secondary structure(s), the ΔG of mRNA folding, and the sequence around the AUG start codon, and we then made nucleotide changes, as necessary, to minimize potential secondary structure.

For an mRNA that encodes a signal peptide, the overall translation efficiency of the mRNA will be affected by codons and a potential secondary structure within the signal peptide sequence. Therefore, to improve the expression efficiency of a target gene by codon optimization, codon usage in the signal peptide also must be considered. The signal peptide of the pPIC9 expression vector, α-factor prepro-leader, encoding ~90 amino acids, was derived from *S. cerevisiae*. Although codon usage is highly similar between *P. pastoris* and *S. cerevisiae*, the codon choice for an individual amino acid is very different. For example, for glutamic acid, *P. pastoris* prefers the codon GAG, whereas *S. cerevisiae* prefers GAA. The α-factor prepro-leader contains nine glutamic acids, accounting for 10% of the total residues in the signal peptide. Therefore, expression in *P. pastoris* could be further enhanced by codon optimization of the α-factor prepro-leader. The codons of the whole mRNA were adjusted to an appropriate proportion according to the codon usage percentage of higher expressed genes in *P. pastoris*, including *AOX1*, *GAP*, and *actin*. For example, in the presence of methanol, alcohol oxidase produced from *AOX1* comprises up to approximately 30% of the total soluble protein in wild-type *P. pastoris* (Sreekrishna et al. 1993); thus, there are no rare codons in *AOX1*, suggesting that any foreign gene having a similar codon composition could potentially express high levels of heterologous protein. In addition, we also eliminated AT-rich stretches and adjusted the G+C content during the optimization process. Using this codon optimization strategy to express heterologous proteins in *P. pastoris*, we have achieved a threefold increase in the expression of phytase from *E. coli* (Luo et al. 2004), as well as high-level expression of phytase from *Citrobacter braakii* (Huang et al. 2006) and thermoacidophilic alpha-amylase from an environmental sample (Yuan et al. 2005). We have now obtained similar results with the expression of the glucanase gene from *F. succinogenes*. In summary, our current work demonstrates that codon optimization according to our novel strategy is an effective method for enhancing expression of glucanase in *P. pastoris*.

In this study, we also optimized the conditions of the induction phase for high-cell-density fermentation. MUT^s strains of *P. pastoris* grow poorly on methanol, and heterologous protein expression is inhibited (Sreekrishna et al. 1997). To solve this problem, a methanol/glycerol-mixed feed method was used as described by Zhang et al. (2003). The addition of glycerol can promote cell growth but partially represses the *AOX1* promoter. Therefore, we adopted a compromise induction strategy involving two different feedings at two stages. For the first 3 days, 5% glycerol+95% methanol-mixed feed was added at 3 ml h^{-1} l^{-1}, and 100% methanol was added at 3 ml h^{-1} l^{-1} during the following 3 days. Compared with methanol feeding and glycerol/methanol-mixed feeding alone, this strategy achieved the highest glucanase activity in the culture medium. A signif icant increase in activity was detected when the glycerol/methanol mixed induction changed to methanol induction (Fig. 5b). This may be attributable to the complete consumption of glycerol with subsequent release of *AOX1* promoter inhibition.

The most striking success in this study was the production of secreted glucanase up to 3 g l^{-1} (15,249±441 U ml^{-1}) after high-cell-density fermentation of *P. pastoris*, which was significantly higher than the truncated 1,3-1,4-β-D-glucanase from *F. succinogenes* (0.11 g l^{-1}, 1,940 U ml^{-1}; Wen et al. 2005) and β-1,3-1,4-glucanase gene from *B. licheniformis* (0.25 g l^{-1}, 256.7 U ml^{-1}) in *P. pastoris* (Teng et al. 2007). The specific activity, 10,800 U mg^{-1} in the report of Wen et al. (2005) was significantly higher than that (5,180±133 U/mg) in our study; however, the discrepancy of specific activity did not influence the high expression of the recombinant glucanase in this study. To our knowledge, this level of 1,3-1,4-β-D-glucanase production is the highest ever reported. The recombinant glucanase accounted for as much as 90% of the total secreted protein (Fig. 6a), which could simplify late processing steps and reduce the cost of large-scale production. These advantages show great potential for industrial production of the truncated 1,3-1,4-β-D-glucanase and perhaps other proteins/enzymes in *P. pastoris*.

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