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Section in which the paper is to be considered: Microbial and Enzyme Technology
Construction of a highly thermostable 1,3-1,4-β-glucanase by combinational mutagenesis and its
potential application in the brewing industry

Chengtuo Niu^{a,b,c} Linjiang Zhu^{a,b} Annie Hill^c R. Alex Speers^c Qi Li^{a,b,*}

^a Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology,
Jiangnan University, Wuxi 214122, China

^b Lab of Brewing Science and Technology, School of Biotechnology,
Jiangnan University, Wuxi 214122, China

^c International Center for Brewing and Distilling,
Heriot-Watt University, Edinburgh EH14 4AS, United Kingdom

*Correspondence to: Qi Li, School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi,
Jiangsu, China 214122 . E-mail: liqi@jiangnan.edu.cn
Tel: +86-510-85918176. Fax: +86-510-85805219.

22 **Abstract**

23 Objectives

24 To improve the thermostability and catalytic property of a mesophilic 1,3-1,4- β -glucanase by
25 combinational mutagenesis and to test its effect in congress mashing.

26 Results

27 The mutant β -glucanase (rE-BglTO) constructed by combinational mutagenesis showed a 25°C
28 increase in optimal temperature, a 19.5°C rise in T_{50} value and a 15.6°C increase in melting temperature
29 (T_m) compared to wild-type enzyme. Its half-life values at 60°C and 70°C were 151.9 min and 98.8 min,
30 which were 367% and 798% higher than those of wild-type enzyme. Besides, its specific activity and
31 k_{cat} value were 4273.7 U mg⁻¹ and 188.6 s⁻¹ while its stability under acidic conditions was also
32 improved. In flask fermentation, the catalytic activity of rE-BglTO reached 2380.9 U ml⁻¹, which was
33 62.9% higher than that of wild-type enzyme. The addition of rE-BglTO in congress mashing decreased
34 the filtration time and viscosity by 21.3% and 9.6%, respectively.

35 Conclusions

36 The mutant β -glucanase showed high catalytic activity and thermostability which indicated that
37 rE-BglTO is a good candidate for application in the brewing industry.

38 **Key words:** 1,3-1,4- β -glucanase; catalytic property; combinational mutagenesis; congress mashing;

39 thermostability

40 **Introduction**

41 1,3-1,4- β -glucanases can hydrolyze β -glucans into oligosaccharides by cleaving the
42 1,4- β -glycosidic bonds in a strict manner (Planas 2000). In the brewing industry uses cereals as raw
43 materials and β -glucanases with high catalytic efficiency and thermostability are required to degrade
44 the β -glucans in the cereal cell walls. This increases extract yields and reduces filtration times in
45 mashing (Chaari et al. 2014) and reduces the chance of haze development in packaging (Jin et al. 2004).
46 The optimal temperatures of *Bacillus* β -glucanases are usually around 45-65°C, which are not able to
47 withstand the heat environment in industry (Planas 2000). Though some thermophilic β -glucanases
48 from thermophiles can hold their optimal temperatures around 80°C (Schimming et al. 1991), their
49 catalytic activities are not suitable for application under industrial conditions with relatively lower
50 temperatures. Besides, the catalytic activities of most β -glucanases from wild-type microbes are usually
51 low (Yang et al. 2008). Therefore, enhancing the thermostability and catalytic activity of mesophilic
52 β -glucanases is required.

53 To enhance the thermostability of β -glucanase, various enzyme engineering strategies, such as
54 directed evolution (Mao et al. 2016) and hybridization (Olsen et al. 1991), were adopted. However,
55 more thermostable β -glucanases are required to meet brewing standard. In addition to thermostability,
56 the yield of β -glucanases from wild-type microbes is usually low, even with process optimization and
57 control (Yang et al. 2008). Therefore, improving the yield of recombinant β -glucanase is important for
58 its application in industry. A combination of thermostability beneficial mutations was reported to be
59 able to further improve the protein thermostability (Zhang et al. 2010). In our previous researches, the
60 thermostability of a mesophilic β -glucanase gene (*bglT*) from *Bacillus terquilensis* CGX 5-2 was
61 improved by lysine-based site-directed mutagenesis (Niu et al. 2015), disulfide bond engineering (Niu

62 et al. 2016) and site-saturation mutagenesis, respectively. In order to further enhance its
63 thermostability, the above beneficial sites were combinational mutated within a mesophilic β -glucanase
64 (BglT) in this study. The optimized gene (*rE-BglTO*)– was constructed by gene synthesis and
65 expressed in *Escherichia coli* BL21(DE3). Its thermostability and catalytic properties were
66 characterized and compared to wild-type enzyme. The effect of rE-BglTO in Congress mashing was
67 determined and compared to commercial enzymes.

68 **Materials and Methods**

69 **Clone Cloning, expression and purification of β -glucanase in *E. coli***

70 The *rE-BglTO* gene which harbored the eleven beneficial mutation, including K20S, N31C, S40E,
71 S43E, E46P, P102C, K117S, N125C, K165S, T187C and H205P, was synthesized (the amino acid
72 sequences of the *BglT* gene and *rE-BglTO* gene were shown in Supplementary Figure 1) and ligated
73 into vector recombinant plasmid pUC57-*rE-BglTO* synthesized by Genewiz (Suzhou, China). The
74 recombinant plasmid pUC57-*rE-BglTO* was digested by the restriction enzymes *Bam*HI and *Xho*I,
75 ligated into vector **pET28a(+)** and transformed into *E. coli* BL21(DE3) competent cells. The positive
76 clones were verified by DNA sequencing analysis. The recombinant *E. coli* cells were cultivated in
77 optimized TB media (20g yeast extract/l; 12.5 g tryptone/l; 14.1 ml glycerol/l; 2.17 g KH₂PO₄/l and
78 2.74 g K₂HPO₄/l) at 37°C. The expression and purification of recombinant β -glucanase were conducted
79 through a Ni-NTA affinity column (Qiagen) according to previous reported methods (Niu et al. 2015).
80 The purity of enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
81 (SDS-PAGE). The protein concentration was estimated by the Bradford method with bovine serum
82 albumin (BSA) as standard.

83 **Activity assay of 1,3-1,4- β -glucanase**

84 The activity of 1,3-1,4- β -glucanase was measured using the improved AZO method (Niu et al.
85 2014) using 1% AZO barley β -glucan (Megazyme, Wicklow, Ireland) as the substrate. The reducing
86 sugar content was measured using the 3,5-dinitrosalicylic acid (DNS) method. One unit of the
87 1,3-1,4- β -glucanase activity was defined as the amount of enzyme that released 1 μ mol reducing sugar
88 from the substrate per minute at pH 6.5. Each measurement was repeated in triplicate. The kinetic
89 parameters of β -glucanase were determined at 40°C and estimated by Eadie-Hofstee plots.

90 **Effects of temperature, pH, metal ions and inhibitors on enzymatic activity and stability**

91 The optimal temperature and pH were determined by measuring the enzymatic activities at a range
92 of parameters while the other conditions remained constant. The pH stability and thermostability were
93 conducted according to a previous reported method (Niu et al. 2016). The midpoint temperature (T_{50})
94 value was defined as the temperature at which β -glucanase lost half of its activity after treatment at
95 40-90°C for 10 min. The half-life value of β -glucanase, designated as $t_{(1/2, X^{\circ}\text{C})}$, was defined the time that
96 the β -glucanase activity decreased to 50% at set temperatures. The melting temperature (T_m), ΔH and
97 ΔG values were determined by a Q2000 differential scanning calorimeter (DSC) (TA, New castle,
98 USA) at a protein concentration of 1 mg ml⁻¹ in 20 mM phosphate buffer (pH6.5).

99 To estimate the effect of metal ions and inhibitors on enzyme activity, the purified β -glucanase
100 was incubated in different metal ion solutions, including KCl, NaCl, CaCl₂, MgCl₂, FeCl₂, FeCl₃,
101 CoCl₂, ZnCl₂, MnCl₂ and CuCl₂ (1 mM and 10 mM) and inhibitor solutions, including EDTA, DTT
102 and β -mercaptoethanol (1 mM and 10 mM) at 40°C for 1 h. After that, the residual activities were
103 measured and compared with the activities of enzyme without any treatment.

104 **Far-UV circular dichroism (CD) analysis and 3D structure analysis**

105 The far-UV spectra of β -glucanases was measured from 190 nm to 250 nm by a MOS-45 circular

106 dichroism spectrometer (Bio-logic, Claix, France). The proportions of secondary structure elements
107 (helix, β -sheet, β -turn and random coil) were estimated by the Dichroism online server (Whitmore and
108 Wallace 2008). The 3D structures of the wild-type BglT and rE-BglTO were homologically modeled in
109 SWISS-MODEL online server with the 3D structure of β -glucanase from *B.subtilis* (PDB code: 3O5S) as
110 template. The amino acid sequence identities between them were 99.53% and 94.37%, respectively.

111 **Effects of β -glucanase on the filtration time and viscosity of mash**

112 Pilsner malt (20 g) was ground to grind 7 in a DLFU disc mill (Bühler Miag, London, UK) and
113 suspended in 100 ml of pre-heated water (45°C). This mash was started with the addition of 1.0 ml
114 wild-type BglT, rE-BglTO or a commercial enzyme (Ultraflo[®]L, Novozymes, Denmark) through EBC
115 standard Congress mashing process (the mashing temperature was maintained at 45°C for 30 min, then
116 raised to 70°C at a rate of 1°C min⁻¹ and added 100 mL water in the mash) (4.5.1 Analytical-EBC 1997).

117 A similar control with 1 ml water instead of enzyme solution was used. The filtration time was
118 determined by filtering 20 ml cooled mash through a quantitative filter paper (Grade 113V, GE
119 Healthcare, Freiburg, Germany) while the filtration of mash without enzyme treatment was used as
120 control. The reduction of filtration time was calculated using the following equation:

$$121 \quad \Delta\psi = (\psi_{\text{control}} - \psi) \times 100 / \psi_{\text{control}}$$

122 where ψ and ψ_{control} are the total flow time of mash with or without enzyme treatment and $\Delta\psi$ is the
123 filtration time reduction.

124 The mash viscosity was determined at 20°C by Wells-Brookfield Cone/Plate digital viscometer
125 (Brookfield AMETEK, MA, USA) with Cone CP-40 at a shear rate of 90 s⁻¹ (Speers et al. 2004). The
126 viscosity reduction was calculated using the following equation:

127
$$\Delta\mu = (\mu_{\text{control}} - \mu) \times 100 / \mu_{\text{control}}$$

128 where μ and μ_{control} are the viscosity of mash with or without enzyme treatment and $\Delta\mu$ is viscosity
129 reduction.

130 **Results and discussion**

131 **Production of recombinant β -glucanase**

132 The optimized gene *rE-BglTO* which harbored K20S, N31C, S40E, S43E, E46P, P102C, K117S,
133 N125C, K165S, ~~F187S-T187C~~ and H205P mutation sites was synthesized, ligated into **pET28a(+)**
134 vector and transformed into *E.coli* BL21(DE3) competent cells. Fig.1a showed that the growth curve of Figure 1
135 recombinant *E.coli* harboring *rE-BglTO* gene was ~~almost the same with~~ similar to that of recombinant *E.*
136 *coli* harboring *BglT* gene. After 9 h shaking at 37°C, the dry cell weight values of the recombinant *E.*
137 *coli* cells harboring *rE-BglTO* and *BglT* gene were ~~both around 2.7-883~~ g l⁻¹ and 2.814 g l⁻¹,
138 respectively, which were slightly higher than that of wild-type *E.coli* cells (2.514 g L⁻¹). The
139 recombinant *E. coli* cells began to secrete β -glucanases when the inducers were added into the media.
140 Fig.1b showed that the highest enzymatic activity of rE-BglTO was 2380.9 U ml⁻¹ when induced ~~for at~~
141 6 h, which was 62.9% higher than that of wild-type BglT (1461.3 U ml⁻¹).

142 The recombinant wild-type BglT and rE-BglTO were then purified and verified by SDS-PAGE
143 analysis (Supplementary Figure ~~4~~2). The catalytic properties of the wild-type BglT and rE-BglTO were
144 determined and compared (Table 1). The specific activity of rE-BglTO was 4273.7 U mg⁻¹, which was Table 1
145 71.6% higher than that of the wild-type BglT (2490.1 U mg⁻¹). The K_m value for rE-BglTO (0.273 mM)
146 was slightly lower than that of the wild-type BglT (0.297 mM), which indicated that rE-BglTO had
147 better binding affinity with substrate barley β -glucan than the wild-type BglT. Besides, the k_{cat} and
148 k_{cat}/K_m values of rE-BglTO were 188.6 s⁻¹ and 690.8 s⁻¹ mM⁻¹, respectively, which were 37.3% and 49.4%

149 higher than those of the wild-type BglT. These results indicated that the combination of beneficial
150 mutations could greatly enhance the catalytic efficiency and binding affinity of β -glucanase.

151 **Effects of temperature and pH on stability of β -glucanase**

152 As shown in Fig.2a, the optimal temperature of rE-BglTO was 70°C, which was 25°C higher than
153 that of the wild-type BglT (45°C). rE-BglTO could maintain more than 80% activity between 35-70°C,
154 while the wild-type BglT could only maintain high activity between 35-55°C. rE-BglTO also showed
155 an improvement in kinetic stability, since its T_{50} value was 18.5°C higher than that of the wild-type
156 BglT (Fig.2b). The changes in enzyme kinetic stability was further confirmed by the half-life values.
157 Fig.2c showed that the wild-type BglT was almost fully inactivated after incubation at 60°C for 140
158 min while rE-BglTO still could maintain around 60% activity after the same treatment. At 70°C, the
159 wild-type BglT was soon inactivated while rE-BglTO still could have more than 60% activity left after
160 80 min treatment (Fig.2d). The half-life values of rE-BglTO at 60°C and 70°C were 151.9 min and 98.8
161 min, respectively, which were 367% and 798% higher than those of the wild-type BglT (32.5 min and
162 11 min). As for enzyme thermodynamic stability, the T_m value of rE-BglTO was 55.9°C, which was
163 15.6°C higher than that of the wild-type BglT (Table 2). DSC method also revealed some
164 thermodynamic stability related parameters. As shown in Table 2, the ΔH value of rE-BglTO was
165 much higher than that of the wild-type BglT, which indicated that more energy was required for
166 denaturation of rE-BglTO. The ΔG value for rE-BglTO was 4.4 kcal mol⁻¹ higher than that for the
167 wild-type BglT. The kinetic and thermodynamic parameters of rE-BglTO were also higher than those
168 of mutants previously reported (Fig.2 and Table 2), which~~These results~~ indicated that the combinational
169 mutagenesis could greatly enhance the kinetic and thermodynamic stability of β -glucanase.

170 The optimal pH value of rE-BglTO was shifted from pH6.5 to pH6.0 (Fig.3). It also showed better

Figure 2

Table 2

Figure 3

171 stability at acidic environments. There were still 74.8% and 92% activities left for rE-BglTO after one
172 hour incubation at pH4.5 and pH5.5 while the remaining activities for the wild-type BglT were 7.9%
173 and 59.9%, respectively. This could improve the performance of rE-BglTO in mashing between mash is
174 a weakly acidic solution (pH5-5.5).

175 **Effects of metal ions and inhibitors on stability of β -glucanase**

176 Metal ions and chemical reagents have been shown to play key roles in protein folding and
177 catalysis (Andreini et al. 2008). The effect of various cations and inhibitors on the activity of the
178 wild-type BglT and rE-BglTO was tested. As shown in Table 3, the presence of Fe^{2+} (10 mM) and Fe^{3+}
179 (10 mM) both strongly inhibited the activities of the wild-type BglT (8.4% and 6.5%) and rE-BglTO
180 (10.4% and 3.5), respectively, while the enzymes were moderately inhibited by Ca^{2+} (84.2% and
181 86.4%), Co^{2+} (93.9% and 94.7%), Li^{2+} (51.3% and 52.0%), Cu^{2+} (86.2% and 89.7%) and Zn^{2+} (47.6%
182 and 43.6%) when the cations were present at 10 mM. Meanwhile, the presence of Cu^{2+} could greatly
183 activate the activities of the wild-type BglT and rE-BglTO to 167.4% and 169.4% at 1 mM. The
184 presence of Mn^{2+} could enhance the rE-BglTO activity to 123.9% at 1 mM while the activity of the
185 wild-type BglT was moderately inhibited to 80.4%. The activities of both enzymes were almost
186 uninfluenced by K^+ , Na^+ and NH_4^+ . Among the inhibitors, the enzymatic activities of the wild-type
187 BglT and rE-BglTO were both greatly inhibited by DTT (34.6% and 30.6%), EDTA (30.9% and 16.7%)
188 and β -mercaptoethanol (40.9% and 30.3%) at 10 mM.

Table 3

189 **Effect of β -glucanase on the filtration time and viscosity of mash**

190 The filtration time and viscosity of mash after treatment with the wild-type BglT and rE-BglTO
191 were measured and compared with a commercial β -glucanase. As shown in Table 4, the addition of the

Table 4

192 wild-type BglT, rE-BglTO and the commercial enzyme reduced the mash filtration time by 8.4%, 21.3%
193 and 16.8%, respectively. The wild-type BglT, rE-BglTO and the commercial enzyme also reduced the
194 mash viscosity by 3.5%, 9.6% and 7.9%. This indicated that the performance of rE-BglTO in β -glucan
195 degradation in mashing was superior to the wild-type BglT and the commercial enzyme.

196 **Conformational changes in molecular structures of β -glucanase**

197 To understand the mechanism for the thermostability improvement, the secondary structures of the
198 wild-type BglT and rE-BglTO were analyzed by CD. Fig.4 showed that both the positive and negative
199 bands in CD spectrum of rE-BglTO shifted to larger wavelengths compared to the wild-type BglT. This
200 indicated that rE-BglTO might had more β -sheet structures, which was confirmed by the secondary
201 structure percentage analysis (Supplementary Table 1). The proportions of helix, β -sheet and β -turn in
202 rE-BglTO were increased by 1%, 12% and 5% compared to the wild-type BglT, respectively, while the
203 percentage of random coil was decreased from 39% to 21%. The results of secondary structures were
204 further confirmed by the 3D structures of wild-type BglT and rE-BglTO. Fig.5 showed that the 3D

Figure 4

205 structures of the wild-type BglT and rE-BglTO was both the identical ~~anti-parallel β -sheet~~
206 ~~jelly-roll β -sandwich structure with anti-parallel β -sheets~~. However, several different places were
207 observed. Two helix structures α 1 (residues No.36-38) and α 2 (residues No.97-99) were lost in
208 rE-BglTO while two new helix structures α 3 (residues No.140-142) and α 4 (residues No.190-193)
209 were formed. Moreover, three new β -sheet structures β 1 (residues No.6-8), β 2 (residues No.18-20) and
210 β 3 (residues No.60-66) were formed while eight β -sheet structures were prolonged (residues No.52 in
211 β 4, —No.73-72 in β 5, No.92-95 in β 6, No.104-107 in β 7, No.122-124 in β 8, No.150-152 in β 9,
212 No.178-180 in β 10 and —No.211-213 in β 11). More structured residues protein structure could result in
213 more rigid protein overall and local structure, which might be the reason for the enhancement of

Figure 5

214 enzyme thermostability (Di Marino et al. 2014). Several residues related to β -glucanase substrate
215 binding, such as Glu63, Arg65, Phe92, Tyr94, Glu105, Asn121 and Tyr123, were also located in the
216 transformed regions. The reason for the enhancement of catalytic properties might be the large-scale
217 secondary structure changes in the substrate binding region, especially Glu105 which was reported to
218 be the key catalytic nucleophile in β -glucanase catalysis process (Planas 2000). More negative surface
219 charge was observed in rE-BglTO compared to the wild-type BglT (Supplementary Figure 23). This
220 might be the reason for the shift of optimal pH and better stability in acidic environment since
221 acidophilic enzymes were reported to contain more acidic residues on their surface (Michaux et al.
222 2010).

223 **Conclusion**

224 In this study, a highly thermostable 1,3-1,4- β -glucanase (rE-BglTO) was constructed by
225 combination of eleven beneficial mutations. The optimal temperature and melting temperature of
226 rE-BglTO were 25°C and 15.6°C higher than those of the wild-type BglT. Its half-life values at 60°C
227 and 70°C were 367% and 798% higher than those of the wild-type BglT. The catalytic activity of
228 rE-BglTO could reach 2380.9 U ml⁻¹, which was 62.9% higher than that of the wild-type BglT. The
229 mutant enzyme also showed good ability to reduce the viscosity and filtration time of mash, indicating
230 its potential value for application in the brewing industry.

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241 **Supporting information**

242 [Supplementary Fig.1-Amino acid sequences of the *BglT* gene and *rE-BglTO* gene.](#)

243 Supplementary Fig.[42](#)-SDS-PAGE analysis of purified [the](#) wild-type and recombinant

244 1,3-1,4- β -glucanases.

245 Supplementary Table 1-Comparison of secondary structure between [the](#) wild-type BglT and rE-BglTO

246 using Dichroweb online software.

247 Supplementary Fig.[23](#)-Comparison of electrostatic surface potential of [the](#) wild-type BglT and

248 rE-BglTO.

249 **References**

- 250 Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM (2008) Metal ions in biological catalysis:
251 from enzyme databases to general principles. *J Biol Inorg Chem* 13:1205-1218. doi:
252 10.1007/s00775-008-0404-5
- 253 Chaari F, Belghith-Fendri L, Blibech M, Driss D, Ellouzi SZ, Ellouz-Chaabouni S (2014) Biochemical
254 characterization of a lichenase from *Penicillium occitanis* Pol6 and its potential application in
255 the brewing industry. *Process Biochem* 49:1040-1046. doi: 10.1016/j.procbio.2014.02.023
- 256 Di Marino D, Achsel T, Lacoux C, Falconi M, Bagni C (2014) Molecular dynamics simulations show
257 how the FMRP Ile304Asn mutation destabilizes the KH2 domain structure and affects its
258 function. *J Biomol Struct Dyn* 32:337-350. doi: 10.1080/07391102.2013.768552
- 259 Jin YL, Speers A, Paulson AT, Stewart RJ (2004) Effects of β -glucans and environmental factors on the
260 viscosities of wort and beer. *J Inst Brew* 110:104-116. doi:
261 10.1002/j.2050-0416.2004.tb00189.x
- 262 Mao S, Gao P, Lu Z, Lu F, Zhang C, Zhao H, Bie X (2016) Engineering of a thermostable
263 β -1,3-1,4-glucanase from *Bacillus altitudinis* YC-9 to improve its catalytic efficiency. *J Sci*
264 *Food Agr* 96:109-115. doi: 10.1002/jsfa.7066
- 265 Michaux C, Pouyez J, Mayard A, Vandurm P, Housen I, Wouters J (2010) Structural insights into the
266 acidophilic pH adaptation of a novel endo-1,4- β -xylanase from *Scytalidium acidophilum*.
267 *Biochimie* 92:1407-1415. doi: 10.1016/j.biochi.2010.07.003
- 268 Niu C, Zhu L, Wang J, Li Q (2014) Simultaneous enhanced catalytic activity and thermostability of a
269 1,3-1,4- β -glucanase from *Bacillus amyloliqueformis* by chemical modification of lysine
270 residues. *Biotechnol Lett* 36:2453-2460. doi: 10.1007/s10529-014-1616-0

271 Niu C, Zhu L, Xu X, Li Q (2016) Rational design of disulfide bonds increases thermostability of a
272 mesophilic 1,3-1,4- β -glucanase from *Bacillus terquilensis*. Plos One 11:e0154036. doi:
273 10.1371/journal.pone.0154036

274 Niu C, Zhu L, Zhu P, Li Q (2015) Lysine-based site-directed mutagenesis increased rigid β -sheet
275 structure and thermostability of mesophilic 1,3-1,4- β -glucanase. J Agr Food Chem
276 63:5249-5256. doi: 10.1021/acs.jafc.5b00480

277 Olsen O, Borriss R, Simon O, Thomsen KK (1991) Hybrid *Bacillus* (1-3,1-4)- β -glucanases:
278 engineering thermostable enzymes by construction of hybrid genes. Mol Gen Genet
279 225:177-185. doi: 10.1007/BF00269845

280 Planas A (2000) Bacterial 1 3-1,4- β -glucanases: structure, function and protein engineering.
281 BBA-Protein Struct Mol Enzymol 1543:361-382. doi: 10.1016/S0167-4838(00)00231-4

282 Schimming S, Schwarz WH, Staudenbauer WL (1991) Properties of a thermoactive
283 β -1,3-1,4-glucanase (lichenase) from *Clostridium thermocellum* expressed in *Escherichia coli*.
284 Biochem Biophys Res Commun 177:447-452. doi: 10.1016/0006-291X(91)92004-4

285 Speers R, Patelakis S, Paulson A, Oonsivilai R (2004) Shear rates during brewing operations. Tech
286 Quart-Master Brewer Assn Am 41:241-247.

287 Whitmore L, Wallace BA (2008) Protein secondary structure analyses from circular dichroism
288 spectroscopy: methods and reference databases. Biopolymers 89:392-400. doi:
289 10.1002/bip.20853

290 Yang S, Qiaojuan Y, Jiang Z, Fan G, Wang L (2008) Biochemical characterization of a novel
291 thermostable β -1,3-1,4-glucanase (lichenase) from *Paecilomyces thermophila*. J Agr Food
292 Chem 56:5345-5351. doi: 10.1021/jf800303b

- 293 Zhang Z-G, Yi Z-L, Pei X-Q, Wu Z-L (2010) Improving the thermostability of *Geobacillus*
294 *stearothermophilus* xylanase XT6 by directed evolution and site-directed mutagenesis.
295 *Bioresource Technol* 101:9272-9278. doi: 10.1016/j.biortech.2010.07.060

296 Table 1 Catalytic properties of the wild-type BglT, K20S/K117S/K165S mutant,
 297 N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO

Enzymes	Specific activity (U mg ⁻¹)	K_m (mM) ^a	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
BglT	2490.1±55.3	0.297±0.005	137.4±1.9	462.5±19.1
<u>K20S/K117S/K165S</u>	<u>3936.4±39.6</u>	<u>0.278±0.004</u>	<u>184.9±2.7</u>	<u>665.2±19.7</u>
<u>N31C-T187C/P102C-N125C</u>	<u>4045.4±47.9</u>	<u>0.277±0.004</u>	<u>186.3±2.2</u>	<u>672.6±17.7</u>
<u>E46P/S43E/H205P/S40E</u>	<u>4093.8±61.1</u>	<u>0.271±0.003</u>	<u>187.4±1.7</u>	<u>691.5±13.6</u>
rE-BglTO	4273.7±26.9	0.273±0.003	188.6±1.3	690.8±12.5

298 ^a The K_m value was calculated according to the Eadie-Hofstee plots. The enzyme concentration was 100
 299 $\mu\text{g ml}^{-1}$ while the substrate concentration varied from 1 to 10 mg ml^{-1} . The experiments were repeated
 300 in triplicate

301 Table 2 Thermodynamic stability parameters of the wild-type BglT, K20S/K117S/K165S mutant,
 302 N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO. ~~The protein~~
 303 ~~melting temperature (T_m) and the thermodynamic stability parameters were determined by a Q2000~~
 304 ~~differential scanning calorimeter (DSC) (TA, New castle, USA) at a protein concentration of 1 mg ml⁻¹~~
 305 ~~in 20 mM phosphate buffer (pH6.5)~~

Enzymes	T_m (°C)	ΔH (kcal mol ⁻¹)	ΔG (kcal mol ⁻¹)
WT	40.3	173.5	6.3
<u>K20S/K117S/K165S</u>	<u>50.8</u>	<u>189.4</u>	<u>8.8</u>
<u>N31C-T187C/P102C-N125C</u>	<u>54.9</u>	<u>207.6</u>	<u>10.3</u>
<u>E46P/S43E/H205P/S40E</u>	<u>54.1</u>	<u>204.7</u>	<u>10.1</u>
rE-BglTO	55.9	205.1	10.7

306

307 Table 3 Effect of metal ions and inhibitors on activities of the wild-type BglT and rE-BglTO (CK are
 308 the activities of β -glucanase samples with same amount of buffer solution instead of the metal ion or
 309 inhibitors)

Metal ions/inhibitors	Relative activity of wild-type BglT (%)		Relative activity of rE-BglTO (%)	
	1 mM	10 mM	1 mM	10 mM
CK	100	100	100	100
CaCl ₂	75.3±1	84.2±1.2	83.1±1.0	86.4±0.8
CoCl ₂	83.8±0.7	93.9±1.2	81.6±0.9	94.7±1.3
CuCl ₂	167.4±1.1	86.2±0.9	169.4±1.3	89.7±0.7
FeCl ₂	44.4±0.8	8.4±0.1	48.7±1.2	10.4±0.7
FeCl ₃	28.7±0.3	6.5±1.1	19.8±0.3	3.5±0.2
KCl	101.3±0.9	99.9±0.3	100.7±0.6	100.1±0.4
LiCl ₂	82.1±0.8	51.3±1.4	84.9±0.7	52.0±1.1
MgCl ₂	189.4±0.6	50.0±0.5	189.6±0.7	53.4±1.1
MnCl ₂	80.4±1.1	55.2±0.6	123.9±1.2	63.9±0.8
NH ₄ Cl	98.7±0.5	100.3±0.4	100.3±0.3	102.1±0.6
NaCl	100.3±0.7	97.8±1.1	101.3±0.9	98.3±1.3
ZnCl ₂	55.8±1.2	47.6±0.4	50.9±0.6	43.6±0.3
DTT	79.8±0.7	34.6±0.9	73.1±0.7	30.6±0.7
EDTA	71.3±1.6	30.9±0.8	39.6±0.2	16.7±0.9
β - mercaptoethanol	73.1±1.2	40.9±1.6	68.7±1.0	30.3±0.8

310

311 Table 4 Effect of the wild-type BglT, rE-BglTO and a commercial β -glucanase (Ultraflo[®] L) on the
 312 viscosity and filtration time of mash

Samples	Filtration time (s)	Filtration time reduction (%)	Viscosity (mPa.s)	Viscosity reduction (%)
Control	202±3	-	1.14±0.01	-
BglT	185±3	8.4	1.10±0.01	3.5
rE-BglTO	159±2	21.3	1.03±0.01	9.6
Commercial enzyme	168±2	16.8	1.05±0.01	7.9

313

314 Figure legends

315 **Fig.1** The growth curves (a) and enzymatic activity curves (b) of the wild-type *E. coli*, recombinant *E.*
316 *coli* harboring the *BglT* and *rE-BglTO* genes. The growth curves were determined by cultivation of *E.*
317 *coli* cells at 37°C, 200 rpm. The enzymatic activities curves were determined by measuring the
318 enzymatic activities at 40°C-different time points since IPTG and α -lactose were added into the media.
319 The data was presented as mean \pm standard deviation from three independent experiments

320 **Fig.2** The optimal temperature curves (a), kinetic stability curves (b), the inactivation curves at 60°C (c)
321 and the inactivation curves at 70°C (d) of the wild-type *BglT*, K20S/K117S/K165S mutant,
322 N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and *rE-BglTO*. The optimal
323 temperature curves was determined by measuring the enzymatic activities at a temperature range of
324 45°C to 80°C with 5°C as interval. The kinetic stability curves was determined by measuring the
325 enzymatic activity after treatment from 40°C to 80°C for 10 min and following 10 min on ice. The
326 inactivation curves were determined by incubation the enzymes at 60°C and 70°C and enzymatic
327 activities were measured at a set of time points. Error bars indicated were obtained from the standard
328 deviation from triplicates. 100% activities of the wild-type *BglT*, K20S/K117S/K165S mutant,
329 N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and *rE-BglTO* were 1530.2 U
330 ml⁻¹, 1897.5 U ml⁻¹, 1903.4 U ml⁻¹, 2515.7 U ml⁻¹ and 2950.3 U ml⁻¹

331 **Fig.3** The optimal pH and pH stability of the wild-type *BglT*, K20S/K117S/K165S mutant,
332 N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and *rE-BglTO*. (a) The optimal
333 pH of the wild-type *BglT*, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant,
334 E46P/S43E/H205P/S40E mutant and *rE-BglTO* were determined by measuring the enzymatic activities
335 in acetic acid/sodium acetate buffer (pH4.0-6.0) and sodium phosphate buffer (pH6.0-8.5) at 40°C; (b)

336 The pH stability was calculated by dividing the residue activities of enzymes after 1 h treatment in
337 different pH buffers by enzymatic activities without any treatment. The data plotted were averages of
338 triplicate experiments. 100% activities of the wild-type BglT, K20S/K117S/K165S mutant,
339 N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO were 1461.3 U
340 ml⁻¹, 1220.1 U ml⁻¹, 1363.5 U ml⁻¹, 2103.8 U ml⁻¹ and 2380.9 U ml⁻¹

341 **Fig.4** The CD spectrums of the wild-type BglT and rE-BglTO in 20 mM sodium phosphate buffer
342 (pH6.5). The Far-UV CD spectrums were measured from 190 nm to 250 nm in a 0.1 cm path length
343 quartz cell with a resolution of 1 nm and corrected by subtracting the proper baseline. The
344 concentrations of β -glucanases were both 100 $\mu\text{g ml}^{-1}$

345 **Fig.5** Comparison of the 3D structures of the wild-type BglT and rE-BglTO. (a) the concave side of the
346 wild-type BglT; (b) the concave side of rE-BglTO; (c) the convex side of the wild-type BglT; (d) the
347 convex side of rE-BglTO. The helix, β -sheet and loop structures were colored red, yellow and green,
348 respectively. The calcium ion was shown in sphere (green dot). The eleven mutation sites were shown
349 in sticks and labeled. The labeled α and β represented the α -helix and β -sheet structures, respectively