Improving the Thermostability and Catalytic Efficiency of *Bacillus deramificans* Pullulanase by Site-Directed Mutagenesis

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Pullulanase (EC 3.2.1.41) is a well-known starch-debranching enzyme. Its instability and low catalytic efficiency are the major factors preventing its widespread application. To address these issues, Asp437 and Asp503 of the pullulanase from *Bacillus deramificans* were selected in this study as targets for site-directed mutagenesis based on a structure-guided consensus approach. Four mutants (carrying the mutations D503F, D437H, D503Y, and D437H/D503Y) were generated and characterized in detail. The results showed that the D503F, D437H, and D503Y mutants had an optimum temperature of 55°C and a pH optimum of 4.5, similar to that of the wild-type enzyme. However, the half-lives of the mutants at 60°C were twice as long as that of the wild-type enzyme. In addition, the D437H/D503Y double mutant displayed a larger shift in thermostability, with an optimal temperature of 60°C and a half-life at 60°C of more than 4.3-fold that of the wild-type enzyme. Kinetic studies showed that the K_m values for the D503F, D437H, D503Y, and D437H/D503Y mutants decreased by 7.1%, 11.4%, 41.4%, and 45.7% and the K_{cat}/K_m values increased by 10%, 20%, 140%, and 100%, respectively, compared to those of the wild-type enzyme. Mechanisms that could account for these enhancements were explored. Moreover, in conjunction with the enzyme glucoamylase, the D503Y and D437H/D503Y mutants exhibited an improved reaction rate and glucose yield during starch hydrolysis compared to those of the wild-type enzyme, confirming the enhanced properties of the mutants. The mutants generated in this study have potential applications in the starch industry.

Starch is the main component of many agricultural products, e.g., corn (maize), potatoes, rice, and wheat; it is one of the most common carbohydrates in nature. Generally, starch contains 20 to 25% amylose and 75 to 80% amylopectin. Amylopectin is a branched polysaccharide in which the linear chains of α -1,4-glucose moieties are joined by α -1,6-glucosidic linkages (1). Of the two components of starch, amylopectin presents the greatest challenge to amylolytic enzymes, since most of these enzymes are specific for α -1,4-glucosidic bonds whereas the α -1,6-glucosidic bonds must also be cleaved for complete utilization of the amylopectin.

Pullulanase (EC 3.2.1.41) is a well-known starch-debranching enzyme. It catalyzes the hydrolysis of the α -1,6 glycosidic linkages in pullulan, amylopectin, and the α - and β -limit dextrins of amylopectin. Pullulanase belongs to the α -amylase family. It was first isolated from a culture of *Klebsiella pneumoniae* by Wallenfels et al. in 1966 (2). To date, many microorganisms, including mesophiles, thermophiles, and hyperthermophiles, have been shown to produce pullulanase, and a large number of pullulanases have been identified and characterized (3–6). In addition, several pullulanase-encoding genes have been cloned and characterized (7–9), and their crystal structures have been determined (10–12).

Due to its ability to hydrolyze the α -1,6 linkages in amylopectin, there is a great commercial interest in potential industrial applications of pullulanase, most importantly the production of high-glucose syrup from starch. Generally, glucose syrup production requires two enzymatic hydrolysis processes: liquefaction and saccharification. During the saccharification process, the single glucose residues from the nonreducing ends of the dextrins, which usually contain both α -1,4-glucosidic linkages and the α -1,6-glucosidic bonds of amylopectin, are removed by glucoamylase (13). The addition of pullulanase,

which can hydrolyze the α -1,6-glucosidic bonds of the branched dextrins, would allow the amount of glucoamylase usage and saccharification time to be reduced and the substrate concentration and conversion rate to be improved. However, for this process to be successful, glucoamylase and pullulanase must act simultaneously, so the two enzymes must be compatible with respect to pH and temperature (13, 14). Currently, the saccharification reaction is usually carried out at 60°C and pH 4.5 over 48 to 60 h to maximize the activity of the glucoamylase; thus, a pullulanase with sufficient thermostability and high catalytic efficiency at 60°C is of special interest (15).

One way to obtain enzymes with desired characteristics is to reconstruct existing enzymes by protein engineering, which has been successfully utilized to enhance the stability or catalytic efficiency of many enzymes. Generally, protein engineering can be classified into three categories (16): rational design (12, 17, 18), irrational design (directed evolution) (19), and a semirational approach (20, 21). Of the rational design methods, a structure-guided consensus approach combines information about the quaternary structure with knowledge of a few sequences that have moderate identity to reduce the number of target residues in a given protein to be mutated. At present, it is considered an effi-

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cient and reliable method for improving enzymatic properties (16, 22–25).

Previously, pullulanase from *Bacillus deramificans* was expressed and characterized in our laboratory, and the results showed that the recombinant enzyme was not stable at 60°C (data not shown), which reduced its practical debranching efficiency in the coupled system. In order to improve the efficiency of the enzyme, in the present study, the structure-guided consensus approach was used to identify promising residues in the conserved region and adjacent site that might improve the thermostability of *B. deramificans* pullulanase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and enzymes. The pullulanase gene (GenBank accession number CAC60157) from *B. deramificans* was synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China), based on the preferred codon usage of *Escherichia coli*. Plasmid pET-20b(+) was used for subcloning, *E. coli* JM109 was the host for cloning work, and *E. coli* BL21(DE3) was the host for the expression of the pullulanase. The α -amylase used was Termamyl120L from *Bacillus licheniformis* (Novozymes, Denmark). Glucoamylase was a commercial Weimei from Sunhy Biology Co., Ltd. (Wuhan, China).

Design and construction of site-directed mutants. ClustalW was utilized for multiple sequence alignment (26). The theoretical structures of the wild-type and mutant pullulanases were obtained by homology modeling from the SwissModel protein-modeling server, with the crystal structure of BaPul13A (PDB ID, 2wan) (12) as model. The mutations were generated by PCR using site-specific mutagenesis by overlap extension (27). The sequences of oligonucleotides containing the appropriate base changes are listed in Table S1 in the supplemental material. Plasmid pET-20b-*pulA* was used as the template.

Expression and purification of wild-type enzyme and mutants. *E. coli* BL21(DE3) harboring recombinant plasmid was cultured in TB medium containing 100 μ g/ml ampicillin and 7.5 g/liter glycine at 30°C until the A_{600} reached 1.0 (28). Protein expression was induced at 25°C with the addition of isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.05 mM for 48 h. The culture supernatants were collected by centrifugation and adjusted to pH 4.5, followed by incubation at 60°C for 20 min. The mixtures were centrifuged at 12,000 rpm and 4°C for 20 min to remove the denatured protein. The supernatant was then subjected to salt fractionation and dialyzed against buffer A (20 mM sodium phosphate buffer, pH 6.8). The sample was then subjected to anionic chromatography on DEAE-Sepharose column (160 mm by 10 mm; Pharmacia Biotech), which was pre-equilibrated with buffer A on a fast protein liquid chromatography system (AKTA FPLC system; GE Healthcare). Finally, a linear gradient of 0 to 1 M NaCl in buffer A was used to elute the adsorbed proteins. The fractions containing pullulanase activity were pooled and dialyzed against buffer A overnight.

Measurement of pullulanase activity and kinetic parameters. Pullulanase activity was measured in 50 mM sodium acetate buffer (pH 4.5) according to Gantelet et al. using 1% (wt/vol) pullulan as the substrate at 60°C for 15 min (29). The amount of enzyme added in the reaction mixture was about 3.0×10^{-10} M. One unit of pullulanase activity was defined as the amount of enzyme that released 1 μ M D-glucose reducing sugar equivalents per minute from pullulan, under the defined assay conditions.

The kinetic parameters (K_m , V_{max} , and K_{cat} values) of the purified pullulanase were determined according to Malle et al. (10) with a slight modification, using pullulan as the substrate (at concentrations of 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 mg/ml) at 60°C in 50 mM sodium acetate buffer (pH 4.5). V_{max} and K_m values were estimated by fitting the initial rate data to the Michaelis-Menten equation using

nonlinear regression with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All kinetic parameters presented in this study are the mean values derived from triplicate measurements.

Effects of pH and temperature on enzyme activity. The optimum pH of the recombinant enzyme was determined by measuring its activity across a pH range of 3.5 to 7.0 using sodium acetate buffer (pH 3.5 to 5.0) and citric acid/sodium phosphate buffer (pH 5.0 to 7.0). The temperature dependence of the recombinant enzyme activity was measured between 45 and 65°C in 50 mM sodium acetate buffer at pH 4.5 using pullulan as the substrate. At each temperature, the buffer and pullulan were preincubated for 10 min. The reaction was initiated by the addition of the enzyme and allowed to proceed for 15 min.

The half-lives of the enzymes at 60°C were used to assess thermostability. Samples diluted in 50 mM sodium acetate buffer (pH 4.5) to a protein concentration of 0.05 mg/ml were incubated at 60°C. After heat treatment, the residual activity for 0.5% pullulan was measured at 60°C in the same buffer. The initial activity before heat treatment at 60°C was taken as 100%. All of the values presented in graphs and tables are the means of three replications.

Glucose production by a combination of glucoamylase and pullulanase. Starch slurries (30%, wt/vol) were prepared by suspending 272.7 g corn starch (88.0%, dry basis) in deionized water and making the volume up to 800 ml. Calcium ions at 1 mM and 2.5 units of α -amylase per gram of starch (dry basis) were added to the starch slurries. This mixture (pH 6.1, at room temperature) was heated with stirring at 95°C for 90 min. Then the pH was adjusted to 4.0, and heating continued at 100°C for 10 min to inactivate the enzyme. A 50-ml portion of substrate (equivalent to 15 g liquefied starch [dry substance]) was transferred to 250-ml Erlenmeyer flasks, which were then placed in a shaking water bath at 60°C. After the pH had been adjusted to 4.5, 100 units of glucoamylase and 0.5 units of pullulanase per gram of dry substance in the starch hydrolysate were used in the saccharification experiments. In order to estimate the performance of the mutated enzymes, the wild-type pullulanase served as a control. Samples were taken at regular intervals and heated in a boiling water bath for 15 min to inactivate the enzymes. After cooling, the samples were diluted 1:20 with deionized water and then filtered through a 0.45- μ m cellulose acetate membrane before being subjected to high-performance liquid chromatography (HPLC) analysis (30). The DX value (D-glucose contents) was defined as the percentage of glucose on a dry basis (31). All of the values presented in the graphs and tables are the means of three replications.

RESULTS AND DISCUSSION

Design of mutations, construction, and purification of mutant enzymes. In the present study, site-directed mutagenesis was used to generate the mutants in order to improve the thermostability and efficiency of the pullulanase. To select the mutation sites, the amino acid sequence of pullulanase from *B. deramificans* was used to identify five homologs in the NCBI database, and they were aligned using ClustalW (see Fig. S1 in the supplemental material). As shown in Fig. S1, the *B. deramificans* pullulanase sequence shared 33%, 38%, 38%, 39%, and 64% identity with those of *B. subtilis*, *B. cereus*, *C. lentocellum*, *C. phytofermentans*, and *B. acidopullulyticus*, respectively.

In addition, a three-dimensional model of the pullulanase from *B. deramificans* was constructed based on the crystal structure of the enzyme from *B. acidopullulyticus* (PDB ID, 2wan), with which it shares the greatest identity, using the SwissModel server (Fig. 1A). The structure model showed that region A (Asp503-Pro509), which is involved in van der Waals interaction with subsite -1 and -2 glucose residues in the substrate (9), is located in a loop (Phe490-Asp513, in green in Fig. 1B). The loop was identified as a significantly disordered

FIG 1 Model structures of wild-type pullulanase and its variants. (A) Overview. The positions of the residues that are critical for substrate binding or catalysis in wild-type pullulanase are shown as “sticks.” (B, C, D, E, and F) Comparison of closeup views of structural changes introduced by mutation at positions 437 and 503. (B) Wild-type enzyme; (C) D503F mutant; (D) D503Y mutant; (E) D437H mutant; (F) D437H/D503Y mutant. The sticks indicate substrate binding residues Trp505 and Tyr507 (C, green; N, blue; O, red) and mutation residues Asp437 and Asp503 (C, yellow; N, blue; O, red). The predicted disorder loop is in green. The hydrogen bonds are indicated as red stippled lines. The figure was produced using PyMol.

region by the disorder prediction tool, RONN software (32). Interestingly, Asp503 in region A of *B. deramificans* pullulanase was clearly discriminated from the phenylalanine or tyrosine residues of the other pullulanases (see Fig. S1 in the supplemental material) or, in the case of *B. acidopullulyticus* pullula-

nase, from Tyr505. In addition, sequence alignment showed that Asp442 of *B. acidopullulyticus* pullulanase is absolutely conserved in all six of the aligned pullulanases, whereas His439 is replaced by Asp437 in the *B. deramificans* pullulanase but conserved in the other five sequences. As shown in Fig. 1B,

although the side chain of Asp437 points toward the side chain of Asp503, there is no interaction between them, and the space around the two residues is large enough to form an internal cavity, which may contain water and thus affect the stability of the pullulanase. Considering that *B. acidopullulyticus* pullulanase has good thermostability at 60°C (33, 34), it seemed possible that the two Asp residues, Asp437 and Asp503, might be key targets for improving the enzymatic properties of the *B. deramificans* pullulanase.

Further analysis showed that mutation of Asp503 to phenylalanine would introduce a bulkier side chain (Fig. 1C). Upon mutation of Asp503 to tyrosine, this residue not only would contain a benzene ring but is also predicted to form three new hydrogen bonds (Fig. 1D). Similarly, mutation of Asp437 to histidine would introduce an imidazole group and a new hydrogen bond (Fig. 1E). In addition to these single mutations, the combination of D503Y and D437H mutants may not only promote van der Waals interaction between the imidazole group of the His437 and the benzene ring of the Tyr503 but also new hydrogen bonds with the neighboring polar and charged atoms (Fig. 1F). Based on the above analysis, D503F, D503Y, D437H, and D437H/D503Y mutants were constructed and characterized. The pullulanase activities in the culture supernatants were assayed, and there was no significant difference between the expression levels of the recombinant wild-type enzyme and the mutants. The purified recombinant pullulanases displayed molecular masses of approximately 101 kDa (see Fig. S2 in the supplemental material). Compared with the wild-type enzyme, D503Y and D503Y/D437H resulted in an increase in specific activity of 37.5% and 4.9%, respectively, whereas the specific activities of the D437H and D503F mutants were similar to that of the wild-type enzyme (see Tables S2 to S6 in the supplemental material).

Effects of pH and temperature on enzyme activity. Industrial-scale production of glucose syrup is usually performed at pH 4.5 to 5.0 and 60°C, which are the conditions under which glucoamylase exhibits its maximum activity. In order to act in conjunction with glucoamylase, pullulanase must display high activity and sufficient stability under these conditions. In the present study, the hydrolysis of pullulan by the wild-type enzyme and the mutants was measured at various pH values (3.5 to 7.0). The results showed that the pH optimum of all mutants was 4.5, which was similar to that of the wild-type enzyme (Fig. 2A). The temperature dependence of the enzyme activities was measured in the range of 45 to 65°C using pullulan as the substrate in sodium acetate buffer (pH 4.5). The optimum temperature of the D503F, D503Y, and D437H mutants was 55°C, which was similar to that of the wild-type enzyme, whereas the D437H/D503Y double mutant showed a shift in optimal temperature from 55 to 60°C (Fig. 2B). At 60°C, wild-type pullulanase exhibited 80% of its maximum activity, whereas the D503F, D503Y, and D437H mutants displayed 82.9%, 94.3%, and 85.1% of their individual maximum activity, respectively. Moreover, at 65°C, wild-type pullulanase displayed only 32.6% of its maximum activity, while the D503F, D437H, D503Y, and D437H/D503Y mutants displayed 33.0%, 58.1%, 79.4%, and 83.1% of their individual maximum activities, respectively. Furthermore, the mutant pullulanases were more thermostable than those from mesophilic bacteria, such as *B. acidopullulyticus* (12), *Bacillus* sp. 18 strain S-1 (35), and *Bacillus* sp. US149 (36), but less stable than those from most of the thermophiles,

FIG 2 Effects of pH and temperature on the activities and stabilities of pullulanases. (A) Optimum pH values of pullulanases. Assays were carried at 60°C for 10 min in buffers of various pH. The activity of pullulanase at pH 4.5 was defined as 100%. (B) Optimum temperatures of pullulanases. The activity was measured in 50 mM sodium acetate buffer (pH 4.5) at 45 to 65°C for 10 min. The activities are expressed as percentages. (C) Thermostabilities of pullulanases. The activity of recombinant enzyme without heat treatment was defined as 100%. Error bars correspond to the standard deviations from three independent determinations.

including *Bacillus thermoleovorans* US105 (3) and *Clostridium thermosulfurigenes* SV2 (5).

To evaluate the thermostabilities, the enzymes were incubated at 60°C and pH 4.5, and the residual activity was measured after different incubation times. As shown in Fig. 2C, the activity of

wild-type enzyme decreased exponentially with a half-life of 22 h. The D503F, D503Y, and D437H mutants displayed longer half-lives, 36 h for D503F and D503Y and 50 h for D437H, which were 1.6- and 2.3-fold greater than that of the wild-type enzyme. In addition to the single mutations, our study also examined the possibility of combining single mutations for synergistic effects. Notably, the D437H/D503Y double mutant was the most stable; it retained more than 60% of its initial activity after incubation at 60°C for 95 h, and its half-life was more than 4.3-fold that of the wild-type enzyme.

It is known that both hydrogen bonding and hydrophobic interactions play important roles in the thermal stability of proteins. Previously, a number of studies have shown that the introduction of these interactions into thermophilic proteins is an efficient approach for further enhancing their stability (37, 38). In the present study, analysis of the structure models of the mutants also showed that new hydrogen bonds and/or hydrophobic interactions were introduced to the disorder loop of Phe490-Asp513 (Fig. 1). This ordering tends to confer reduced flexibility of the loop and thus increasing the thermal stability of the pullulanase.

Kinetic parameters. The wild-type enzyme and mutants were subjected to kinetic analysis at 60°C (Table 1). The results showed that, compared to the wild-type enzyme, the K_m values of the D503F, D437H, D503Y, and D437H/D503Y mutants decreased by 7.1%, 11.4%, 41.4%, and 45.7%, respectively. In addition, the catalytic constants of all the mutants were enhanced, and the D503Y mutant had the highest K_{cat} . As shown in Table 1, the K_{cat} values of the D503F, D437H, D503Y, and D437H/D503Y mutants increased by 3.0%, 10.2%, 40.0%, and 6.9%, respectively. Moreover, compared to that of the wild-type, all mutants displayed enhanced catalytic efficiency. The K_{cat}/K_m values of the D503F, D437H, D503Y, and D437H/D503Y mutants were 1.1-, 1.2-, 2.4- and 2.0-fold that of the wild-type enzyme, respectively.

Due to the complexity of protein structures as well as the very limited knowledge of how they perform their functions, in most cases site-directed mutagenesis solely based on protein structure analysis has an adverse impact on the kinetic parameters. The consensus method, however, assumes that nature has efficiently optimized the protein sequence space, and this approach has previously been proved to be beneficial in improving kinetic parameters (16). Furthermore, a careful comparison of the D503Y and D437H/D503Y mutants showed that the double mutant exhibited greater thermostability and substrate affinity than D503Y, but the catalytic constant (K_{cat}) of the D437H/D503Y mutant was lower than that of the D503Y mutant. This could be explained by the fact that the proper conformational flexibility is critical to the catalytic activity, whereas the superposition of mutations tends to confer a reduced flexibility of the residues in the substrate binding site, which results in the lower catalytic constant of the double mutant compared to its parent (th D503Y mutant).

Glucose production using glucoamylase in combination with pullulanase. Based on the thermostabilities and catalytic dy-

FIG 3 Glucose production by a combination of glucoamylase and pullulanase. Starch (30%) was incubated with 100 U of glucoamylase and 0.5 U of pullulanase at 60°C (pH 4.5). The amount of glucose generated was measured by HPLC. The time course of saccharification was followed at 60°C for 64 h. Error bars correspond to the standard deviations of three independent determinations.

namic parameters, the D503Y and D437H/D503Y mutants, among the four mutants, were chosen for these further studies with the wild-type enzyme as control. The results demonstrated that when pullulanase and glucoamylase were combined, the value of DX increased rapidly within the first 16 h. After the DX value reached 70 to 80%, it then increased slowly during the next 36 h (Fig. 3). After 52 h of incubation, the maximum glucose yield for the wild-type enzyme was obtained, and the DX value reached 92.7%. For D503Y, a maximum DX value of 95.5% was achieved after 52 h of incubation. The maximum DX value (95.0%) for the D437H/D503Y mutant was obtained after 40 h of incubation, which was shorter by about 12 h. Considering the huge commodity scale production of sugars from starch, an increase of just 2.3 to 2.8% in DX value obtained in this study is predicted to have a significant effect in the syrup industry. In addition, the shortened reaction time also allows energy consumption to be reduced effectively.

In summary, in the present study, the mutation of Asp503 to phenylalanine or tyrosine and Asp437 to histidine led to improved thermostability, substrate binding affinity, and catalytic efficiency to various extents. Notably, the D503Y and D437H/D503Y mutants exhibited the highest catalytic efficiency and thermostability, respectively. Significantly, this work demonstrates that the structure-guided consensus approach is an efficient strategy for identifying “hot residues” important for the thermostability and catalytic properties of an enzyme.

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REFERENCES

- Tester R, Karkalas J, Qi X. 2004. Starch structure and digestibility enzyme-substrate relationship. *World Poultry Sci. J.* 60:186–195.
- Wallenfels K, Bender H, Rached J. 1966. Pullulanase from *Aerobacter*

TABLE 1 Kinetic parameters of wild-type enzyme and mutants

Enzyme	K_{cat} (s^{-1})	K_m (mg ml^{-1})	K_{cat}/K_m (ml mg^{-1} s^{-1})
Wild-type	1,900.4 ± 103.5	0.70 ± 0.02	2,712.9 ± 121.6
D503F	1,958.3 ± 95.3	0.65 ± 0.02	3,003.9 ± 130.2
D503Y	2,660.3 ± 121.1	0.41 ± 0.01	6,512.5 ± 260.5
D437H	2,093.3 ± 85.7	0.62 ± 0.02	3,384.0 ± 149.7
D437H/D503Y	2,031.0 ± 104.8	0.38 ± 0.01	5,403.0 ± 227.1

- aerogenes*: production in a cell-bound state. *Biochem. Biophys. Res. Commun.* 22:254–261.
3. Gantelet H, Duchiron F. 1999. A new pullulanase from a hyperthermophilic archaeon for starch hydrolysis. *Biotechnol. Lett.* 21:71–75.
 4. Ben Messaoud E, Ben Ammar Y, Mellouli L, Bejar S. 2002. Thermostable pullulanase type I from new isolated *Bacillus thermoleovorans* US105: cloning, sequencing and expression of the gene in *E. coli*. *Enzyme Microb. Technol.* 31:827–832.
 5. Gomes I, Gomes J, Steiner W. 2003. Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization. *Bioresource Technol.* 90: 207–214.
 6. Reddy RM, Reddy PG, Seenayya G. 1999. Enhanced production of thermostable β -amylase and pullulanase in the presence of surfactants by *Clostridium thermosulfurogenes* SV2. *Process Biochem.* 34:87–92.
 7. Bertoldo C, Armbricht M, Becker F, Schäfer T, Antranikian G, Liebl W. 2004. Cloning, sequencing, and characterization of a heat-and alkali-stable type I pullulanase from *Anaerobranca gottschalkii*. *Appl. Environ. Microbiol.* 70:3407–3416.
 8. Zouari Ayadi D, Ben Ali M, Jemli S, Ben Mabrouk S, Mezghani M, Ben Messaoud E, Bejar S. 2008. Heterologous expression, secretion and characterization of the *Geobacillus thermoleovorans* US105 type I pullulanase. *Appl. Microbiol. Biotechnol.* 78:473–481.
 9. Kang J, Park KM, Choi KH, Park CS, Kim GE, Kim D, Cha J. 2011. Molecular cloning and biochemical characterization of a heat-stable type I pullulanase from *Thermotoga neapolitana*. *Enzyme Microb. Technol.* 48:260–266.
 10. Malle D, Itoh T, Hashimoto W, Murata K, Utsumi S, Mikami B. 2006. Overexpression, purification and preliminary X-ray analysis of pullulanase from *Bacillus subtilis* strain 168. *Acta Crystallogr. B* 62:381–384.
 11. Mikami B, Iwamoto H, Malle D, Yoon HJ, Demirkan-Sarikaya E, Mezaki Y, Katsuya Y. 2006. Crystal structure of pullulanase: evidence for parallel binding of oligosaccharides in the active site. *J. Mol. Biol.* 359: 690–707.
 12. Turkenburg JP, Brzozowski AM, Svendsen A, Borchert TV, Davies GJ, Wilson KS. 2009. Structure of a pullulanase from *Bacillus acidopullulyticus*. *Proteins* 76:516–519.
 13. Roy I, Gupta MN. 2004. Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. *Enzyme Microb. Technol.* 34:26–32.
 14. Crabb WD, Shetty JK. 1999. Commodity scale production of sugars from starches. *Curr. Opin. Microbiol.* 2:252–256.
 15. Philippe D, Antoine A. December 1993. Pullulanase, micro-organisms producing the same, method for preparation thereof as well as its use. Europe patent 0,605,040 A1.
 16. Vázquez-Figueroa E, Chaparro-Riggers J, Bommarius AS. 2007. Development of a thermostable glucose dehydrogenase by a structure-guided consensus concept. *Chembiochem* 8:2295–2301.
 17. Joo JC, Pack SP, Kim YH, Yoo YJ. 2011. Thermostabilization of *Bacillus circulans* xylanase: computational optimization of unstable residues based on thermal fluctuation analysis. *J. Bacteriol.* 151:56–65.
 18. Korkegian A, Black ME, Baker D, Stoddard BL. 2005. Computational thermostabilization of an enzyme. *Science* 308:857–860.
 19. Bershtein S, Tawfik DS. 2008. Advances in laboratory evolution of enzymes. *Curr. Opin. Chem. Biol.* 12:151–158.
 20. Chica RA, Doucet N, Pelletier JN. 2005. Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design. *Curr. Opin. Biotechnol.* 16:378–384.
 21. Spadiut O, Leitner C, Salaheddin C, Varga B, Vertessy BG, Tan TC, Divne C, Haltrich D. 2008. Improving thermostability and catalytic activity of pyranose 2-oxidase from *Trametes multicolor* by rational and semi-rational design. *FEBS J.* 276:776–792.
 22. Anbar M, Gul O, Lamed R, Sezerman UO, Bayer EA. 2012. Improved thermostability of *Clostridium thermocellum* endoglucanase Cel8A by using consensus-guided mutagenesis. *Appl. Environ. Microbiol.* 78:3458–3464.
 23. Polizzi KM, Chaparro-Riggers JF, Vazquez-Figueroa E, Bommarius AS. 2006. Structure-guided consensus approach to create a more thermostable penicillin G acylase. *Biotechnol. J.* 1:531–536.
 24. Viader-Salvadó JM, Gallegos-López JA, Carreón-Treviño JG, Castillo-Galván M, Rojo-Domínguez A, Guerrero-Olazarán M. 2010. Design of thermostable beta-propeller phytases with activity over a broad range of pHs and their overproduction by *Pichia pastoris*. *Appl. Environ. Microbiol.* 76:6423–6430.
 25. Blum JK, Ricketts MD, Bommarius AS. 2012. Improved thermostability of AEH by combining B-FIT analysis and structure-guided consensus method. *J. Biotechnol.* 160:214–221.
 26. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948.
 27. Li Z, Zhang J, Wang M, Gu Z, Du G, Li J, Wu J, Chen J. 2009. Mutations at subsite –3 in cyclodextrin glycosyltransferase from *Paenibacillus macerans* enhancing alpha-cyclodextrin specificity. *Appl. Microbiol. Biotechnol.* 83:483–490.
 28. Li Z, Gu Z, Wang M, Du G, Wu J, Chen J. 2010. Delayed supplementation of glycine enhances extracellular secretion of the recombinant alpha-cyclodextrin glycosyltransferase in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 85:553–561.
 29. Gantelet H, Duchiron F. 1998. Purification and properties of a thermoactive and thermostable pullulanase from *Thermococcus hydrothermalis*, a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Appl. Microbiol. Biotechnol.* 49:770–777.
 30. Lee HS, Hong J. 2001. Kinetics of glucose isomerization to fructose by immobilized glucose isomerase: anomeric reactivity of D-glucose in kinetic model. *J. Biotechnol.* 84:145–153.
 31. Linko YY, Wu XY. 1993. Improvement and estimation of enzymic starch saccharification process. *Biotechnol. Techniques* 7:551–556.
 32. Yang ZR, Thomson R, McNeil P, Esnouf RM. 2005. RONN: the bio-basis function neural network technique applied to the detection of natively disordered regions in proteins. *Bioinformatics* 21:3369–3376.
 33. Kusano S, Nagahata N, Takahashi SI, Fujimoto D, Sakano Y. 1988. Purification and properties of *Bacillus acidopullulyticus* pullulanase (biological chemistry). *Agric. Biol. Chem.* 52:2293–2298.
 34. Schüle M, Højer-Pedersen B. 1984. Characterization of a new class of thermophilic pullulanases from *Bacillus acidopullulyticus*. *Ann. N. Y. Acad. Sci.* 434:271–274.
 35. Lee MJ, Lee YC, Kim CH. 1997. Intracellular and extracellular forms of alkaline pullulanase from an alkaliphilic *Bacillus* sp. S-1. *Arch. Biochem. Biophys.* 337:308–316.
 36. Roy A, Messaoud EB, Bejar S. 2003. Isolation and purification of an acidic pullulanase type II from newly isolated *Bacillus* sp. US149. *Enzyme Microb. Technol.* 33:720–724.
 37. Vogt G, Woell S, Argos P. 1997. Protein thermal stability, hydrogen bonds, and ion pairs. *J. Mol. Biol.* 269:631–643.
 38. Zhang W, Mullaney EJ, Lei XG. 2007. Adopting selected hydrogen bonding and ionic interactions from *Aspergillus fumigatus* phytase structure improves the thermostability of *Aspergillus niger* PhyA phytase. *Appl. Environ. Microbiol.* 73:3069–3076.