Research Article

Production Optimization of an Active β-Galactosidase of Bifidobacterium animalis in Heterologous Expression Systems

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β-Galactosidase (E.C.3.2.1.23) catalyzes the hydrolysis of lactose into glucose and galactose and the synthesis of galacto-oligosaccharides as well. The β-galactosidases from bacteria, especially lactobacilli, and yeast have neutral pH and are much more likely to be developed as food additives. However, the challenges of cumbersome purification, product toxicity, and low yield in protein production have limited the commercialization of many excellent candidates. In this study, we identified a β-galactosidase gene (bg42-106) in Bifidobacterium animalis ACCCC05790 and expressed the gene product in Escherichia coli BL21(DE3) and Pichia pastoris GS115, respectively. The recombinant bG42-106 purified from E. coli cells was found to be optimally active at pH 6.0 and 60°C and had excellent stability over a wide pH range (5.0–8.0) and at high temperature (60°C). The specific activity of bG42-106 reached up to 2351 U/mg under optimal conditions. The galacto-oligosaccharide yield was 24.45 g/L after incubation with bG42-106 at 60°C for 2 h. When recombinant bG42-106 was expressed in Pichia pastoris GS115, it was found in the culture medium but only at a concentration of 1.73 U/ml. To increase its production, three strategies were employed, including codon optimization, disulfide formation, and fusion with a Cherry tag, with Cherry-tag fusion being most effective. The culture medium of P. pastoris that expressed Cherry-tagged bG42-106 contained 24.4 U/mL of β-galactosidase activity, which is 14-fold greater than that produced by culture of P. pastoris harboring wild-type bG42-106.

1. Introduction

The β-galactosidase (EC 3.2.1.23) is an enzyme that breaks the β-1,4-d-galactosidic linkages of lactose to produce glucose and galactose. This enzymatic action makes it a food supplement to make lactose-free milk special for the lactose-intolerant people, which comprise more than 50% of the world’s population [1]. Moreover, the enzyme has transglycosylation activity to synthesize galacto-oligosaccharides, the prebiotics to stimulate the growth and/or activity of beneficial bacteria in the colon [2]. Besides the application in food industry, β-galactosidase is attracting much attention owing to its ability to synthesize β-galactosyl derivatives that are involved in many biological processes [3]. Thus β-galactosidase represents a high-value biocatalyst, and its mass production is of importance for the commercial purpose.

Commercial β-galactosidases are mainly from microorganisms, especially from yeast and bacteria. Of them, Kluyveromyces lactis represents the major microbial source due to the high lactose hydrolysis activity of its β-galactosidase in milk. However, K. lactis β-galactosidase has poor thermal and pH stabilities [4, 5] and is not a secreted protein, which means that its large-scale application is hampered by the high purification cost. Therefore, identification of β-galactosidases that have good stability and excellent catalytic properties is of practical significance. β-Galactosidases from many different species, including Aspergillus niger [6], Thermus sp. [7], Lactobacillus reuteri [8], and Bifidobacteria infantis [9], have been found. The excellent stability under a wide range of conditions and substantial catalytic activity make Bifidobacteria β-galactosidase a compelling candidate for application in food industry. The only bottleneck is its
intracellular enzyme in nature, and its removal would present a further stage of cells disruption and purification, which are a costly process. Production of Bifidobacteria β-galactosidase in a recombinant expression system, e.g., Escherichia coli or Pichia pastoris, may overcome these limitations.

For the study reported herein, bg42-106 from Bifidobacterium animalis ACCCC05790, which encodes a β-galactosidase with advantageous properties, was cloned and expressed in E. coli and P. pastoris. To improve gene expression and recombinant protein secretion in P. pastoris, several strategies were employed, including codon optimization and coexpression with a protein disulfide isomerase and fusion with a Cherry tag. Among these, codon optimization is widely and successfully used to increase the expression levels of foreign proteins in P. pastoris [10]. Protein disulfide isomerase is an endoplasmic reticulum-associated molecular chaperone involved in the rearrangement of incorrect disulfide pairings via its isomerase activity and also facilitates folding, assembly, and posttranslational modifications of eukaryotic proteins in general [11]. Several studies have shown that coexpression of pdi in P. pastoris improves heterologous protein expression [12, 13]. The commercial Cherry tag is a portion of the cytochrome heme-binding domain that can increase the solubility of the tagged protein. Our results showed that all these strategies increased the yield of B. animalis β-galactosidase, with Cherry-tag fusion being most effective at protein secretion level.

2. Materials and Methods

2.1. Strains and Growth Conditions. B. animalis ACCCC05790 from the Agricultural Culture Collection of China (Beijing, China) was grown anaerobically at 37°C in deMan, Rogosa and Sharpe (MRS) medium (2% glucose, 1% peptone, 1% meat extract, 0.5% yeast extract, 0.5% sodium acetate, 0.2% K₂HPO₄, 0.2% diaminobutyric acid, 0.1% (v/v) Tween 80, 0.02% MgSO₄·7H₂O, and 0.005% MnSO₄·H₂O, pH 6.2). E. coli strains TOP10 (TransGen, Beijing, China) and BL21(DE3) (Novagen, Darmstadt, Germany) were cultured in Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) containing 100 μg/mL ampicillin. Saccharomyces cerevisiae and P. pastoris GS115 were cultivated at 30°C in yeast extract/peptone dextrose medium (2% peptone, 2% glucose, and 1% yeast extract). Buffered glycerol complex medium, buffered methanol complex medium, regeneration dextrose medium, minimal dextrose medium, and minimal methanol medium were prepared according to manuals of Pichia expression kits (Invitrogen, Carlbad, CA).

2.2. Plasmids, Enzymes, and Chemicals. pEASY-T3 (Transgen) was used for gene cloning, pET-30a(+) (Novagen), pPICZA (Invitrogen), and pPIC9 (Invitrogen) served as expression vectors. Restriction and other enzymes used for DNA manipulations were obtained from TaKaRa (Dalian, China). Chemicals and reagents for high-performance liquid chromatography (HPLC) were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. o-Nitrophenyl-β-d-galactopyranoside (oNPG) was purchased from AppliChem (Gatersleben, Germany).

2.3. Cloning of the β-Galactosidase Gene bg42-106. Standard procedures for DNA extraction, plasmid isolation, restriction-enzyme digestion, and ligation were performed as described [26]. Bacterial β-galactosidase sequences were retrieved from GenBank by an Entrez search (http://www.ncbi.nlm.nih.gov/Entrez/); consensus regions in an alignment of these sequences were identified by ClustalW [27] and used to design the degenerate primers bg42F and bg42R (Table 1).

The core region of bg42-106 was obtained via touchdown PCR with the B. animalis genomic DNA (50 ng) as the template and primers bg42F and bg42R (final concentrations, 5 μM each primer). The PCR program was 94°C for 3 min; 10 cycles of 94°C for 30 s, 55°C for 30 s with a decrease of 1°C per cycle, and 72°C for 30 s; 30 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s. The gene fragment was purified using the reagents of a gel extraction kit (Tiangen, Beijing, China) and ligated into pEASY-T3 vectors, which were individually transformed into E. coli TOP10. Positive transformants were screened on LB agar plates containing 0.8 mg/mL X-gal, 3 mM isopropyl-β-d-thiogalactopyranoside (IPTG), and 100 μg/mL ampicillin for 16 h at 37°C. White colonies containing the bg42-106 fragment were confirmed by DNA sequencing. Based on the known sequence, the up- and down-stream flanking regions of bg42-106 were obtained with genome-walking thermal asymmetric interlaced (TAIL)-PCR [28] with specific primers Dsp1, Dsp2, Dsp3, Usp1, Usp2, and Usp3 (Table 1).

2.4. Sequence Analysis. Verification of the nucleotide and deduced amino acid sequences, an open-reading frame search, multiple sequence alignment, and sequence assembly were performed using Vector NTI 10.3 software. The sequences of the DNA fragments obtained by touchdown PCR were compared with those of known β-galactosidases in GenBank by BLASTx. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) was used to determine if bg42-106 contained a signal sequence.

2.5. Expression and Purification of Recombinant bg42-106 in E. coli. Primers bg42-106f and bg42-106r (Table 1) harboring the restriction sites EcoRV and NotI were used to amplify the full-length bg42-106 from B. animalis genomic DNA. The PCR product was purified, enzyme digested, and inserted into pET-30a(+) to construct the recombinant plasmid pET30-bg42-106, which was further transformed into chemically competent E. coli BL21(DE3) cells by heat-shock at 42°C. Positive E. coli cells were cultured at 37°C in LB medium, 50 μg/mL kanamycin until the OD₆₀₀ nm of the culture reached 0.6. The cells were then induced with 0.4 mM IPTG at 28°C for an additional 4 h. Recombinant bg42-106 carried a C-terminal (His)₉ tag.

The cells were harvested by centrifugation (12,000 × g, 5 min, 4°C), washed with 50 mM sodium phosphate (pH 6.5), and disrupted by ultrasonication. Cell debris was removed
by centrifugation (12,000 × g, 10 min, 4°C), and the crude enzyme extract was loaded onto a Ni²⁺-NTA affinity column (Qiagen, Hilden, Germany) that had been equilibrated with start buffer (20 mM sodium phosphate pH 6.5, 500 mM NaCl, and 5 mM imidazole). The protein was eluted at 1 mL/min with elution buffer (20 mM sodium phosphate pH 6.5, 500 mM NaCl, and 200 mM imidazole). Fractions containing β-galactosidase activity were pooled, desalted, and concentrated.

Protein concentration was determined using Bradford protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The contents of total cellular protein and purified bG42-106 were examined by SDS-PAGE (12%, w/v). To estimate the native molecular mass, purified bG42-106 was detected in an 8% (w/v) nondenaturing PAGE. The content of total cellular protein was measured at 60°C by incubating the gel in an X-gal (4 mg/mL) solution.

### 2.6. β-Galactosidase Activity Assay

The β-galactosidase activity was monitored with oNPG hydrolysis at OD₄₂₀nm. Reactions contained 200 μL of enzyme solution and 800 μL of substrate (0.25% (w/v) oNPG in 100 mM Na₂HPO₄-citric acid, pH 6.0). Each reaction was incubated at 60°C for 15 min and terminated by addition of 1 mL of 10% (w/v) trichloroacetic acid and 2 mL of 1 M Na₂CO₃. One unit of β-galactosidase activity was defined as the amount of enzyme that liberated 1 μmol of o-nitrophenol per min. The specific activity of the enzyme was expressed as units/mg protein.

### 2.7. Characterization of Recombinant bG42-106 from E. coli

To determine the pH optimum of bG42-106, its activity was measured at 60°C between pH 3.0 and 7.0 in 100 mM Na₂HPO₄-citric acid, pH 7.0 and 9.0 in 100 mM Tris-HCl, and pH 9.0 and 10.0 in 100 mM Na₂CO₃-NaHCO₃. To measure its pH stability, the enzyme was first incubated in the aforementioned buffers at 37°C for 60 min and then assayed under standard conditions (pH 6.0, 60°C, 15 min). To determine the optimal temperature for enzyme activity, reactions were measured between 30 and 75°C at optimal pH for 15 min. The effect of temperature on stability was measured by incubating bG42-106 at 60°C or 65°C. Samples were removed at various times and assayed under standard conditions. To determine the half-life of bG42-106, the enzyme was incubated at 60°C, and activity was determined periodically for 72 h under standard conditions.

The effects of various reagents on enzyme activity were examined by assaying the enzyme in the presence of 1 or 10 mM of Na₂, K⁺, Ca²⁺, Cu²⁺, Mn²⁺, Co²⁺, Cd²⁺, Fe²⁺, Ni²⁺, Mg²⁺, Zn²⁺, Pb²⁺, Ag⁺, Triton X-100, Na₃EDTA, SDS, or CTAB in 100 mM Na₂HPO₄-citric acid, pH 6.0. Enzyme activity measured in the absence of any reagent was defined as 100%. Kinetic parameters (Kₘ, Vₘₐₓ and kₗ) were obtained from the Michaelis-Menten equation using GraphPad Prism software. The substrate (oNPG) concentration ranged from 1.38 to 25 mM. All experiments were carried out at 60°C and pH 6.0 in triplicate.

### 2.8. Lactose Hydrolysis and Galacto-Oligosaccharide Synthesis by Recombinant bG42-106 from E. coli

To assess the extent of lactose hydrolysis by bG42-106, reactions were performed at 50°C or 60°C in 100 mM Na₃HPO₄-citric acid (pH 6.0) containing 200 g/L lactose and purified bG42-106 for various times and terminated by heating for 10 min at 100°C. Formation of galacto-oligosaccharides and other saccharides was quantified by HPLC through a 6.5 × 300 mm Sugar-Pak I column (Waters, Milford, MA). This column can separate
oligosaccharide molecules of the same size but different linkage type. Samples were eluted in 50 mg/mL CaNaEDTA at 500 μL/min and 85°C. The yield of galacto-oligosaccharides (g/L) was calculated as $[\text{GOS}] = [\text{Lac}_\text{initial}] - [\text{Lac}_\text{final}] - [\text{Glu}_\text{final}] - [\text{Gal}_\text{final}]$ [29], in which GOS is the yield of galacto-oligosaccharides, Lacinitial is the initial amount of lactose, Lacfinal is the final amount of lactose, Glufinal is the yield of glucose, and Galfinal is the final yield of galactose.

2.9. Expression of bg42-106 in *P. pastoris*. Restriction enzyme-digested bg42-106 as described above was ligated into pPIC9 to form the recombinant plasmid pPIC9-bG42-106, which was then transformed into *E. coli* TOP10 to maintain the plasmid. The construct pPIC9-bG42-106 was linearized by BglII and then transformed into *P. pastoris* GS115 competent cells by electroporation. Transformed cells were selected by G418 (Invitrogen). Recombinant *P. pastoris* cells were incubated in 25 mL of buffered methanol complex medium at 30°C, followed by 48-h induction with 0.5% methanol. The culture supernatant was assayed for ß-galactosidase activity. ß-galactosidase activity was also measured. The *P. pastoris* strain containing the highest ß-galactosidase was denoted as wild-type GS115/ScPDI-bG42-106.

2.10. Codon Optimization and Expression of Codon-Optimized bg42-106m. To improve bg42-106 expression in *P. pastoris*, low-usage (<15% frequency) codons were replaced with the common ones found in the *P. pastoris* genome [30]. In addition, the G+C content of bg42-106 was adjusted to be similar to those of highly expressed *P. pastoris* genes. The optimized gene, denoted as bg42-106m, was synthesized by AugCt (Beijing, China). bg42-106m was ligated into pPIC9 and expressed in *P. pastoris* as described above. Intra- and extra-cellular ß-galactosidase activities were assayed. The yeast strain containing bg42-106m was denoted as GS115/bG42-106m.

2.11. Coexpression of scpdi in *P. pastoris* GS115/bG42-106. The gene coding for the protein disulfide isomerase of *Sacharomyces cerevisiae* (scpdi) was PCR amplified with FastPfu DNA polymerase (Transgen) using *S. cerevisiae* genomic DNA as the template and primers ScPDI-F and ScPDI-R (Table 1). The PCR product was purified and ligated into pPICZα to form pPICZα-scpdi, which was transformed into *E. coli* TOP10 competent cells for sequencing. Recombinant pPICZα-scpdi was electroporated into *P. pastoris* GS115/bG42-106. The transformants were screened on yeast extract/peptone dextrose agar plates that contained 100 μg/mL Zeocin. The *P. pastoris* strain that contained both scpdi and bg42-106 was denoted as GS115/ScPDI-bG42-106. After coexpression of both proteins as described above for bgF42-106 expression, bG42-106 activity was measured as described above.

2.12. Fusion of bg42-106 with a Cherry Tag. Primers CherryF and CherryR (Table 1) were used to clone the Cherry-tag coding sequence in the Cherry Express vector pSCherry1 (Delphi Genetics SA, Gosselies, Belgium). A 15-bp extension homologous to the sequence flanking the multiple cloning site in pPIC9-bG42-106 was added to both ends of the Cherry-tag sequence by PCR. The primer pairs P1 and P2 and P3 and P4 (Table 1) were used to individually amplify pPIC9/bG42-106. The 12- to 19-bp overlaps at the ends of the vector were used as the homologous recombinant arms. Using the CloneEZ recombinant cloning kit (Genscript, Nanjing, China), the Cherry tag and the vectors were fused via the Cherry-tag extensions and the homologous sequences in the vector to yield pPIC9-Cherry-bG42-106. In addition, a KEX2 site (Glu-Lys-Arg * Glu-Ala-Glu-Ala, where * is the cleavage site) coding sequence was inserted between *Cherry* and bg42-106 genes for subsequent removal of the Cherry tag during secretion from *P. pastoris*. The plasmid pPIC9-Cherry-bG42-106 was electroporated into *P. pastoris* GS115 as described above. ß-Galactosidase activity in the culture medium and yeast cells was measured, respectively. The yeast strain containing pPIC9-Cherry-bG42-106 is denoted as GS115/Cherry-bG42-106.

2.13. Nucleotide Sequence Accession Number. The nucleotide sequence for the *B. animalis* ACCC05790 ß-galactosidase gene bg42-106 was deposited in the GenBank database under accession number JX188444.

3. Results

3.1. Cloning and Sequence Analysis of bg42-106. The full-length bg42-106 was obtained from the genomic DNA of *B. animalis* ACCC05790 through touchdown PCR and TAIL-PCR. The open-reading frame contained 2088 bp that encoded a polypeptide of 695 amino acids and a stop codon. No signal peptide sequence was identified. The calculated molecular mass of bg42-106 was 78 kDa. Sequence comparison indicated that bg42-106 belonged to family 42 of glycosyl hydrolases (GH). The deduced amino acid sequence was 98% and 33% identical to that of a putative ß-galactosidase from *B. animalis* subsp. *lactis* HN019 (ZP_02963414) and a characterized ß-galactosidase from *Bifidobacterium adolescentis* DSM20083 [16].

3.2. Expression and Purification of Recombinant bg42-106 from *E. coli*. The recombinant plasmid, pET30-bG42-106, was constructed by cloning bg42-106 into vector pET-30(+) and then transferred into *E. coli*. After induction with IPTG for 4 h, cells were sonicated, and the intracellular ß-galactosidase activity was determined to be ~50 U/mL. The crude enzyme extract was purified to apparent homogeneity with Ni2+-NTA affinity chromatography (Figure 1(a)). Purified bG42-106 had an apparent molecular mass of ~80 kDa on SDS-PAGE, which was consistent with the theoretical mass of bG42-106 (78 kDa). The native molecular mass of bG42-106 was also analyzed, and the result showed that purified bG42-106 migrated one single band of ~180 kDa on native
3.3. Characterization of Recombinant bG42-106 from E. coli.

The pH and temperature optima for bG42-106 activity were pH 6.0 and 60°C, respectively (Figures 2(a) and 2(b)). The enzyme retained almost all of the activity after incubation for 1 h between pH 5.0 and 8.0 at 37°C, but lost activity sharply below pH 4.0 or above pH 9.0 (Figure 2(c)). bG42-106 was thermostable at 60°C, retaining approximately 100% of the activity after 1-h incubation at 60°C and pH 6.0 (Figure 2(d)). The half-life at 60°C was determined to be 53.7 h. After incubation at 65°C for 30 min, the enzyme only retained about 50% activity.

The effects of various metal ions and reagents on bG42-106 activity were assessed (Table 2). Cu²⁺, Cd²⁺, Fe²⁺, and SDS (each at 10 mM) inhibited the enzymatic activity by 44% to 65%, but only SDS at lower concentration (1 mM) affected the activity. Conversely, 1 mM Pb²⁺ and Ag⁺ completely inhibited the activity. Other metal ions (K⁺, Na⁺, Co²⁺, Zn²⁺, and Ni²⁺) and reagents (CTAB and Na₂EDTA) had no substantial effects on the activity.

With the use of oNPG as the substrate for a steady-state kinetic study, the Vₐₚₓ, Kₘ, and kₐₜ/Kₘ values were found to be 720 μmol/min/mg, 5.14 mM and 186.77 s⁻¹ mM⁻¹, respectively. The specific activity of purified bG42-106 was 2351 U/mg as determined under standard assay conditions.

3.4. Lactose Hydrolysis and Galacto-Oligosaccharide Synthesis of Recombinant bG42-106 from E. coli.

The hydrolysis ability of bG42-106 to degrade lactose into galactose and glucose was assessed at two temperatures (50°C or 60°C) and different enzyme concentrations (2 to 40 U/mL) for 24 h. When the initial concentration of lactose was >200 g/L, hydrolysis was nearly complete after 24 h in the presence of 40 U/mL bG42-106 (Figure 3(a)). The rate of lactose hydrolysis was also monitored at different enzyme concentrations for up to 24 h.

Table 2: Effect of metal ions and chemical reagents on the β-galactosidase activity of purified recombinant bG42-106.

| Metal ions and reagents | 1 mM  | 10 mM |
|-------------------------|-------|-------|
| Control                 | 100.0 | 100.0 |
| Na⁺                     | 97.9 ± 1.0 | 95.6 ± 2.0 |
| K⁺                      | 98.6 ± 2.0 | 94.4 ± 1.0 |
| Ca²⁺                    | 97.6 ± 3.0 | 97.5 ± 1.0 |
| Cu²⁺                    | 90.6 ± 1.0 | 44.3 ± 4.0 |
| Mn²⁺                    | 100.9 ± 3.0 | 93.2 ± 2.0 |
| Co²⁺                    | 99.4 ± 2.0 | 96.0 ± 1.0 |
| Cd²⁺                    | 96.7 ± 2.0 | 59.3 ± 2.0 |
| Fe²⁺                    | 96.9 ± 1.3 | 65.3 ± 3.0 |
| Ni²⁺                    | 102.3 ± 1.0 | 96.2 ± 0.0 |
| Mg²⁺                    | 102.3 ± 2.0 | 87.2 ± 1.0 |
| Zn²⁺                    | 104.2 ± 2.0 | 95.4 ± 2.0 |
| Ag⁺                     | 0      | 0     |
| Pb²⁺                    | 0      | 0     |
| Triton                  | 103.2 ± 4.0 | 94.1 ± 2.0 |
| EDTA                    | 100.3 ± 3.0 | 91.6 ± 2.0 |
| SDS                     | 55.5 ± 2.0 | 39.3 ± 4.0 |
| CTAB                    | 90.8 ± 1.0 | 91.8 ± 2.0 |

*Values represent the means of triplicates relative to the untreated control samples.
Figure 2: Biochemical characterization of recombinant bG42-106 from E. coli. (a) Effects of pH on the enzyme activity. (b) Effects of temperature on the enzyme activity. (c) pH stability of bG42-106. (d) Thermal stability of bG42-106. Closed squares, 100 mM Na$_2$HPO$_4$-citric acid (pH 3.0–7.0); closed triangles, 100 mM Tris-HCl (pH 7.0–9.0); closed circles, 100 M Na$_2$CO$_3$-NaHCO$_3$ (pH 9.0–10.0). The error bars represent the mean ± standard deviation of three replicates.

(Figure 3(b)). The lactose concentration decreased from the initial 200 g/L to ∼150 g/L at 24 h in the presence of 2 or 4 U/mL of bG42-106. At a concentration of 40 U/mL, however, bG42-106 degraded ∼50% of the lactose within 2 h; then, the lactose hydrolysis rate decreased, and nearly ∼90% of the lactose was hydrolyzed at 16 h.

The production of galacto-oligosaccharides in the presence of different amounts of bG42-106 was also assessed at 60°C (Figure 3(b)). In the presence of 2 or 4 U/ml of bG42-106 and 200 g/L lactose, the amount of galacto-oligosaccharides increased up to 15 g/L at 24 h. The maximal amount of galacto-oligosaccharides (24.45 g/L) was achieved with 40 U/ml bG42-106 at 60°C for 2 h. Thereafter, the amount of galacto-oligosaccharides decreased to almost zero at 24 h. When the bG42-106 concentration was 10 U/mL, a hydrolysis and synthesis balance of galacto-oligosaccharides was obtained, in which the amount of galacto-oligosaccharides was ∼10 g/L.

3.5. Effect of Codon Optimization. A total of 451 nucleotides of bg42-106 involving 387 amino acids were replaced according to the optimized codon usage (Supplementary Table 1). The nucleotide sequence identity of wild-type and modified bg42-106 was 77.9%. The G+C content was adjusted from 61.1% to 50.2% to mimic that of the P. pastoris genome. bg42-106 and bg42-106m were expressed in P. pastoris GS115 with methanol induction. The highest enzyme activity found in the culture medium was 1.73 U/mL for bG42-106 and 2.64 U/ml
for bG42-106m (Figure 4), respectively, reflecting a 1.5-fold improvement in the yield.

3.6. Effect of Coexpression of ScPDI and bG42-106. ScPDI and bG42-106 were successfully coexpressed in P. pastoris. After induction as described above, the \( \beta \)-galactosidase activity in the culture medium was 3.15 U/mL, 1.8 folds of that of bG42-106 alone. The intracellular \( \beta \)-galactosidase activity for ScPDI-bG42-106 was 9.23 U/ml, which was less than that of bG42-106 (11.97 U/mL) (Figure 4). ScPDI, therefore, acted as a molecular chaperone that may assist bG42-106 folding and subsequent secretion.

3.7. Effect of Fusing a Cherry Tag. A 326-bp Cherry-tag sequence was PCR amplified from pSCherry1 and fused with bg42-106 in vector pPIC9. The Cherry tag was removed from bg42-106 upon secretion. The extracellular activity of Cherry-bG42-106 was 24.4 U/mL, which was 14-fold greater than that for bG42-106. Moreover, the intracellular activity of Cherry-bG42-106 was less than that of its extracellular activity (Figure 4). The Cherry tag, therefore, significantly improved the yield of bG42-106 in P. pastoris. When bg42-106, bg42-106m, and bg42-106 were coexpressed with ScPDI, their intracellular levels were 3- to 7-fold higher than the extracellular ones (Figure 4).

4. Discussion

In this study we identified a \( \beta \)-galactosidase in B. animalis ACCC05790 and successfully produced it in both prokaryotic and eukaryotic expression systems. Compared with the \( \beta \)-galactosidases of various microbial sources that have been characterized (Table 3), bG42-106 under study represents relatively high \( \beta \)-galactosidase activity. Moreover, it has excellent enzyme properties, such as stability over a wide pH range (5.0–8.0) and at high temperature (60°C), and high yield of galacto-oligosaccharides (24.45 g/L). These features are particularly useful for biotechnological and industrial applications.

*P. pastoris* is well established for the rapid and cost-effective expression of recombinant proteins from discovery.
Table 3: Enzymatic properties of bG42-106 and its microbial counterparts.

| Microbial source | pH<sub>opt</sub> | T<sub>opt</sub> (°C) | Specific activity (U/mg) | References |
|------------------|-----------------|----------------------|-------------------------|------------|
| B. animalis ACCC05790 | 6.0 | 60 | 2351 | This study |
| B. breve B24 | 7.0 | 45 | 8073.60 | [14] |
| Thermus sp. T2 | 6.5 | 80–90 | 900<sup>a</sup> | [7] |
| Paeccilomyces aeruginus | 4.5 | 60 | 820 | [15] |
| B. infantis HL96 | 7.5 | 60 | 569 | [9] |
| B. adolescentis DSM20083 | 6.0 | 50 | 526 | [16] |
| Lactobacillus delbrueckii | 5.0–5.5 | 35–50 | 430 | [17] |
| Kluyveromyces fragilis | 6.5 | — | 250–290 | [18] |
| L. reuteri | 7.0 | 50 | 180–190 | [8, 19] |
| L. plantarum WCFS1 | 7.5 | 55 | 154 | [20] |
| Bacillus stearothermophilus | 7.0 | 70 | 125 | [21] |
| Archaeabacterium sulfolobus | — | — | 116 | [22] |
| Arthrobacter sp. | 7.0 | 18 | 115 | [23] |
| Halorubrum lacusprofundi | 6.5 | 50 | 110.83 | [24] |
| Aspergillus niger van Tiegh | 2.0–4.0 | 65 | 69.3 | [6] |
| Streptococcus mitis | 6.0–6.5 | 30–40 | 2.5<sup>a</sup> | [25] |

<sup>a</sup> These data were converted from nmol for comparison purpose.

Through commercialization, proteins are secreted from P. pastoris under a strictly regulated control system. To improve the yield of bG42-106 in P. pastoris, we employed three strategies including codon optimization, coexpression of a protein disulfide isomerase, and fusion of a Cherry tag. Of them, protein disulfide isomerase has unpredictable effect on heterologous protein secretion from P. pastoris [31]. For example, coexpression of a protein disulfide isomerase increased the secretion of a malaria vaccine candidate (Pfs25) by 2- to 5-fold [32] and increased the yield of a human parathyroid hormone (hPTH) from 127 mg/L to 349 mg/L [33]. Because hPTH contains no cysteine, the protein disulfide isomerase may act as a molecular chaperone instead of a disulfide isomerase. In other studies, however, protein disulfide isomerase had little effect on the expression of targeted protein, and in one instance the production level of an immunoglobulin-binding protein was actually reduced [34]. bG42-106 contains 11 cysteines, some of which might form disulﬁdes and thus require protein disulfide isomerase for formation of disulfide bonds, protein folding, and export from the cell. Thus we coexpressed bg42-106 and a protein disulfide isomerase from S. cerevisiae in P. pastoris and increased the yield of bG42-106 to 1.8 folds. Compared with previous studies, i.e., references [19, 20], the effect of protein disulfide isomerase on bG42-106 is less significant; it may be because this is not the most important limiting factor affecting the secretion of bG42-106 and other strategies are also expected to improve the yield of bG42-106.

Gene fusion, with glutathione S-transferase, maltose-binding protein, as examples, has been used to promote secretion of heterologous proteins in recombinant expression systems. Still, each fusion is assessed empirically, and thus the optimum results for any single case are not necessarily generalizable to other heterologously expressed proteins [35]. The Cherry tag, a red light-producing polypeptide composed of the cytochrome heme-binding domain, has been used to attain high levels of soluble protein in E. coli when fused to the N-terminus of a targeted protein. In some cases, Cherry tags have been used as a cost-efficient tool for protein purification by imparting heat stability [36]. In our study, the Cherry-tag gene was fused with bg42-106 in vector pPIC9. Cherry-bg42-106 was secreted at a higher level, 14-fold greater than that produced by culture of P. pastoris harboring wild-type bg42-106. This favorable secretion level may be a consequence of the relatively high solubility of the Cherry tag itself.

In certain cases the conformation of an expressed protein may preclude its passage through cell membrane, suggesting the need to compare the conformation of the targeted protein with that of proteins secreted naturally. In addition, the expression of exogenous protein in P. pastoris usually depends on the natural host of the target gene; the wild-type unsecreted protein is often difficult to achieve secretion expression in recombinant host. bG42-106 from B. animalis ACCC05790 is an intracellular protein, which may explain its relatively low-level secretion in P. pastoris in the absence of the Cherry tag or another expression “trick” for heterologous proteins.

5. Conclusions

A novel β-galactosidase was identified in B. animalis ACCC05790 and expressed in both prokaryotic and eukaryotic systems. The recombinant protein showed high specific activity and good pH and thermal stability and produced high galacto-oligosaccharide yield. Three strategies including codon optimization, coexpression of a protein disulfide
isomerase, and fusion of a Cherry-tag expression were carried out to promote the yield of bg42-106 from \( P. \) pastoris. Fusion protein approach improved the secretion level of Cherry-tagged bg42-106.

**Data Availability**

No data were used to support this study.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

**Authors’ Contributions**

Xinxin Xu and Xiaohu Fan are equal contributors.

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**Supplementary Materials**

Supplementary Table 1. Comparison of the codon usage of \( bg42-106 \) and the optimized \( bg42-106m \) gene. (Supplementary Materials)

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