Enhancing the Maltose Affinity of β-CGTase from Bacillus circulans 251 and Its Application in Trehalose Production Postprint

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Abstract

Application of B. circulans 251 β-CGTase to trehalose preparation increased the trehalose conversion rate from 50.4% to 71.9%. To further improve the substrate conversion rate, error-prone PCR-based high-throughput screening technology was employed to screen for B. circulans 251 β-CGTase mutants with enhanced affinity for maltose as a disproportionation reaction acceptor. Using a low-substrate-concentration 96-well plate 4,6-ethylidene-p-nitrophenyl-α-D-maltoheptaoside (EPS) chromogenic method, a mutant M234I with improved maltose affinity was finally obtained. The wild-type β-CGTase and mutant enzyme M234I were purified, and their enzymatic properties were determined. The results showed: the specific activity of the mutant was 345.25 U/mg, while that of the wild-type was 357.63 U/mg; the Km of mutant M234I for maltose was 0.2582 mM, which was only 54.4% of that of the wild-type (0.4749 mM), indicating a significant improvement in maltose affinity; the optimum temperature and optimum pH of the mutant did not change significantly compared with the wild-type. Using maltodextrin (DE value 16) as substrate, mutant M234I was applied in a multi-enzyme composite system for trehalose production. The enzymatic reaction results showed that the trehalose conversion rate reached a maximum of 74.9%, representing an increase of approximately 3% compared with the wild-type β-CGTase.

Full Text

Enhancing Maltose Affinity of β-CGTase from *Bacillus circulans* 251 and Its Application in Trehalose Production

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Abstract

The β-CGTase from *Bacillus circulans* 251 was applied to trehalose preparation, increasing the trehalose conversion rate from 50.4% to 71.9%. To further improve substrate conversion, error-prone PCR combined with high-throughput screening was employed to identify mutants with enhanced affinity for maltose as a disproportionation acceptor. Using a colorimetric assay with low substrate concentrations of 4,6-ethylidene-p-nitrophenyl-α-D-maltoheptaoside (EPS) in 96-well plates, a mutant M234I with improved maltose affinity was successfully isolated. After purification of both wild-type and mutant enzymes, characterization revealed that the specific activity of mutant M234I was 345.25 U/mg, compared to 357.63 U/mg for the wild type. The Km value of mutant M234I for maltose was 0.2582 mM, only 54.4% of that of the wild type (0.4749 mM), indicating significantly enhanced maltose affinity. The optimal temperature and pH of the mutant remained largely unchanged compared to the wild type. When applied to trehalose production using a multi-enzyme system with maltodextrin (DE 16) as substrate, the mutant M234I achieved a maximum trehalose conversion rate of 74.9%, representing an approximately 3% improvement over the wild-type β-CGTase.

Keywords: β-CGTase, error-prone PCR, molecular modification, affinity, trehalose conversion

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Introduction

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) belongs to glycoside hydrolase family 13 and is an extracellular multifunctional enzyme capable of catalyzing various reactions, including three transglycosylation reactions (coupling, cyclization, and disproportionation) and a weak hydrolytic reaction. Based on their product specificity in cyclization reactions, CGTases are commonly classified as α-CGTase, β-CGTase, and γ-CGTase. Among the three transglycosylation reactions, disproportionation dominates, representing an intermolecular transglycosylation that transfers a cleaved portion of a linear maltooligosaccharide to another acceptor, thereby elongating the acceptor chain. CGTase transglycosylation activity can utilize various dextrins, sugar chains, and starch molecules as glycosyl donors to improve the properties of acceptor molecules through disproportionation or coupling reactions, such as enhancing the sweet-

ness characteristics of stevioside, improving the solubility of chalcone sweeteners, and producing vitamin C derivatives (AA-2G) with enhanced stability, as well as preparing turanose and coupled sugars. This transglycosylation capability also plays a crucial auxiliary role in multi-enzyme reaction systems, significantly improving substrate utilization. For example, incorporating CGTase in trehalose production systems can elongate the sugar chains of remaining small-molecule sugar acceptors, enabling them to be further utilized by maltooligosyl trehalose synthase for synthesizing maltooligosyl trehalose, thereby enhancing substrate utilization. The CGTase from *B. circulans* 251 is a typical β-CGTase that exhibits high acceptor affinity for small-molecule sugars in disproportionation reactions. This study investigated its application in a multi-enzyme system for trehalose production and performed directed evolution through error-prone PCR combined with high-throughput screening to obtain improved mutants for enhanced performance.

Materials and Methods

1.1.1 Strains and Plasmids *Escherichia coli* JM109 and *E. coli* BL21(DE3) strains were preserved in our laboratory. The pMD19-T(simple) cloning vector was purchased from Takara (Dalian, China). The pET-20b(+) expression vector and the β-CGTase gene (β-cgt) from *B. circulans* 251 were also preserved in our laboratory.

1.1.2 Culture Media LB liquid medium, LB solid medium, and TB medium were prepared according to standard protocols.

1.1.3 Enzymes and Reagents Restriction endonucleases (NdeI, HindIII), rTaq DNA polymerase, protein molecular weight markers, DL5000 and DL10000 DNA markers were purchased from Takara (Dalian, China). T4 DNA ligase was obtained from New England Biolabs (USA). Plasmid mini-prep and gel extraction kits were purchased from CW Biotech. Trehalose, maltose, glucose, and maltodextrin (DE 16) were obtained from Sigma-Aldrich. Acetonitrile (HPLC grade) was purchased from Shanghai ANPEL Scientific Instrument Co., Ltd. EPS, kanamycin, and ampicillin were obtained from J&K Scientific. Other routine reagents were purchased from Sinopharm Chemical Reagent Co., Ltd.

1.2.1 Error-Prone PCR of β-CGTase Gene The β-cgt gene from *B. circulans* 251 was used as the template for error-prone PCR. Primers were designed as CGT-ePCR-F: 5' -CATGCCATGGCACCGGATACCAGCGTT-3' and CGT-ePCR-R: 5' -GGGAAGCTTACGGTTGCCAATTCACGT-3' , with restriction sites underlined. The PCR reaction (50 L) contained: ddH_2O (30.7 L), Mg^{2+} (25 mM, 8.0 L), Mn^{2+} (10 mM, 0.3 L), 10× rTaq buffer (5 L), plasmid DNA template (0.5 L), primers CGT-ePCR-F and CGT-ePCR-R (10 M each, 0.5 L), dNTP mix (2.5 mM, 4 L), and rTaq DNA polymerase (5 U/ L, 0.5 L). The

PCR program consisted of initial denaturation at 94°C for 4 min, followed by 30 cycles of 98°C for 30 s, 55°C for 10 s, and 72°C for 2 min 10 s, with a final extension at 72°C for 10 min and holding at 4°C.

1.2.2 Construction of Mutant Library The error-prone PCR products were gel-purified and ligated into the pMD19-T(simple) cloning vector. The ligation mixture was transformed into *E. coli* JM109 competent cells and plated on LB agar containing 100 g/mL ampicillin. After overnight incubation at 37°C, all positive clones were washed off the plate with LB liquid medium containing 100 g/mL ampicillin and cultured at 37°C, 200 rpm for 3-5 h. Mixed plasmids were extracted using a plasmid prep kit, double-digested with NdeI and HindIII, and ligated into the pET-20b expression vector. The ligation products were transformed into *E. coli* BL21(DE3) expression host and plated on LB agar containing 100 g/mL kanamycin to complete the mutant library construction.

1.2.3 Screening of Mutant Library A high-throughput colony picker (Molecular Devices QPix 2) was used to transfer individual colonies from LB agar plates into 96-deep-well plates containing 600 L LB medium with 100 g/mL kanamycin. The plates were incubated at 37°C, 900 rpm for 8-10 h. Then, 50 L of seed culture was transferred to fresh 96-deep-well plates containing 600 L TB medium and cultured at 37°C, 900 rpm for 2 h. Protein expression was induced by adding IPTG to a final concentration of 5 mM, followed by incubation at 25°C for 48 h. The cultures were centrifuged at 4000 rpm, 4°C for 20 min, and the supernatants were collected for enzyme activity assays after appropriate dilution. The assay mixture contained 200 L of 0.5 mM EPS and 0.25 mM maltose, pre-incubated at 50°C for 10 min. Then, 30 L of diluted crude enzyme was added and reacted for exactly 75 min. The reaction was terminated by boiling for 10 min. After cooling, 40 L of α-glucosidase and 130 L deionized water were added, mixed, and incubated at 60°C for at least 60 min. Finally, 100 L of 1 M Na_2CO_3 solution was added, mixed, and 200 L was transferred to a microplate for absorbance measurement at 400 nm. Mutants showing higher activity than the wild type under these substrate concentrations were selected for further screening. The Km values of improved mutants for maltose were determined, and mutants with reduced Km values were sent to Genewiz (Suzhou, China) for sequencing.

1.2.4 β-CGTase Disproportionation Activity Assay The disproportionation activity assay was modified from the method described by van der Veen et al. Briefly, 12 mM EPS and 20 mM maltose solutions were prepared in 50 mmol/L phosphate buffer (pH 5.5). Equal volumes (300 L each) were pre-incubated at 50°C, then 100 L of diluted enzyme solution was added. After exactly 10 min, the reaction was terminated by boiling for 10 min. Subsequently, 30 L of diluted crude enzyme was added and reacted for 75 min, followed by boiling for 10 min. After cooling, 100 L of α-glucosidase and 100 L deionized water were added, mixed, and incubated at 60°C for at least 60 min. Then, 100

L of 1 M Na_2CO_3 solution was added, mixed, and the absorbance was measured at 400 nm. One unit of CGTase disproportionation activity was defined as the amount of enzyme that converted 1 mol of EPS per minute under the specified conditions at 50°C.

1.2.5 Expression and Purification of β-CGTase *E. coli* BL21(DE3) cells harboring wild-type or mutant β-CGTase were inoculated into 10 mL LB medium containing 100 g/mL kanamycin and cultured at 37°C for 8-10 h. A 5% inoculum was transferred to TB medium containing 100 g/mL kanamycin and 7.5 g/L glycine, cultured at 37°C for 2 h, then induced with 5 mM IPTG and incubated at 25°C for 48 h. The culture was centrifuged at 8000 rpm for 15 min, and the supernatant was collected as crude enzyme.

The crude enzyme was subjected to 30% ammonium sulfate precipitation, followed by dialysis and purification using a Mono Q anion-exchange chromatography column. Protein concentration was determined using the Bradford method. The purified β-CGTase and its mutant were used for enzymatic characterization and kinetic parameter determination.

1.2.6 Enzymatic Characterization (1) Optimal Temperature: Substrates were pre-incubated at temperatures ranging from 35°C to 70°C (5°C intervals), and enzyme activity was measured using the method described in section 1.2.4. The highest activity was defined as 100%.

(2) Temperature Stability: Enzyme solution was diluted with pH 6.0 buffer and incubated at 50°C. Samples were taken at regular intervals to measure residual activity using the method in section 1.2.4, with initial activity (0 h) defined as 100%.

(3) Optimal pH: Substrates were dissolved in 50 mmol/L buffers ranging from pH 4.0 to 8.0 (0.5 pH unit intervals) to prepare 6 mM EPS and 20 mM maltose solutions. After pre-incubation at 50°C, activity was measured as described in section 1.2.4, with maximum activity defined as 100%.

(4) pH Stability: Enzyme was incubated in 50 mmol/L buffers (pH 4.0–8.0, 0.5 intervals) at 4°C for 24 h, then residual activity was measured using the method in section 1.2.4, with initial activity defined as 100%.

(5) Determination of Km and kcat Values: Maltose solutions ranging from 0.25 mM to 100 mM were prepared in 50 mmol/L phosphate buffer (pH 5.5). After pre-incubation at 50°C, enzyme activity was measured as described in section 1.2.4. Kinetic parameters were calculated by fitting the data using GraphPad Prism software.

1.2.7 Application of β-CGTase in Two-Enzyme System for Trehalose Production A 20% (w/w) maltodextrin solution (DE 16) was prepared in sodium dihydrogen phosphate-potassium dihydrogen phosphate buffer. The reaction mixture contained 5 U/g substrate pullulanase, 2.5 U/mL maltooligosyl

trehalose synthase, 2.5 U/mL maltooligosyl trehalose hydrolase, and 0.6–2.4 U/mL CGTase (based on disproportionation activity). The pH was adjusted to 5.5, and the reaction was carried out at 45°C with shaking at 120–180 rpm for 30–35 h. The reaction was terminated by boiling, yielding reaction mixture A. This mixture was then adjusted to pH 4.4 and treated with compound glucoamylase at 60°C for 24 h. The products were analyzed by high-performance liquid chromatography (Agilent 1260) to calculate trehalose conversion. HPLC analysis was performed using an amino column with acetonitrile:water (80:20) as mobile phase at 30°C, flow rate of 0.8 mL/min, and refractive index detection (RID).

1.2.8 Structural Prediction and Mutation Site Analysis The crystal structure of wild-type β-CGTase was obtained from the Protein Data Bank (PDB ID: 1CXK). Average structures of the mutant and wild-type enzymes were calculated through Amber MD simulations.

Results and Discussion

2.1 Application of β-CGTase from *B. circulans* 251 in Trehalose Preparation Trehalose enzymatic conversion was performed as described in section 1.2.7. Without CGTase addition, HPLC analysis after saccharification showed a trehalose yield of only 75.6 g/L, corresponding to a conversion rate of 50.4%. However, when β-CGTase from *B. circulans* 251 was added, trehalose yield increased to 143.8 g/L, achieving a conversion rate of 71.9%. Analysis of the reaction products revealed that the system containing β-CGTase still contained substantial residual maltose (21.9 g/L). We hypothesized that improving the affinity of β-CGTase for maltose as an acceptor in disproportionation reactions would enable utilization of this residual maltose to elongate sugar chains for further trehalose synthesis, thereby increasing both substrate utilization and trehalose conversion. Therefore, directed evolution of the *B. circulans* 251 CGTase was performed using error-prone PCR combined with high-throughput screening to obtain improved mutants.

2.2 Optimization of Error-Prone PCR Conditions Error-prone PCR requires supplementation with additional Mg^{2+} and Mn^{2+} ions. Mg^{2+} stabilizes the nucleic acid backbone and affects DNA polymerase activity, where low concentrations reduce PCR efficiency while excessive concentrations cause nonspecific amplification. Mn^{2+} decreases template specificity of DNA polymerase and increases the error rate of base pairing during PCR. By sequencing PCR products obtained with different Mg^{2+} and Mn^{2+} concentrations and following the principle that 1–3 amino acid mutations are optimal, we selected final concentrations of 0.15 mM Mn^{2+} and 4 mM Mg^{2+} for the reaction system.

2.3 Construction and Screening of the Mutant Library After optimizing error-prone PCR conditions, the β-cgt gene was amplified and subjected to initial screening as described in sections 1.2.2 and 1.2.3. Mutants showing absorbance values 0.1 higher than the wild-type CGTase were selected for secondary screening in deep-well plates. After 11 rounds of screening approximately 3000 clones, a positive transformant H-7 exhibiting improved activity at low substrate concentrations was identified. For confirmation, H-7 was inoculated into TB medium (100 g/mL kanamycin) at 5% inoculum, cultured at 37°C, 200 rpm for 2 h, induced with 5 mM IPTG, and expressed at 25°C, 200 rpm for 48 h. Enzyme activity assays using 20 mM or 0.5 mM maltose with 6 mM EPS revealed that H-7 showed 1.4-fold higher activity than the wild type at 0.5 mM maltose, but only 95% of wild-type activity at 20 mM maltose, suggesting improved maltose affinity.

2.4 Sequencing of the Mutant Sequencing of the improved mutant H-7 revealed mutations at three nucleotide positions: 624, 702, and 1110. Only the mutation at position 702 resulted in an amino acid change, converting methionine to isoleucine at residue 234. The sequencing results are summarized in Table 1 , confirming that mutant H-7 is the M234I variant.

Table 1 Sequencing results of the mutant

Mutant	Mutated nucleotide bases	Mutated amino acids
M234I	T624C / G702A / C1110T	M234I

2.5 Purification of Wild-Type and Mutant β-CGTase The crude enzyme was precipitated with 30% ammonium sulfate and centrifuged at 8000 rpm for 20 min to collect the protein pellet. The pellet was resolubilized and dialyzed against 20 mM sodium dihydrogen phosphate-potassium dihydrogen phosphate buffer (pH 7.5), then purified using a Mono Q anion-exchange column. SDS-PAGE analysis [Figure 1: see original paper] showed that the target protein achieved electrophoretic purity. Protein content and enzyme activity were measured at each purification step, with results indicating that the specific activity (disproportionation) of recombinant β-CGTase was 357.63 U/mg, while that of mutant M234I was 345.25 U/mg, showing no significant change after mutation.

Figure 1 SDS-PAGE analysis of wild-type and mutant M234I β-CGTase
M: Protein Marker (High)
1: Purified wild-type β-CGTase
2: Purified mutant M234I

Table 2 Purification results of wild-type and mutant (M234I) β-CGTase

Enzymes	Purification steps	Total protein	Total activity	Specific activity (U/mg)	Recovery rate (%)	Purification fold
M234I β-CGT	Crude extract					
	Salt precipitation					
	Mono Q				10/100	
Wild-type β-CGT	Crude extract					
	Salt precipitation					
	Mono Q				10/100	

2.6 Kinetic Analysis Kinetic parameters determined using purified wild-type and mutant M234I β-CGTase are shown in Table 3 . The Km value of wild-type β-CGTase for maltose was 0.4749 mM, while that of mutant M234I was 0.2582 mM, demonstrating significantly improved substrate affinity. The kcat values for wild-type and mutant M234I were 488.7 s^{-1} and 453.2 s^{-1}, respectively.

Table 3 Kinetic parameters of wild-type and mutant M234I β-CGTase

Enzyme	kcat (s^{-1})	Km (mM)	kcat/Km ($mM^{-1} \cdot s^{-1}$)
Wild type		0.4749	
M234I		0.2582	

2.7 Enzymatic Properties of Wild-Type and Mutant β-CGTase 2.7.1 Optimal Temperature and Thermal Stability

The optimal reaction temperature and thermal stability of mutant M234I and wild-type β-CGTase are shown in Figures 2 [Figure 2: see original paper] and 3 [Figure 3: see original paper]. Both enzymes exhibited an optimal temperature of 60°C, indicating no significant change in the mutant. However, as shown in Figure 3, the half-lives at 45°C, 50°C, 55°C, and 60°C were 10.2 h, 1.9 h, 15 min, and 3 min for the mutant, compared to 13.1 h, 2.9 h, 39 min, and 5 min for the wild type, respectively. Thus, the thermal stability of mutant M234I β-CGTase was slightly reduced compared to the wild type, though both showed poor stability at high temperatures.

Figure 2 [Figure 2: see original paper] Optimal temperature of wild-type and mutant M234I β-CGTase

Figure 3 Temperature stability of wild-type and mutant M234I β-CGTase at (a) 45°C, (b) 50°C, (c) 55°C, and (d) 60°C

2.7.2 Optimal pH and pH Stability

The optimal pH and pH stability results are presented in Figures 4 [Figure 4: see original paper] and 5 [Figure 5: see original paper]. Both mutant M234I and wild-type β-CGTase showed an optimal pH of 6.0. The enzymes maintained over 90% relative activity at pH 5.5–8.0, but both were rapidly inactivated below pH 5.5, indicating similar pH stability profiles.

Figure 4 [Figure 4: see original paper] Optimal pH of wild-type and mutant M234I β-CGTase

Figure 5 [Figure 5: see original paper] pH stability of wild-type and mutant M234I β-CGTase

2.8 Application of Mutant M234I in Trehalose Production Trehalose enzymatic conversion was performed as described in section 1.2.7. As shown in Figure 6 [Figure 6: see original paper], when the enzyme dosage was 2.4 U/mL, the wild-type β-CGTase produced 143.8 g/L trehalose (71.9% conversion), while mutant M234I produced 149.8 g/L trehalose, achieving a conversion rate of 74.9%. The glucose, maltose, and trehalose contents in the reaction products are shown in Table 4 . The mutant showed reduced maltose content and increased glucose and trehalose levels compared to the wild type.

Figure 6 [Figure 6: see original paper] Trehalose yield under different enzyme dosages

Table 4 Glucose, maltose, and trehalose content in the reaction system before saccharification

Enzyme	Glucose (g/L)	Maltose (g/L)	Trehalose (g/L)
Wild type			
M234I			

2.9 Mutation Site Analysis The three-dimensional structure of wild-type β-CGTase was obtained from the PDB database (PDB ID: 1CXK). The mutation site M234I is located in a loop region near the +1 subsite of the substrate binding site, with H233 (also in this loop) participating in acceptor glucose residue binding. Average structures of the wild-type and mutant M234I were obtained through Amber MD simulations [Figure 7: see original paper], and docking with substrate molecules revealed that the M234I mutation altered the loop conformation, bringing it closer to the acceptor binding region. The hydrogen bond distance between H233 and the acceptor substrate maltose was 2.9 Å in the mutant [FIGURE:8(A)], shorter than the 3.3 Å distance in the

wild type [FIGURE:8(B)], potentially improving CGTase binding to maltose acceptor.

Figure 7 [Figure 7: see original paper] Average structures of wild-type and mutant M234I β-CGTase kinetic trajectories. The green region shows the wild-type loop and residues, while the blue region shows the mutant M234I loop and residues.

Figure 8 [Figure 8: see original paper] Docking models of acceptor substrate with average structures of (a) mutant M234I and (b) wild-type β-CGTase

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Conclusion

This study applied β-CGTase from *B. circulans* 251 to trehalose production, increasing the conversion rate from 50.4% to 71.9%. Analysis of the reaction products revealed substantial residual maltose, suggesting that improved maltose affinity could further enhance substrate utilization. Using error-prone PCR and high-throughput screening, we successfully obtained mutant M234I with enhanced maltose affinity in disproportionation reactions, showing a Km of 0.2582 mM (54.4% of the wild-type value). The mutant maintained similar optimal temperature and pH, though with slightly reduced thermal stability. Application of M234I in a multi-enzyme trehalose production system reduced residual maltose content and increased trehalose conversion to 74.9%, representing a 3% improvement over the wild type. These findings contribute to the development of more efficient enzymatic processes for trehalose production from starch.

References

[1] Wu J, Gu ZB, Chen J. Preparation and application of cyclodextrin glycosyltransferase. Beijing: Chemical Industry Press, 2011: 3-4.

[2] Ba VD V, Leemhuis H, Kralj S, et al. Hydrophobic amino acid residues in the acceptor binding site are main determinants for reaction mechanism and specificity of cyclodextrin-glycosyltransferase. *Journal of Biological Chemistry*, 2001, 276(48): 44557-44562.

[3] Kelly RM, Dijkhuizen L, Leemhuis H. The evolution of cyclodextrin glucanotransferase product specificity. *Applied Microbiology and Biotechnology*, 2009, 84(1): 119-133.

[4] Li ZF, Gu ZB, Du GC, et al. Structural characteristics and catalytic mechanisms of cyclodextrin glycosyltransferase. *China Biotechnology*, 2010, 30(6): 144-150.

[5] Brunei C, Lamare S, Legoy MD. Studies of specific cyclodextrin production starting from pure maltooligosaccharides using *Thermoanaerobacter* sp. cy-

clodextrin glycosyltransferase. *Biocatalysis and Biotransformation*, 1998, 16(4): 317-327.

[6] Jemli S, Messaoud EB, Ayadi-Zouari D, et al. A β-cyclodextrin glycosyltransferase from a newly isolated *Paenibacillus pabuli* US132 strain: purification, properties and potential use in bread-making. *Biochemical Engineering Journal*, 2007, 34(1): 44-50.

[7] Lee YH, Baek SG, Shin HD, et al. Transglycosylation reaction of cyclodextrin glucanotransferase in the attrition coupled reaction system using raw starch as a donor. *Korean Journal of Applied Microbiology & Biotechnology*, 1993, 6(27): 801-815.

[8] Li CM. Secretory expression of β-cyclodextrin glycosyltransferase in *Bacillus subtilis* and thermostability studies. Wuxi: Jiangnan University, 2014.

[9] Plou FJ, Martín MT, de Segura AG, et al. Glucosyltransferases acting on starch or sucrose for the synthesis of oligosaccharides. *Canadian Journal of Chemistry*, 2002, 80(6): 743-752.

[10] Martín MT, Cruces MA, Alcalde M, et al. Synthesis of maltooligosyl fructofuranosides catalyzed by immobilized cyclodextrin glucosyltransferase using starch as donor. *Tetrahedron*, 2004, 60(3): 529-534.

[11] Mukai K, Tabuchi A, Nakada T, et al. Production of trehalose from starch by thermostable enzymes from *Sulfolobus acidocaldarius*. *Starch-Stärke*, 1997, 49(1): 45-49.

[12] van der Veen BA, Leemhuis H, Kralj S, et al. Hydrophobic amino acid residues in the acceptor binding site are main determinants for reaction mechanism and specificity of cyclodextrin-glycosyltransferase. *Journal of Biological Chemistry*, 2001, 276(48): 44557-44562.

[13] Yang YL, Wang L, Chen S, et al. Optimization of β-cyclodextrin production by recombinant β-cyclodextrin glycosyltransferase. *Biotechnology Bulletin*, 2014(8): 175-181.

[14] Uitdehaag JCM, Mosi R, Kalk KH, et al. X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the α-amylase family. *Nature Structural Biology*, 1999, 6(5): 432-436.

[15] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 1976, 72(1-2): 248-254.

Note: Figure translations are in progress. See original paper for figures.