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# Expression of *Bacillus subtilis* MA139 β-mannanase in *Pichia pastoris* and the Enzyme Characterization

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**Abstract** The 1014 nucleotides long gene-encoding β-mannanase from *Bacillus subtilis* strain MA139 was cloned using PCR. To obtain high expression levels in *Pichia pastoris*, the β-mannanase gene was optimized according to the codon usage bias of *P. pastoris* and fused downstream of GAP promoter. The reconstituted plasmid pGAP-mann was transformed into *P. pastoris* X-33 strain to constitutively express β-mannanase. When cultured at 28 °C for 3 days protein yields up to 2.7 mg/mL was obtained with the enzyme activity of up to 230 U/mL. In comparison, wild-type gene product yielded 1.9 mg/mL and 170 U/mL, respectively indicating that the protein yield and enzyme activity were significantly improved by codon modification. After purification, the enzyme properties were characterized. The optimal activity was at pH 6.0 and 50 °C. In the pH range of 3.0 to 9.0, β-mannanase showed above 60% of its peak activity. Among the numerous ions tested copper significantly inhibited the enzyme activity. These results suggested that codon-optimized β-mannanase expressed in *P. pastoris* could potentially be used as an additive in the feed for monogastric animals.

**Keywords** β-mannanase · *Bacillus subtilis* · Codon optimization · *Pichia pastoris* · Expression

## Introduction

Mannan, consisting of β-1,4-linked D-mannopyranose residues, is a major hemi-cellulose present in the cell walls and in the seeds of plants [1]. β-mannanase (EC.3.2.1.78) is a key

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enzyme to catalyze the random hydrolysis of β-mannosidic linkages in mannan and heteromannan [2]. β-Mannanase can be produced by various microorganisms, such as *Aeromonas hydrophila* [3], *Bacillus* sp. [4–7], *Enterococcus casseliflavus* [8], *Streptomyces* sp. [9], *Klebsiella oxytoca*, and *Acinetobacter* sp [10]. β-mannanase has extensively been used in paper industry. Recently, β-mannanase is being used as an animal feed supplement to aid in better digestion. Due to the low abundance and high expense involved in the purification of naturally occurring β-mannanase, ectopic expression of this gene in a host system is considered as a pragmatic option.

*Bacillus subtilis* strain MA139 which was demonstrated to be capable of secreting several active components, such as β-mannanase, xylanase, β-glucanase, and carboxymethyl cellulose was successfully isolated in our previous study [11]. All the above-mentioned enzymes are excellent food additives for monogastric animals (our unpublished observation). In this study, we cloned β-mannanase gene from *B. subtilis* MA139 and expressed it in *Pichia pastoris* X-33 rather than in *Escherichia coli* because of its potential application as a food supplement. To obtain high expression levels, this wild-type β-mannanase gene was codon-optimized according to the known *P. pastoris* codon usage bias. After purification, the enzyme properties are characterized.

## Materials and Methods

### Strains, Vectors, and Chemicals

*P. pastoris* X-33 and pGAPzαA were purchased from Invitrogen (San Diego, CA, USA). *Escherichia coli* Top10 was used as the host strain. Yeast extract and Tryptone were purchased from OXOID (Hampshire, England). All other chemicals used in this study were of analytical grade commercially available.

### Cloning of the β-Mannanase Gene from *B. subtilis* MA139

The genomic DNA was extracted from *B. subtilis* MA139 that was cultured overnight at 37°C in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl). The β-mannanase gene was amplified using the primers 5′-GCGCATACTGTGTCGCCT-3′ and 5′-TCACTCAACGATTGGCGTTA-3′ with LA Taq™ DNA polymerase (TaKaRa, Japan) through 35 cycles of denaturation (94°C 30 s), annealing (50°C 30 s), and extension (72°C 1 min). The polymerase chain reaction (PCR) product was purified with a UNIQ-10 DNA purification kit (Sangon, Shanghai, China), ligated into pUCm-T vector (Sangon, Shanghai, China), and then transformed into *E. coli* TOP10 (Tianwei, China) using the calcium chloride method [12]. Positive clones were screened by PCR method using the above-mentioned primers and PCR conditions. The selected positive recombinant plasmids were confirmed by sequencing with M13 forward and reverse primers in a Model 377 Automated DNA sequencer (Applied Biosystems, CA, USA) with dideoxy chain terminator chemistry.

### Construction of an Expression Plasmid

The *B. subtilis* MA139 β-mannanase gene was amplified with primers of BS-mann-F' (5′-GGGGTACCGCGCATACTGTGTCGCCT-3′, *Kpn*I site is underlined) and BS-mann-R' (5′-GCTCTAGATCACTCAACGATTGGCGTTA-3′, *Xba*I site is underlined) using pUC-

mann as the template following the above condition. The PCR product digested by *Kpn*I/*Xba*I was ligated into the constitutive expression vector pGAPZαA to form the plasmid of pGAP-wt. The cloned *B. subtilis* MA139 β-mannanase gene was optimized according to the codon usage bias of *P. pastoris* [13–15]. This gene sequence was designed with two restriction enzyme sites on either ends (*Eco*RI/*Xba*I) and de novo synthesized by Sangon (Sangon, Shanghai, China). After digesting the synthetic DNA with *Eco*RI and *Xba*I (TakaRa, Shiga, Japan), the expression plasmid pGAP-mann was generated by ligating it into the pGAPZαA vector digested with the same two enzymes. As a control, the wild-type β-mannanase gene was cloned using the same procedure, and named pGAP-WT. The constructed plasmids were transformed into *E. coli* Top10 and amplified by selection of the positive transformants on a low-salt LB plates (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract, 15 g/L agar) containing 25 μg/mL Zeocin™ (Invitrogen, USA). The pGAP-mann or pGAP-WT plasmids were confirmed by restricted digestion and sequencing. Due to the presence of a signal peptide sequence in the pGAPzαA plasmid upstream of the inserted gene, the β-mannanase was designed to be extracellularly produced.

## Transformation and Expression of β-mannanase Gene in *P. pastoris*

The recombinant plasmid pGAP-mann or pGAP-WT was linearized by digestion with BspHI (New England BioLabs, USA), then transformed into *P. pastoris* X-33 cells by electroporation (2,000 V, 5 ms). Transformants were selected by growing the cells on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) plates containing 100 μg/mL Zeocin™ for 3–4 days. A single colony was inoculated into 10 mL of YPD medium and cultured overnight at 28 °C. One milliliter of this culture was then inoculated into 50 mL of YPD medium in a 250-mL flask and incubated at 28 °C for 3 days at 250 rpm.

## Purification of β-mannanase from *P. pastoris*

The culture supernatant was collected by centrifugation at 2,500×*g* for 10 min, which was then treated with ammonium sulfate at room temperature in a two-step procedure using 40% and 70% saturation. The precipitate at 70% saturation was collected by centrifugation at 6,500×*g* for 10 min, and dissolved in 5 ml of buffer A (20 mM Tris–HCl buffer, pH 8.5), and dialyzed overnight at 4 °C using Snakeskin™ pleated dialysis tubing (Pierce, USA) against buffer A. The solution obtained from dialysis was loaded on an anion-exchange column (DEAE-Sephadex A-50, 2.6 cm×20 cm) pre-equilibrated with buffer B (30 mM NaCl–20 mM Tris–HCl buffer, pH 8.5). The column was then washed with buffer B to remove un-adsorbed proteins. Proteins were eluted with a linear 800 mL gradient of buffer C (1 M NaCl–20 mM Tris–HCl buffer, pH 8.5) at a flow rate of 1 mL/min. The active fractions were pooled and concentrated for subsequent use. The empty vector pGAPZαA tranformant was used as the negative control in the whole process.

## Protein Electrophoresis Analysis and Enzyme Activity Assay

To identify the purified enzyme, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12.5% gel on a Mini-II apparatus (Bio-Rad, USA). After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 (Sigma USA). Protein concentration was determined with Micro-BCA Protein Assay Reagent (Pierce, USA) according to the manufacturer's instructions.

The assay for β-mannanase activity was conducted according to the method of Chen et al. [16]. One unit (U) of β-mannanase activity was defined as that amount of enzyme liberating 1 μmol of reducing sugar per minute from a substrate solution at pH 6.0 and 50°C. The reaction mixture contained 2 mL of 0.8% (*w*/*v*) galactomannan (Sigma G-0753) solution and 2 mL of diluted enzyme in buffer at a specific pH and was incubated at 50°C for 20 min. The liberated reducing sugar was measured by the dinitrosalicylic acid method [16, 17]. Each experiment was done in triplicates. In the following determination, each strain was conducted in three independent shake flask experiments.

### Assay of Enzyme Properties

The optimal temperature was determined by the standard enzyme activity assay method as described above at the temperature range of 20 to 80°C. To estimate thermal stability, the enzyme was pre-incubated in disodium hydrogen phosphate–citric acid buffer (pH 6.0) for 30 min and then assayed for residual activity at 50°C and pH 6.0, as described above.

The optimal pH was determined by measuring the enzyme activity in the buffers whose pH ranged from 4.0–9.0. The buffers were disodium hydrogen phosphate–citric acid buffer (pH 4.0–8.0) and glycine–sodium hydroxide (pH 9.0). To estimate pH stability, the enzyme was pre-incubated in the buffer for 1 h at room temperature and then assayed for residual activity at 50°C and pH 6.0, as described above.

To assay the enzyme properties in the presence of metal ions, the enzyme was pre-incubated with 10 mM metallic ion solution in 100 mM sodium citrate buffer (pH 6.0) at room temperature for 30 min. The residual enzyme activity was assayed at 50°C and pH 6.0, as described.

## Results

### Cloning, Sequencing of β-mannanase Gene from *B. subtilis* MA139

The DNA fragment encoding the mature β-mannanase was amplified from the genomic DNA of *B. subtilis* MA139 using the primers designed on the basis of the nucleotide sequence of β-mannanases from *B. subtilis* reported in GenBank (AY827489, DQ309335, DQ351940). The 1014 nucleotides long β-mannanase gene encodes a protein of 337 amino acids with an empirical molecular weight of 38 kDa (Fig. 1). Compared to the reported β-mannanase (AY827489), this new β-mannanase shows 97.53% of similar nucleotide sequence and 99.11% of amino acid sequence. Compared to DQ309335, the similarities of nucleotide sequence and amino acid sequence of our β-mannanase are 97.53% and 98.81%, respectively. And compared to DQ351940, the similarities of nucleotide sequence and amino acid sequence are 97.34% and 97.92%, respectively. The nucleotide sequence of β-mannanase from *B. subtilis* MA139 has been assigned to GenBank (accession number: EU918394).

### Significant Improvement of Expression Level by Codon Optimization

To study the effect of codon bias on the expression of heterologous gene, the wild-type β-mannanase gene was optimized by substitution of 258 nucleotides (nts) with their corresponding counterparts, which had no change on the β-mannanase amino acid sequence. This synthetic DNA has 47.6% G+C content, which shared 74.56% similarity

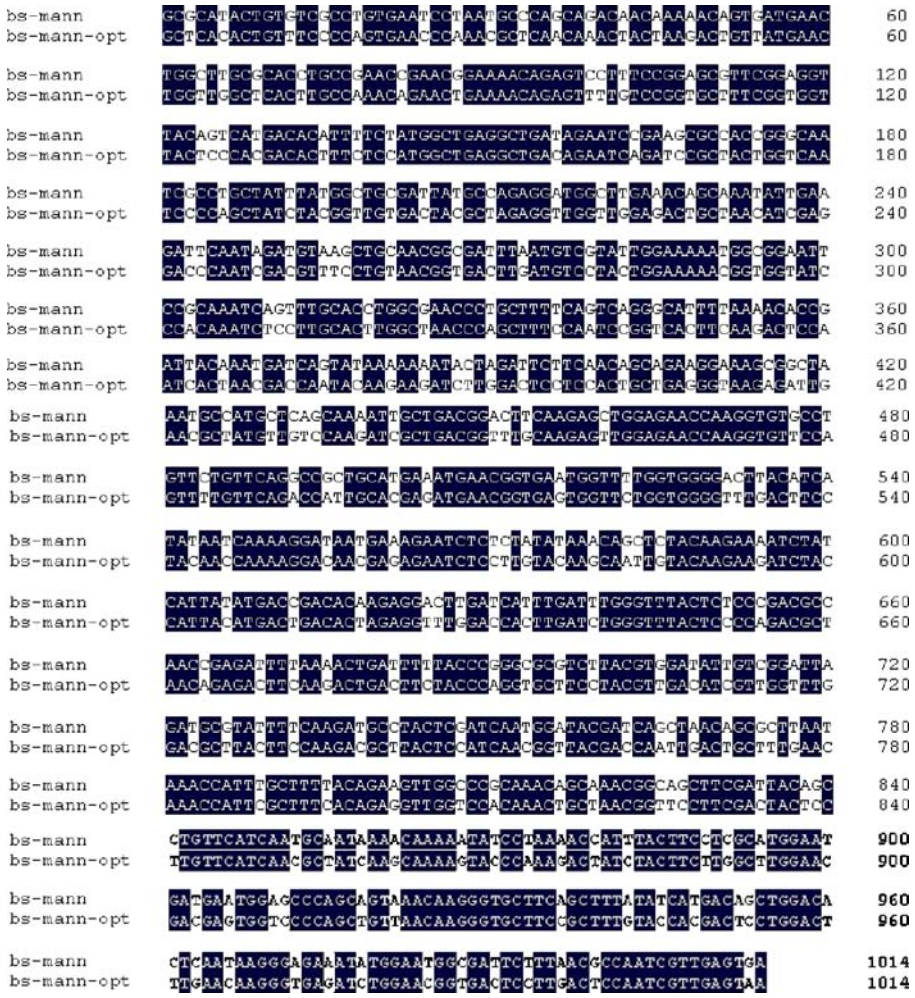


**Fig. 1** Alignment of *B. subtilis* wild-type β-mannanase gene (bs-mann) with optimized β-mannanase gene (bs-mann-opt). The figure was generated by DNAMAN (Lynnon Biosoft, version 5.2.2). Identical residues are *highlighted in black*

to the wild-type β-mannanase gene (Fig. 1). The wild-type and optimized β-mannanase genes were fused into the *P. pastoris* expression vector of pGAPzαA respectively, in which the heterologous gene was driven by a constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase gene (GAP). The recombinant plasmids pGAP-wt and pGAP-mann were transformed into *P. pastoris* X-33 cells and screened by Zeocin, respectively.

The recombinant yeast, X-33/pGAP-wt and X-33/pGAP-mann strains, were cultured in YPD medium at 28.5 °C for 3 days. The $OD_{600}$ values of the cultures were similar to each other which were both over 30. The comparison of enzyme activity in different time points was demonstrated in Fig. 2, which demonstrated significant difference in enzyme activity as the fermentation prolonged. At the third day of fermentation, the enzyme activity of the culture supernatant, X-33/pGAP-mann reached 230 U/mL. Compared to the activity of X-33/pGAP-wt (170 U/mL), the expression enzyme of the optimized β-mannanase gene

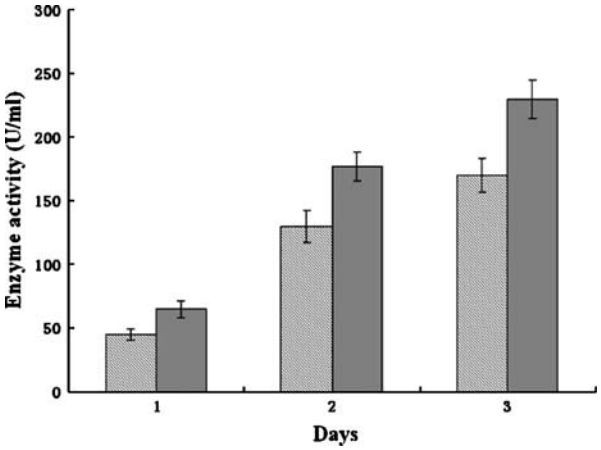


**Fig. 2** The comparison of enzyme activity between X-33/pGAP-wt and X-33/pGAP-mann products at different time points. Values are means±SD (*bars*). Each experiment was conducted in triplicate. : X-33/pGAP-wt; : X-33/pGAP-mann

acquired approximately 35% more activity. As a control, the supernatant of *P. pastoris* X-33 transformed with native pGAPzαA showed no detectable activity. After purification, the yield of β-mannanase in cultured X-33/pGAP-mann reached 2.7 mg/mL. Compared to 1.9 mg/mL of the β-mannanase in X-33/pGAP-wt, the protein yield was increased by 42%. The β-mannanase from crude culture supernatant and purified solution of X-33/pGAP-mann was detected by SDS-PAGE (Fig. 3).

## Enzyme Properties

As a potential feed additive, the enzyme properties of the β-mannanase is of importance in its optimal temperature, pH, and its temperature stability because of various processing techniques applied in feed manufacture. As shown in Fig. 4, the optimal catalytic temperature was 50°C. At 40°C, the enzyme decreased its activity by approximately 50%

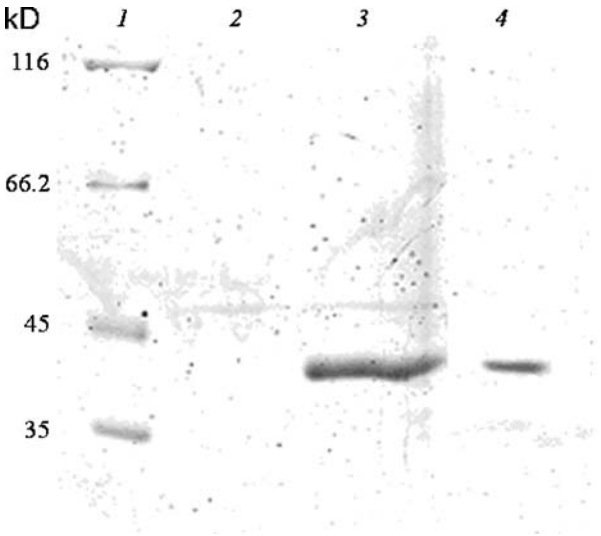


**Fig. 3** SDS-PAGE analysis of purified proteins of *B. subtilis* β-mannanase gene in *P. pastoris*. The empty plasmid transformed in *P. pastoris* X-33 was the negative control. Lane *1* molecular mass standard protein, lane *2* negative control, lane *3* supernatant of pGAP-mann transformant (crude β-mannanase), lane *4* purified β-mannanase

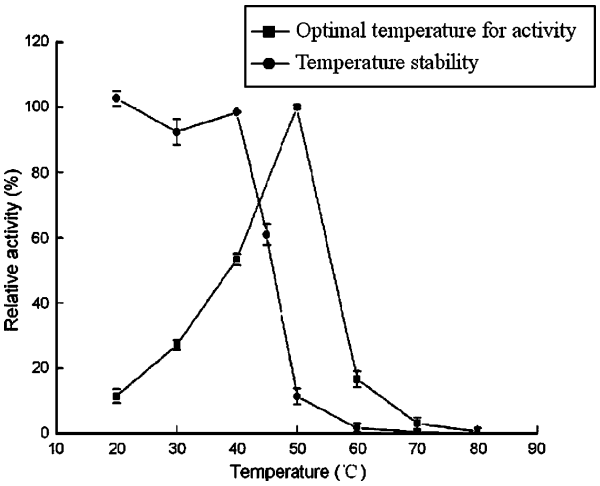


**Fig. 4** Effect of temperature on the activity of the β-mannanase. The optimal temperature was determined by the standard enzyme activity assay method at series of temperatures. To estimate thermal stability, the recombinant enzyme was pre-incubated at various temperatures for 30 min at pH 6.0 and the residual activity was determined at 50°C and pH 6.0. The activity determined under the standard condition of pH 6.0 and 50°C was defined as 100% relative activity. Values are means±SD (*bars*). Each experiment was conducted in triplicate

compared to the activity at 50°C. When the temperature exceeded 50°C, the activity of the β-mannanase quickly declined. Almost no activity was detected at 80°C. To determine the temperature stability of the enzyme, the residual activity was measured at 50°C and pH 6.0 after the enzyme was maintained at the temperatures ranging from 20 to 80°C for 30 min. The enzyme displayed no less than 60% peak activity after being heated at 45°C for 30 min, whereas no activity was observed after excess heating of 60°C for 30 min.

The functional pH values of the recombinant β-mannanase were presented in Fig. 5 as well. The enzyme showed high catalytic activity in pH 5.0–7.0, with the highest activity at pH 6.0 (as filled squares showed). As the pH went to 8.0, nearly 50% activity of the enzyme was lost (as filled circles showed). Overall, the β-mannanase was relatively stable in the pH range of 3.0 to 9.0 (Fig. 5).

As demonstrated in Table 1, like other enzymes, metal ions and EDTA affected β-mannanase activity. Mg2+ and Fe2+ ions did not significantly affect the β-mannanase activity whereas Cu2+ inhibited the enzyme activity by more than 60%.

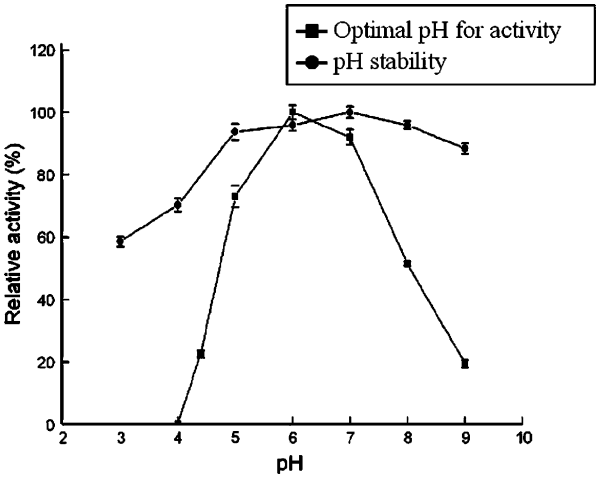


**Fig. 5** Effect of pH on the activity of the β-mannanase. The optimal pH was determined using the standard activity assay and the different pH buffers. To estimate pH stability, the recombinant enzyme was incubated in the different pH at room temperature for 1 h, and then the residual β-mannanase activity was determined at pH 6.0 and 50°C. The activity determined under the standard condition of pH 6.0 and 50°C was defined as 100% relative activity. Values are means±SD (*bars*). Each experiment was conducted in triplicate

**Table 1** Effects of chemical reagents on the β-mannanase activity.

| Reagents | Relative activity[a] (%) |
|---|---|
| $FeSO_4$ | 101.32±0.86 |
| $CuSO_4$ | 37.79±1.79 |
| $MnSO_4$ | 53.41±0.46 |
| $ZnSO_4$ | 79.22±2.71 |
| $MgSO_4$ | 97.09±2.18 |
| $Na_2SO_4$ | 93.91±2.32 |
| EDTA | 63.73±1.39 |

[a] Mean±standard error. Experiments were determined in triplicate. Chemical reagents were individually added up to final concentration of 10 mmol/L

## Discussion

The yeast *P. pastoris* has been routinely used as a heterologous expression system because of its efficient secretion, high expression level, proper protein folding, and very high cell density [18, 19]. The bias of codon usage is directly related to gene expression level in a heterologous expression system [20]. Several studies have revealed the importance of code usage frequency to the expression level in *P. pastoris* [14–16]. For example, CCG (for proline), CGC (for arginine), and GGG (for glycine) have very low usage frequency. Hu et al. [21] acquired three- to fivefold improvement of a single-chain antibody in *P. pastoris* than the wild-type gene by codon optimization. In our lab, a β-mannanase gene from *Aspergillus sulphureus* was highly expressed with a yield of 3 mg/mL in *P. pastoris* after codon optimization, and its enzyme activity was increased by 35% [22]. In this study, the gene sequence of β-mannanase from *B. subtilis* MA139 was optimized which elevated the enzyme activity from 170 U/mL to 230 U/mL.

Two systems of *P. pastoris* for expression of heterologous proteins have been well developed, including the inducible and constitutive systems. In the inducible expression system, the expression of a foreign gene is under the control of alcohol oxidase I promoter which can be only induced by methanol. This causes relative longer fermentation course. The constitutive system was later developed, in which a foreign gene was driven by GAP promoter [23]. This promoter allows the heterologous protein express constitutively without the induction process.

The characteristics of β-mannanase differ in its sources. A much detailed comparison was reviewed previously [24]. In general, the optimal enzyme temperature is in the range of 45°C to 92°C, and the optimal pH ranges in 3.0 to 7.5. For example, the β-mannanase from *A. sulphureus* displays the highest catalytic ability at pH 2.4, which is so far the most acidic functioning β-mannanase [16]. Even when the β-mannanases are from *Bacillus* sp., they are different in the characteristics in terms of the optimal temperature and pH. For example, *B. subtilis* WY34 sourced β-mannanase [25], its optimal temperature and pH were 65°C and 6.0, respectively, and the *Bacillus* sp. N16-5 sourced β-mannanase [26] showed the optimal temperature and pH at 70°C and 10.0 respectively, which was a basic functioning enzyme. A *B. subtilis* KU-1 sourced β-mannanase functions in the range of 50 to 55°C, and optimally catalyzes in pH 7.0 [27]. A β-mannanase from *B. subtilis* NM-39 was optimal at 50°C and pH 5.5 [28], which showed the most similar property as our β-mannanase at this point. Our β-mannanase expressed in *P. pastoris* X-33 optimally functions at 50ºC, which is the closest temperature to the body temperature compared to other reported β-mannanases [25–28]. Its optimal pH 6.0 is also in the pH range of gastric intestinal tract. These characteristics allow the enzyme to be a prospecting additive in feed industry.

A study on *Bacillus* sp. N16-5 sourced β-mannanase, which expressed in *P. pastoris*, acquired 32.2 U/mL of activity after120-h fermentation [26], while our β-mannanase reached the activity at 230 U/mL after 3-day fermentation. Collectively, the β-mannanase gene from *B. subtilis* MA139 was efficiently expressed in *P. pastoris* by codon optimization in this study. Further studies will be focused on pilot scaled-up fermentation of the enzyme.

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## References

1. Matheson, N. K., & McCleary, B. V. (1985). In G. O. Aspinall (Ed.), *The Polysachharides*, *vol. 3* (p. 1). New York: Academic.
2. Singh, S., Madlala, A. M., & Prior, B. A. (2003). *FEMS Microbiology Reviews*, *27*, 3–16.
3. Rättö, M., & Poutanen, K. (1988). *Biotechnology Letters*, *10*, 661–664.
4. Abe, J., Hossain, Z. M., & Hizukuri, S. (1994). *Journal of Fermentation and Bioengineering*, *3*, 259–261.
5. Araujo, A., & Ward, O. P. (1990). *Journal of Applied Bacteriology*, *68*, 253–261.
6. Araujo, A., & Ward, O. P. (1990). *Applied and Environmental Microbiology*, *56*, 1954–1956.
7. Khanongnuch, C., Ooi, T., & Kinoshita, S. (1999). *World Journal of Microbiology and Biotechnology*, *15*, 249–258.
8. Oda, Y., Komaki, T., & Tonomura, K. (1993). *Journal of Fermentation and Bioengineering*, *76*, 14–18.
9. Takahashi, R., Kusakabe, I., Kobayashi, H., Murakami, K., Maekawa, A., & Suzuki, T. (1984). *Agricultural and Biological Chemistry*, *48*, 2189–2195.
10. Titapoka, S., Keawsompong, S., Haltrich, D., & Nitisinprasert, S. (2008). *World Journal of Microbiology and Biotechnology*, *24*, 1425–1433.
11. Guo, X. H., Li, D. F., Lu, W. Q., Piao, X Sh, & Chen, X. L. (2006). *Antonie Van Leeuwenhoek*, *90*, 139–146.
12. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). New York: Cold Spring Harbor Laboratory Press.
13. Zhao, X., Huo, K. K., & Li, Y. Y. (2000). *Chinese Journal of Biotechnology*, *16*, 308–311.
14. Sreekrishna, K. (1993). Strategies for optimizing protein expression and secretion in the methylotrophic yeast *Pichia pastoris*. In R. H. Baltz, G. D. Hegeman & P. L. Skatrud (Eds.), *Industrial microorganism: basic and applied molecular genetics* (pp. 119–126). Washington, DC: American Society of Microbiology.
15. Sinclair, G., & Choy, F. Y. M. (2002). *Protein Expression and Purification*, *26*, 96–105.
16. Chen, X. L., Cao, Y. H., Ding, Y. H., Lu, W. Q., & Li, D. F. (2006). *Journal of Biotechnology*, *128*, 452–461.
17. Miller, G. L. (1959). *Analytical Chemistry*, *31*, 426–428.
18. Kim, T. R., Goto, Y., Hirota, N., Dawata, K., Denton, H., Wu, S. Y., et al. (1997). *Protein Engineering*, *10*, 1339–1345.
19. Reverter, D., Ventura, S., Villegas, V., Vendrell, J., & Avilés, F. X. (1998). *Journal of Biological Chemistry*, *273*, 3535–3541.
20. Sharp, P. M., & Li, W. H. (1986). *Journal of Molecular Evolution*, *24*, 28–38.
21. Hu, S. Y., Li, L. W., Qiao, J. J., Guo, Y. J., Cheng, L. S., & Liu, J. (2006). *Protein Expression and Purification*, *47*, 249–257.
22. Chen, X. L., Qiao, J. Y., Yu, H. F., & Cao, Y. H. (2009). *Biologia*, *64*, 235–238.
23. Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V., & Cregg, J. M. (1997). *Gene*, *186*, 37–44.
24. Moreira, L. R. S., & Filho, E. X. F. (2008). *Applied Microbiology and Biotechnology*, *79*, 165–178.
25. Jiang, Z. Q., Wei, Y., Li, D. Y., Li, L. T., Chai, P. P., & Kusakabe, I. (2006). *Carbohydrate Polymers*, *66*, 88–96.
26. He, X. P., Liu, N., Li, W. W., Zhang, Zh Y, Zhang, B. R., & Ma, Y. H. (2008). *Enzyme and Microbial Technology*, *43*, 13–18.
27. Zakaria, M. M., Yamamoto, S., & Yagi, T. (2006). *FEMS Microbiology Letters*, *158*, 25–31.
28. Mendoza, N. S., Arai, M., Kawaguchi, T., Yoshida, T., & Joson, L. M. (1994). *World Journal of Microbiology and Biotechnology*, *10*, 551–555.