Research Article

Codon Optimization Significantly Improves the Expression Level of α-Amylase Gene from Bacillus licheniformis in Pichia pastoris

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Received 4 August 2014; Accepted 31 October 2014

Academic Editor: Marimuthu Citartan

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α-Amylases (E.C.3.2.1.1) are classified as a member of family 13 of the glycosyl hydrolases and catalyze the hydrolysis of internal α-1,4-O-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products [1]. α-Amylases are one of the most important industrial enzymes that have a wide variety of applications in starch processing, paper industries, detergent, and so on [2, 3]. α-Amylases are ubiquitous enzymes produced by plants, animals, and microorganisms. Although there are many sources of α-amylases, microorganisms are the most important sources of α-amylases for industrial purposes due to advantages such as less time and space required for production, cost effectiveness, and ease of process modification and optimization [4].

In recent years, many kinds of α-amylase have been isolated from various microorganisms, such as bacteria and fungi. Among bacteria, Bacillus sp. is widely used for α-amylase production to meet industrial needs. Bacillus subtilis (B. subtilis), Bacillus stearothermophilus (B. stearothermophilus), and Bacillus licheniformis (B. licheniformis) are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications [5]. So far, a number of α-amylase genes have been isolated and characterized from Bacillus sp., including B. licheniformis, B. stearothermophilus, and B. subtilis [6–8]. In previous studies, a gene encoding α-amylase from B. licheniformis was cloned and expressed in E. coli and B. subtilis [9]. However, the low expression level does not allow the recombinant protein to be applied practically and economically in industry. For commercial exploitation of the recombinant α-amylase, it is essential to achieve high yield of the protein.

The methylotrophic yeast P. pastoris has many advantages as a host for production of recombinant heterologous proteins, such as high cell density, high levels of productivity,
ease of genetic manipulation, the ability to perform complex posttranslational modifications, and very low secretion levels of endogenous proteins [10]. To improve heterologous expression of genes, many strategies have been developed in P. pastoris. It includes high copy number of heterologous gene, appropriate signal peptide in expression vector, high efficient transcriptional promoters, and optimization of cell cultivation [11–13]. However, these optimization strategies did not universally result in high protein production for every recombinant protein as expected. It has now been shown that the difference of codon usage between the native gene sequence and expression host has significant impact on the expression level of recombinant protein [14, 15]. Therefore, the codon optimization is a promising technique for increasing foreign protein expression level.

In this study, we describe the high-level expression of B. licheniformis α-amylase (BlAmy) in P. pastoris and this is the first report about high cell density fermentation for production of recombinant B. licheniformis α-amylase (rBlAmy) in 5- and 50-L bioreactor. Furthermore, the α-amylase gene from B. licheniformis (BlAmy) was modified and expressed according to its preferred codon usage in P. pastoris. To our knowledge, this is also the first report to improve B. licheniformis α-amylase (BlAmy) production by codon optimization strategies in P. pastoris.

2. Materials and Methods

2.1. Strains, Plasmids, Reagents, and Media. The P. pastoris strain GS115 and the expression vector pPIC9K were purchased from Invitrogen (Carlsbad, CA, USA). The E. coli strain Top10 is routinely conserved in our laboratory. Restriction enzymes, T4-DNA ligase, and Pfu DNA polymerase were purchased from Sangon Biotech (Shanghai, China). All other chemicals used were analytical grade reagents unless otherwise stated. Yeast extract peptone dextrose (YPD) medium, buffered glycerol complex (BMGY) medium, and buffered methanol complex (BMMY) medium were prepared according to the manual of Pichia Expression Kit (Version F; Invitrogen). Fermentation Basal Salts (BSM) Medium and PTM1 Trace Salts used for fermentation were prepared according to the Pichia Fermentation Process Guidelines (Invitrogen).

2.2. Codon Optimization and Synthesis of the Gene. The codon usage of BlAmy (GenBank M38570) from B. licheniformis was analyzed using Graphical Codon Usage Analyser (http://gcua.schoedl.de/) and was optimized by replacing the codons predicted to be less frequently used in P. pastoris with the frequently used ones by (http://www.dna20.com/). Signal peptide was analyzed by SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The optimized gene (BlAmy-opt) was synthesized by Sangon (Shanghai, China).

2.3. Vector Construction. The synthetic gene encoding the mature region of α-amylase without the predicted signal sequence was digested by EcoRI and NotI and then ligated into pPIC9K, forming pPIC9K-BlAmy-opt. The native α-amylase gene (BlAmy) from B. licheniformis was cloned into pPIC9K using primers BlF (5’-CATCGAATTCGCAATTTAATGGGACGGCTG-3’) and BIR (5’-CATAGGGCGCCCTATCTTTTGAAATTTGA-3’), resulting in the recombinant plasmid pPIC9K-BlAmy. The recombinant plasmids were checked by DNA sequencing.

2.4. Transformation of P. pastoris and Screening Transformants. P. pastoris GS115 was transformed with 10 μg of Sacl-linearized pPIC9K-BlAmy-opt and pPIC9K-BlAmy vector by electrotransformation, according to Invitrogen's recommendations. Transformants were initially selected by MD medium (1.34% yeast nitrogen base, 4 × 10^{-2} % biotin, 2% dextrose) plates and then checked by colony PCR. The insertion copy number of transformants was determined by their resistance to G418 and transformants with the same copy number were selected. The recombinants carrying BlAmy and BlAmy-opt were screened by a new plate assay method. The recombinants from 1.0, 2.0, and 4.0 mg/mL G418-YPD plate were picked and cultured in 96-deep-well microplates containing 200 μL/well BMGY medium at 30°C for 24 h. After this, the cells were harvested by centrifugation, resuspended, and cultured in 400 μL/well BMMY medium. After 24 h, plates were subjected to centrifugation again and supernatants were used in subsequent activity assays. Qualitative α-amylase activity was determined by a halo plate assay containing 3% (w/v) agar and 5% (w/v) soluble starch (Sangon, China). Supernatants (20 μL) were loaded into wells and plates were incubated at 60°C for 30 min. The clones were selected according to the size of the halos and their activities were checked by shaking flask fermentation.

2.5. Expression of BlAmy-opt and BlAmy in P. pastoris Shake-Flask Cultures. The transformants were selected and cultivated in shaking flask. The seeds were inoculated in 10 mL of BMGY medium in a 100 mL shake flask and incubated at 30°C and 250 rpm until the culture reached an OD600 = 2.0–6.0. The cells were harvested by centrifugation and resuspended in 50 mL of BMMY medium and incubated at 30°C and 200 rpm. The methanol induction temperature was set at 30°C, and 0.7% (v/v) methanol was fed at 24 h intervals for 5 days. The activities of the α-amylase were checked at 24, 48, 72, 96, 120, 144, and 168 h. The colony with the highest activity was selected as the strain to ferment in 5- and 50-L bioreactor.

2.6. High Cell Density Fermentation. The transformed strain showing the highest α-amylase activity in shake-flask culture was cultivated in high cell density fermentor. High cell density fermentation was carried out in 5- and 50-L bioreactor (Baoxing Co., Shanghai, China). The cultivation conditions and medium composition was the same as the previous described method [16]. Inoculum was cultured in BMGY medium. Cells were grown for 18–20 h at 30°C on shaker of 200 rpm. Then, 10% (v/v) of the inoculum was inoculated into the 5- and 50-L bioreactors containing 2- and 20-L basal
salt medium, made of 0.47 g/L CaSO₄⋅2H₂O, 9.1 g/L K₂SO₄, 7.5 g/L MgSO₄⋅7H₂O, 6.2 g/L KOH, 13.35 mL/L H₃PO₄ (85%), 20.0 g/L glycerol, and 1.5 mL Pichia trace metal 1 (PTM1). One liter PTM1 consists of 6 g CuSO₄⋅5H₂O, 0.08 g NaI, 3 g MnSO₄⋅H₂O, 0.5 g CoCl₂, 20 g ZnCl₂, 0.02 g H₂BO₃, 0.2 g Na₂MnO₄⋅2H₂O, 65 g FeSO₄⋅7H₂O, 0.2 g biotin, and 30 mL 6 N H₂SO₄. The temperature was controlled at 30°C and the pH was maintained at 5.0 using NH₄OH (28%) and H₃PO₄ (10%). For 5 L bioreactor, the agitation rate was set at 600 rpm and the aeration rate was 30 L/min. For 50 L bioreactor, the agitation rate was set at 500 rpm and the aeration rate was 40 L/min. When glycerol was used up, as indicated by an abrupt increase in DO, 0.5% (v/v) methanol was kept stable by monitoring the dissolved oxygen (DO). When the initial methanol 0.5% (v/v) was depleted (indicated by an abrupt increase in DO), 100% methanol solution containing 1.2% methanol was added automatically. The concentration of methanol was kept stable by monitoring the dissolved oxygen (OD) and maintaining it at greater than 20%. The enzyme activity of the supernatant and dry cell weight were monitored throughout the cultivation.

2.7. Purification, Deglycosylation, and SDS-PAGE Analysis of Recombinant BlAmy. After fermentation, cells from the cultures were removed by centrifuging at 6000 × g for 10 min. The supernatant was concentrated by ultrafiltration using a Millipore set-up according to the manufacturer's instructions with a membrane of 10 kDa cut-off. The supernate containing recombinant BlAmy was purified by 2 mL Ni²⁺-chelating chromatography according to the manuals (Biorad, USA). The elution buffer containing purified recombinant BlAmy was used for further analysis. Purified recombinant BlAmy was deglycosylated using 300 U of Endo H for 3 h at 37°C according to the manufacturer's instructions (NEB, USA). The deglycosylated and untreated were analyzed by SDS-PAGE. SDS-PAGE was carried out on a 12% running gel and stained with Coomassie Blue.

2.8. Assay of α-Amylase Activity and Protein Determination. α-Amylase activity was assayed according to the method described by previous studies [17]. One unit of α-amylase was defined as the amount of amylase needed to complete the liquefaction of 1 mg of starch into dextrin per minute at 70°C and pH 6.0. The protein content was determined according to the Bradford method using BSA as standard.

2.9. Characterization of the Recombinant BlAmy and Deglycosylated Recombinant BlAmy. The relative enzyme activity was determined at various pH values using 100 mM buffers, pH ranging from 4.0 to 11.0. Buffers used as standard were sodium acetate buffer (pH 4–6), sodium phosphate buffer (pH 6–8), and sodium carbonate buffer (pH 9–11). To evaluate the pH stability, aliquots of enzyme samples were incubated at 30°C for 24 h with respective pH buffers. Remaining enzyme activity was measured under standard assay protocol and calculated considering the initial activity. The optimal temperature of the enzyme was determined by measuring the enzyme activity at various temperatures (40–100°C) in 100 mM of sodium phosphate buffer, pH 7.0. Thermal stability was determined by incubating the purified enzyme in 100 mM of sodium phosphate buffer (pH 7.0) for 1 h at the desired temperatures (60–100°C) followed by measuring the residual activity.

3. Results and Discussion

3.1. Sequence Optimization and De Novo Synthesis of BlAmy. P. pastoris has been routinely used as a heterologous expression system because of its efficient secretion, high expression level, and high cell density [10]. However, the bias of codon usage between the native gene sequence and P. pastoris has significant impact on the expression level of recombinant protein. Codon optimization by using frequently used codons in the host is an efficient measure to improve the expression level of heterologous gene. Generally, this is accomplished by replacing all codons with preferred codons, eliminating AT-rich stretches and adjusting the G+C content [18, 19]. Analysis of the DNA sequence of native α-amylase gene (BlAmy) using Graphical Codon Usage Analyser revealed that some amino acid residues were encoded by codons that are rarely used in P. pastoris, codons like GGC (Gly), GCG (Ala), AGC (Ser), TCG (Ser), and CCG (Pro); most of them are shared less than 15% of usage percentage, which may result in a much lower expression level in P. pastoris. In order to achieve a high-level expression of BlAmy in P. pastoris, the codons of BlAmy were replaced with those more frequently used by P. pastoris (Table 1). The codon adaptation index (CAI) of the native BlAmy was improved from 0.74 to 0.86. Furthermore, the G+C content was increased from 47.6 to 49.2%, which was closer to the G+C content of other high-expression genes in P. pastoris. The nucleotides A, T, G, and C dispersed evenly in the synthesized gene to eliminate AT- or GC-rich motifs, codons containing both AT and GC were selected when the differences between the codon frequencies were not significant. Totally, the codons encoding 305 amino acids were optimized in which a total of 328 nucleotides were changed (Table 1). The optimized gene (BlAmy-opt) shared 77% of nucleotide sequence identity with that of the native gene (BlAmy) (Figure 1).

3.2. Vector Construction and Selection of Producing Clones. The recombinant plasmids pPIC9K-BlAmy-opt and pPIC9K-BlAmy were linearized and transformed into P. pastoris GS115 and several thousands of transformants were obtained on MD plates. In this study, the putative multicopy inserts were selected for expression by screening with the same concentration of G418. The positive clones (from 1.0, 2.0, and 4.0 mg/mL G418-YPD plate) were cultured in 96-deep-well microplates and further screened by a new halo plate assay (Figure 2). According to the size of the halos, twenty clones (ten isolated from recombinants carrying BlAmy-opt and ten from recombinants carrying BlAmy, resp.) from 2.0 mg/mL G418-YPD plate were selected for shake-flask cultures. The plate assay is a simple, rapid, and well adapted method for screening of large number of samples [20]. The diameter of
the halo zone is very useful for predicting the enzyme yield as an aid to select strains with a high level of α-amylase production.

3.3. Expression of BlAmy-opt and BlAmy in P. pastoris at Shaking Flask Level. Twenty colonies with larger halos were selected and cultivated in shaking flask. In shaking flask, the α-amylase activity increased gradually and reached the highest activity after 144 h of cultivation. After 144 h of cultivation under inducing conditions, the α-amylase activity of the supernatant from different clones carrying BlAmy-opt varied between 310 and 420 U/mL (enhanced 5.25-fold compared with the wild strain Bacillus licheniformis), while the recombinants carrying BlAmy varied between 150 and 230 U/mL (enhanced 2.87-fold compared with the wild strain Bacillus licheniformis), respectively. Two clones (one carrying BlAmy-opt, the other carrying BlAmy) fromYPD plate containing 2.0mg/mL showed the highest α-amylase activity of 420 U/mL and 230 U/mL in shaking flask culture was chosen for high cell density fermentation.

3.4. High Cell Density Fermentation. To obtain a large amount of active protein, fed-batch studies were carried out in 5- and 50-L fermentor. Upon methanol induction, the maximum α-amylase activity and protein concentration produced by recombinant strain GS115 carrying BlAmy-opt reached 8100 U/mL and 8.3 g/L, respectively, in the 5-L fed batch bioreactor (Figure 3(a)). Compared with

**Figure 1:** Sequence comparison between the original (BlAmy) and the optimized (BlAmy-opt) genes. Identical residues are marked in black background.
Table 1: Comparison of the codon usage for wild-type and synthetic α-amylase gene targeted at *P. pastoris* for expression.

| AA   | Codon | Host fraction | BlAmy | BlAmy-opt |
|------|-------|---------------|-------|-----------|
| Gly  | GCG   | 0.06          | 9     | 0         |
|      | GCA   | 0.23          | 10    | 0         |
|      | GGT   | 0.45          | 10    | 19        |
|      | GCC   | 0.26          | 7     | 17        |
| Glu  | GAG   | 0.16          | 4     | 0         |
|      | AGA   | 0.48          | 3     | 15        |
|      | CGG   | 0.05          | 3     | 0         |
|      | CGA   | 0.10          | 3     | 0         |
|      | CGT   | 0.16          | 2     | 7         |
|      | CGC   | 0.05          | 7     | 0         |
| Ala  | AAG   | 0.53          | 11    | 20        |
|      | AAA   | 0.47          | 17    | 8         |
| Arg  | AGT   | 0.15          | 2     | 0         |
|      | AGC   | 0.09          | 4     | 0         |
|      | TGG   | 0.09          | 8     | 0         |
|      | TCA   | 0.19          | 4     | 0         |
|      | TCT   | 0.29          | 3     | 9         |
|      | TCC   | 0.20          | 5     | 17        |
| Ser  | TAA   | 0.53          | 1     | 1         |
|      | AAT   | 0.49          | 11    | 5         |
|      | AAC   | 0.51          | 14    | 20        |
| Met  | ATG   | 1.00          | 7     | 7         |
| Ile  | AAT   | 0.19          | 1     | 0         |
|      | ATT   | 0.50          | 13    | 10        |
|      | ATC   | 0.30          | 6     | 10        |
| Thr  | ACG   | 0.11          | 7     | 0         |
|      | ACA   | 0.24          | 13    | 0         |
|      | ACT   | 0.40          | 3     | 12        |
|      | ACC   | 0.25          | 4     | 15        |
| Trp  | TGG   | 1.00          | 17    | 17        |
| Cys  | TGT   | 0.65          | 0     | 0         |
|      | TGC   | 0.35          | 0     | 0         |
| Tyr  | TAT   | 0.46          | 18    | 5         |
|      | TAC   | 0.55          | 12    | 25        |

Table 1: Continued.

| AA   | Codon | Host fraction | BlAmy | BlAmy-opt |
|------|-------|---------------|-------|-----------|
| Leu  | TTG   | 0.33          | 11    | 23        |
|      | TTA   | 0.16          | 3     | 0         |
|      | CTG   | 0.16          | 6     | 0         |
|      | CTA   | 0.11          | 0     | 0         |
|      | CTT   | 0.16          | 7     | 5         |
|      | CTC   | 0.08          | 1     | 0         |
| Phe  | TTT   | 0.54          | 16    | 5         |
|      | TTC   | 0.46          | 4     | 15        |
| Gln  | CAG   | 0.39          | 9     | 15        |
|      | CAA   | 0.61          | 11    | 5         |
| His  | CAT   | 0.57          | 17    | 6         |
|      | CAC   | 0.43          | 7     | 18        |
| Pro  | CCC   | 0.15          | 3     | 0         |
|      | CCG   | 0.09          | 8     | 0         |
|      | CCA   | 0.41          | 1     | 9         |
|      | CCT   | 0.35          | 3     | 6         |

Figure 2: Screening of strains with higher level of α-amylase production by plate assay. The agar plates contain 3% (w/v) agar and 5% (w/v) soluble starch.

the expression of the native gene in *P. pastoris* (3500 U/mL), the expression level of codon optimized gene was increased by 2.31-fold (Figure 3(a)). The maximum α-amylase activity and protein concentration of recombinant strain GS115 carrying *BlAmy-opt* obtained in the 50-L fed-batch bioreactor were 11000 U/mL and 12.2 g/L, respectively (Figure 3(b)). Compared with the expression of the native gene in *P. pastoris* (4200 U/mL), the expression level of codon optimized gene was increased by 2.62-fold (Figure 3(b)). The recombinant protein accounted for 86% of the total protein in the medium as estimated by the Software Quantity One (Figure 4(a)).
Figure 3: α-Amylase activity and total protein content in basal salt medium at 30°C and pH 5.0 during fed-batch fermentation in 5-L (a) and 50-L (b) bioreactor. α-Amylase activity was determined by starch-iodine color method; the protein content was determined according to the Bradford method using BSA as standard. All measurements were carried out in triplicate.

Figure 4: SDS-PAGE analysis of recombinant BlAmy. (a) SDS-PAGE of recombinant BlAmy in fermentation broth from 5- and 50-L bioreactor after methanol induced for 168 h. M: protein MW markers; lane 1 and lane 2: recombinant BlAmy and BlAmy-opt from 5-bioreactor, respectively. Lane 3 and lane 4: recombinant BlAmy and BlAmy-opt from 50-bioreactor, respectively. (b) Analysis of purified recombinant BlAmy and N-deglycosylated recombinant BlAmy by Endo H. Lane 1: purified recombinant BlAmy; lane 2, lane 3, and lane 4: the N-deglycosylated recombinant BlAmy and Endo H; lane 5: Endo H.

As an easy and simple system, *P. pastoris* is now widely used for heterologous production of recombinant proteins [21]. Due to the difference of codon usage between the native gene sequence and expression host, researchers have used codon optimization to increase the expression level of heterologous genes in *P. pastoris*. By codon optimization, the expression of xylanase gene from *Thermotoga maritime* and *Aspergillus sulphureus* was improved 2.8- and 5-fold, respectively [18, 22]. The optimization of glucanase gene from *B. licheniformis* and *Fibrobacter succinogenes* resulted in a 10- and 2.34-fold increase of target protein production [15, 23]. In this study, the BlAmy-opt was expressed in *P. pastoris* at a significantly higher level (12.2 g/L) with α-amylase activity of 11000 U/mL in 50-L fermentor after 168 h induction through codon optimization. These results showed that codon optimization is an effective method to increase the expression of foreign protein in *P. pastoris*. Meanwhile, the codon optimized recombinant α-amylase has a great potential use in industrial application due to its high-expression level.
3.5. SDS-PAGE Analysis of Recombinant BlAmy. As shown in Figure 4(a), the purified recombinant BlAmy showed two forms of BlAmy with molecular masses close to 70 kDa, which is about 11 kDa larger than 58.5 kDa, the calculated molecular weight of the nonglycosylated BlAmy. As shown in Figure 4(b), Endo H treatment of recombinant BlAmy resulted in a shift in the protein band on SDS-PAGE and yielded a single band of 58 kDa, suggesting that the two forms of recombinant BlAmy contained different degree of glycosylation.

3.6. Characterization of the Recombinant BlAmy and Deglycosylated Recombinant BlAmy. The influence of pH on recombinant BlAmy-opt and deglycosylated recombinant BlAmy activity and stability are presented in Figure 5. The activity
of recombinant BlAmy-opt and deglycosylated recombinant BlAmy were measured over a pH range of 4.0–11.0. As shown in Figure 5(a), the recombinant BlAmy-opt remained active at a pH range of 5.0–9.0 and showed maximum activity at pH 7.0, which is similar to the deglycosylated recombinant BlAmy (Figure 5(b)). In the pH stability study, the recombinant BlAmy-opt and deglycosylated recombinant BlAmy are stable at a broad range of pH values between pH 6.0 and 10.0 after 24 h incubation at 30°C, retaining over 78% of its initial activity.

The activity of recombinant BlAmy-opt and deglycosylated recombinant BlAmy were also determined at different temperatures. As shown in Figures 6(a) and 6(b), the recombinant BlAmy-opt and deglycosylated recombinant BlAmy showed an optimum activity at 90°C and activity dropped above 100°C. Thermostability was examined by incubating the recombinant BlAmy-opt and deglycosylated recombinant BlAmy at different temperatures for 1 h, and the residual activity was measured at 70°C under the conditions mentioned above. The activity of the recombinant BlAmy-opt was almost not affected by a temperature below 70°C, but it decreased dramatically when the temperature was above 90°C. The thermostability of BlAmy was higher than deglycosylated recombinant BlAmy. BlAmy showed 71% and 53% residual activity after 1 h incubation at 90 and 100°C, whereas deglycosylated recombinant BlAmy showed only 62% and 43%.

4. Conclusions

In this study we report the high-level expression of BlAmy in P. pastoris. The results showed that P. pastoris is an excellent host to production of BlAmy. To our knowledge, this is the first report about high cell density fermentation for production of recombinant BlAmy in 5- and 50-L bioreactor. Meanwhile, we developed a new simple and quick plate assay for screening of strains with higher level of BlAmy. To our knowledge, this is the first report about high cell density fermentation for BlAmy. To our knowledge, this is the first report about high cell density fermentation for BlAmy. Meanwhile, we developed an enzyme specificity found in various families of glycoside hydrolases, “Cellular and Molecular Life Sciences, vol. 71, no. 7, pp. 1149–1170, 2013.

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4. Conclusions

In this study we report the high-level expression of BlAmy in P. pastoris. The results showed that P. pastoris is an excellent host to production of BlAmy. To our knowledge, this is the first report about high cell density fermentation for production of recombinant BlAmy in 5- and 50-L bioreactor. Meanwhile, we developed a new simple and quick plate assay for screening of strains with higher level of BlAmy. As shown in Figures 6(a) and 6(b), the recombinant BlAmy-opt and deglycosylated recombinant BlAmy showed an optimum activity at 90°C and activity dropped above 100°C. Thermostability was examined by incubating the recombinant BlAmy-opt and deglycosylated recombinant BlAmy at different temperatures for 1 h, and the residual activity was measured at 70°C under the conditions mentioned above. The activity of the recombinant BlAmy-opt was almost not affected by a temperature below 70°C, but it decreased dramatically when the temperature was above 90°C. The thermostability of BlAmy was higher than deglycosylated recombinant BlAmy. BlAmy showed 71% and 53% residual activity after 1 h incubation at 90 and 100°C, whereas deglycosylated recombinant BlAmy showed only 62% and 43%.

Conflict of Interests

The authors declared that they have no conflict of interests.

Authors’ Contribution

Jian-Rong Wang and Yang-Yuan Li contributed equally to this paper.
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