Improvement of thermal stability of β-1,3-1,4-glucanase from *Bispora* sp. MEY-1 by directed evolution

Yanghao Hu[1], Wenxin Zhang [1], Ziqian Zha [1], Yan Ge [1], Xue Wang [1], Sheng Zhang [1], Jun Wang [1, 2], Shuai You[1,2,*]

([1] School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, China;

[2] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu 212018, China.)

Keywords: glucanase, thermostability, directed evolution, feed.

Presenting author email: youshuai@just.edu.cn

β-D-glucans consist of a heterogeneous group of glucose polymers, comprising β-D-glycosyl residues linked via β-glycosidic bonds, present as structural elements in the cell walls of yeast, fungi and cereal. According to the type of glycosidic bond in a β-glucan, It can be divided into β-1,4-glucan (cellulose), β-1,3-1,4-glucan (lichen chitosan), β-1,3 (4)-glucan, and β-1,3-glucan (seaweed polysaccharide) [1].

β-1,3-1,4-Glucanase produced by bacteria, fungi, plants and animals (or lichenanase; EC 3.2.1.73) is one of the major enzymes used in industry in recent decades. β-1,3-1,4-Glucanase can hydrolyze glucans containing 1,3 and 1,4 bonds, such as barley glucan and lichen polysaccharide. Currently, glucanase produced by microorganisms has potential applications in beer brewing, feed, food and washing industries [2].

In order to obtain glucanase resistant to high temperature, the most common methods are to excavate new genes from thermophilic bacteria or to modify their protein structure by enzyme engineering. Thermophilic fungi *Penicillium* sp. SPC-F 20 (60°C), Thermoascus aurantiacus CBMAI-756 (65°C), and Sclerotium rolfsii (60°C) have been identified as the most excellent microbial sources of thermostable Glucanase. Currently, a variety of protein engineering strategies have been adopted. The T_m value of xylanase was increased by 25°C by directional evolution. The half-life of glucanase was extended three times by surface charge optimization strategy. B-factor value optimization, increasing hydrophobic forces and introducing disulfide bonds are also common methods to improve the thermal stability of enzymes. However, the above methods improve the thermal stability of the enzyme by enhancing the rigidity of the enzyme structure, while losing the catalytic activity of the enzyme [3]. Therefore, how to obtain glucanase with improved thermal stability and catalytic efficiency is very important for the industrial application of enzyme.

In this study, BisGlu16B_ΔC, derived from *Bispora* sp. MEY-1, with catalytic efficiency of 18000 $mL \cdot s^{-1} \cdot mg^{-1}$, was used as the starting material to improve its thermal stability through errble-prone PCR strategy. The obtained dominant mutants with improved thermal stability were tested and compared for their enzymatic properties. This study provided reference for the industrial production of glucanase and its application in feed.

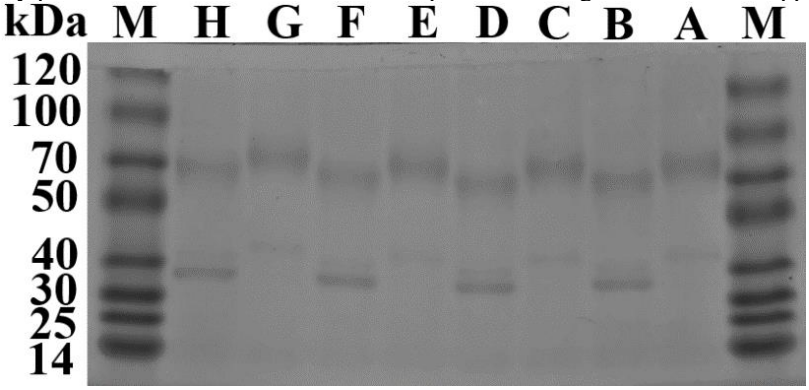

Fig. 1 SDS–PAGE analysis of the purified BisGlu16B_ΔN and its three mutants. Lanes: M, the standard protein molecular weight markers; A, C, E, and G: the purified WT, and three mutants T40K, Q53L, S311Y; B, D, F, and H: deglycosylated the WT and three mutants.

The yeast expression mutant library was constructed by error-prone PCR, and the thermostability of the three mutants T40K, Q53L, and S311Y were confirmed to be significantly higher than that of the WT by high-throughput screening under high temperature conditions. After purifying the WT and three mutants, the purity of glucanase was more than 95%, and the molecular weight of glucanase decreased significantly after the removal of N-glycogroup (Fig. 1). The enzymatic properties of WT and mutant were determined by using lichen polysaccharide as substrate. As shown in Fig. 2A, The optimal pH of wild-type WT and the three mutants was 4.0, and the pH range was similar, maintaining more than 40% relative enzyme activity between ph2.5-5.0. In terms of pH stability, the pH stability of the three mutants in alkaline environment (pH7.0-9.0) was significantly better than that of the wild type. For example, at pH8.0, the relative enzyme activity of all three mutants was above 75%, while that of the wild type was only 20%. The optimal temperature of T40K and S311Y was 60℃, but the optimal temperature of Q53L was 70℃, 10℃ higher than that of wild type, and the relative enzyme activity of Q53L was 26% higher

than that of wild type (1%) at 75℃. In terms of thermal stability, the half-lives of T40K, Q53L and S311Y at 60℃ were 51 min, 240 min and 66 min, respectively, which were 35 min, 224 min and 50 min longer than those of wild type (16 min), respectively. The three mutants showed great application potential in feed industry because of their good thermal stability.

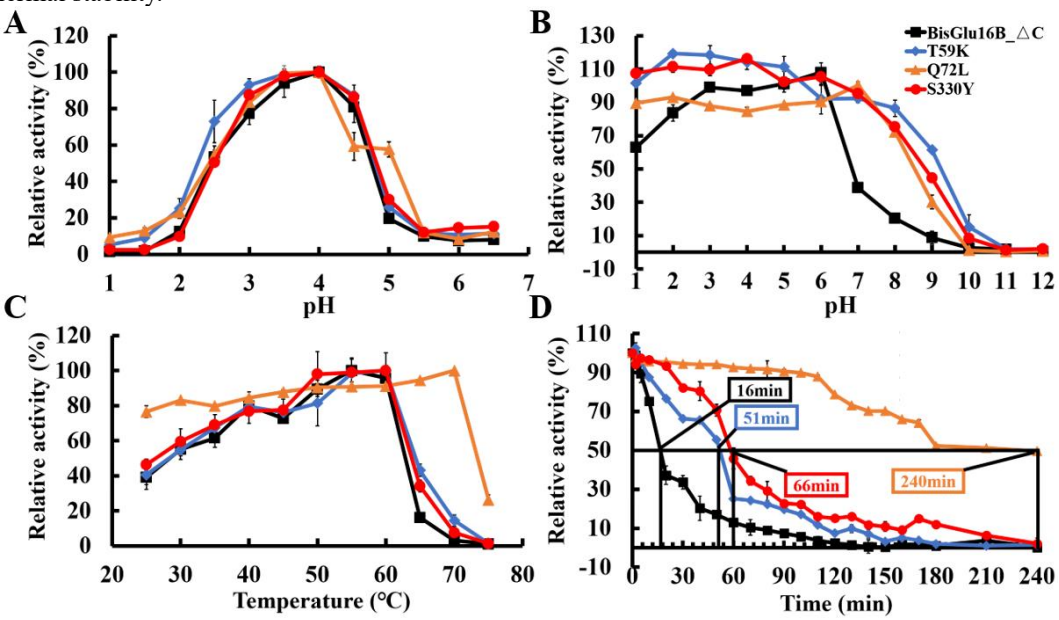

Fig. 2 Properties of the purified recombinant WT and its three mutants. A pH–activity profiles of each enzyme tested at the respective optimal temperatures; B pH–stability profiles. After the enzymes were incubated for 1 h at 37 °C in buffers with pH ranging from 1.0 to 12.0, the residual activities were determined in 100 mM McIlvaine buffer at the optimal temperature and pH of each enzyme; C temperature–activity profiles tested at pH4.0 of each enzyme; D half-lives of the WT and its mutants at 60 °C.

The kinetic parameters and specific activity of glucanase were determined by using lichenin as substrate at pH4.0 and 55℃. As shown in Table 1, the specific activity and catalytic efficiency of the mutant Q53L were 65% and 22% higher than those of the wt, respectively. The enzyme activities of the other two mutants A43P and T59I were not significantly different from those of the WT.

Table 1 Kinetic parameters and specific activity of glucanases against lichenin at 55 °C.

Enzymes	K_m (mg/mL)	V_{max} (μmol/min·mg)	k_{cat}/K_m (mL/s·mg)	Specific activity (U/mg)
WT	3.14 ± 0.29	64600 ± 4400	11900 ± 260	62000 ± 1300
T40K	2.63 ± 0.24	43200 ± 2100	9500 ± 410	46000 ± 2500
Q53L	3.98 ± 0.73	99600 ± 7400	14500 ± 840	102000 ± 830
S311Y	2.76 ± 0.09	60300 ± 1500	12700 ± 150	60000 ± 960

In this study, the thermostability of GH16 β-1,3-1,4-glucanase was improved by directed evolution for the first time. Three mutants with improved thermostability were obtained by high-throughput screening. Enzymatic properties showed that the thermostability and catalytic activity of mutant G165K were improved simultaneously. The dominant mutants obtained in this study show great application potential in the feed industry, and provide a strong reference for the molecular improvement of enzymes.

Acknowledgments This work was supported by the Natural Science Foundation of Jiangsu Province (BK20190957), the National Natural Science Foundation of China (21978121), China Agriculture Research System of MOF and MARA, and Key Projects of International Scientific and Technological Innovation Cooperation of National Key R&D Program of China (2021YFE0111100).

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