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Efficient Production of γ-CD from Starch by γ-CGTase Heterologously Produced in *Pichia pastoris*, Assisted by β-CGTase Liquefaction and Pullulanase Debranching

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Abstract

Cyclodextrins (CDs) are cyclic oligosaccharides composed of α(1 → 4) linked glucose units, which are widely used as solubilizers and stabilizers in the food, pharmaceutical and cosmetic industries. Among the CDs, γ-CD has attracted much attention due to its larger hydrophobic cavity and higher solubility. However, the industrial production of γ-CD is limited by lack of suitable enzymes and production process shortcomings. In this study, various strategies of improving heterologous enzyme production and optimization of the starch conversion process were applied to increase the production of γ-CD. A γ-cyclodextrin glucanotransferase with good product specificity from *Bacillus* sp. FJAT-44876 (BFγ-CGTase) and a liquefying β-CGTase from *Bacillus* sp. 1011 (Bsβ-CGTase) were successfully secreted by *Pichia pastoris*. After codon optimization and using the one-factor-at-a-time (OFAT) principle to improve the fermentation, the yield of recombinant BFγ-CGTase was increased 13.3 times to 463 U/L. Next a process was established involving Bsβ-CGTase-assisted starch liquefaction and simultaneous pullulanase debranching and BFγ-CGTase production of γ-CD. The yield of γ-CD increased by 17.67% via optimizing the amounts of BFγ-CGTase and BtPul used for the reaction. Overall, combination of the various improvements provided a new process for efficient preparation of γ-CD.

Keywords: cyclodextrin; starch; codon optimization; fermentation optimization; conversion rate; industrialization

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1. Introduction

Cyclodextrins (CDs) are a class of cyclic oligosaccharides produced by the action of cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19) on starch [1,2]. They mainly include α-, β-, and γ-CDs, which are composed of six, seven and eight D-glucopyranose residues connected by α(1 → 4) linkages, respectively [3]. CDs possess an internal hydrophobic cavity and have an hydrophilic external surface, making them widely used as solubilizers or stabilizers hosting functional compounds used in the food, pharmaceutical and cosmetic industries [4]. Among them, γ-CD has attracted more attention due to its larger hydrophobic cavity and higher solubility [5]. The price of γ-CD, reaching 700 RMB/kg, constrains its widespread application in foods.

γ-CD held considerable promise for a wide range of industrial applications, creating an urgent need for cost-effective strategies for its production. However, CGTase-catalyzed reactions typically generated cyclodextrin mixtures with highly variable yields and ring-size selectivity (α/β/γ-CD ratios), which posed a major challenge. Therefore, most studies focused on modifying CGTases through screening and genetic engineering to improve their product specificity and stability. In addition, the high production cost of CGTase remained a major barrier to its broader application. A range of approaches—including heterologous expression, fermentation optimization, and cell immobilization—had therefore been proposed to boost enzyme yields and reduce production costs. For example, the β-γ-CGTase from alkaliphilic *Bacillus* sp. SD5 was screened and heterologously expressed in *Pichia pastoris* to improve its recombinant production level [6]. Conventional cyclodextrin manufacturing typically involves α-amylase-mediated liquefaction of starch, CGTase-driven cyclization to form a cyclodextrin mixture, and a subsequent glucoamylase treatment to hydrolyze residual dextrins and maltooligosaccharides. The resulting cyclodextrins were then recovered by organic–solvent precipitation, further purified, and dried to obtain the final product. Nevertheless, achieving high conversion of starch to CDs remained challenging. This inefficiency largely arose from the limited ability of CGTase to process the α(1 → 6) branch points in amylopectin, which compromised catalytic performance. The use of debranching enzymes, including isoamylase and pullulanase, has been demonstrated to enhance CGTase accessibility to amylopectin, leading to improved cyclodextrin formation from starch. For instance, isoamylase had been used for starch debranching in γ-CD preparation, increasing the yield by 22.1% [7]. In summary, achieving efficient preparation of γ-CD through screening and modification of a highly specific γ-CGTase, high-efficiency production of γ-CGTase and process optimization has long been a pressing issue [8].

Our team has been dedicated to accomplishing efficient preparation of γ-CD. Previously we screened and characterized a γ-CGTase from *Bacillus* sp. FJAT-44876 (BFγ-CGTase) with excellent product specificity [9]. Different strategies were also tried for process optimization. Firstly, the BFγ-CGTase was used to pretreat the cassava starch under swelling condition to address the problem that this enzyme cannot perform a liquefaction function during the gelatinization process. The conversion rate of γ-CD reached up to 21.8% [10]. Secondly, a process for simultaneously modifying starch by pullulanase and CGTases to improve yields of CD was also developed and applied, effectively enhancing the conversion rate of α-CD and β-CD [11].

The above-mentioned research achievements provided tools and a technological basis for the large-scale production of γ-CD. In the present work, BFγ-CGTase was produced in *P. pastoris* with increased expression level due to optimized fermentation conditions. To address the issue that BFγ-CGTase cannot play a liquefying function in the gelatinization of highly concentrated starch, a novel step is introduced utilizing β-CGTase for liquefaction then followed by simultaneous pullulanase-catalyzed debranching and BFγ-CGTase production of γ-CD. The presence of a specific complexing agent for γ-CD and the four reactions of β-CGTase ensured that γ-CD became the predominant product in this multi-enzyme process, as the cyclization reaction of β-CGTase was negligible. This study provided a novel route for efficient preparation of γ-CD, which supports initiatives towards its large-scale production and application.

2. Material and Methods

2.1. Strains, Plasmids and Gene Origin

The *P. pastoris* GS115 strain was utilized as host for heterologous production of *Bacillus* sp. FJAT-44876 BFγ-CGTase (NCBI accession number: WP_096185680.1) [9] and *Bacillus* sp. 1011 β-CGTase (Bsβ-CGTase, NCBI accession number: AAA22308.1) [12]. The plasmid

was used as an expression vector. *P. pastoris* GS115 and pPIC9K were a kind gift from Professor Gao Minjie's research team (Jiangnan University, Wuxi, Jiangsu, China). *E. coli* DH5α was used as the host for cloning. The codon optimization of the target gene for the *Pichia pastoris* system and gene synthesis were performed by Genewiz (Suzhou, China). *E. coli* BL21 (DE3) strain carrying the plasmid pET-28a-BtPul (*Bacillus thermoleovorans* US105 pullulanase, BtPul, GenBank accession number: AJ315595) was a precious gift from the group of Professor Yao Nie (Jiangnan University, Wuxi, China) [13].

2.2. Media

The screening of *E. coli* DH5α was carried out using LB medium (0.5% yeast extract, 1% peptone, 1% NaCl and 50 μg/mL kanamycin) and LB agar plates (prepared by adding 1.5% agar to liquid LB medium). The following media were used for fermentation of *P. pastoris* GS115: YPD medium (1% yeast extract, 2% peptone and 2% dextrose); BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate buffer at pH 6.0, 1% glycerol and 4×10^{-5}% biotin); and BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate buffer at pH 6.0, 0.5% methanol and 4×10^{-5}% biotin). YPD agar plates (prepared by adding 1.5% agar to YPD medium) were used for the activation of *P. pastoris* GS115, while minimal dextrose (MD) agar plates (1.34% yeast nitrogen base, 2% dextrose, 1.5% agar and 4×10^{-5}% biotin) were used for the screening of *P. pastoris* GS115 transformants [14].

2.3. Construction of Recombinant Plasmids

The recombinant plasmids were constructed by utilizing homologous recombination assembly. Primers containing homologous arms (Table 1) were designed to amplify both the BFγ-CGTase/Bsβ-CGTase gene fragment and the linearized pPIC9K vector through Polymerase Chain Reaction (PCR) [15]. The DNA fragments were then assembled into the recombinant plasmid using a homologous recombination enzyme at 37 °C and incubated for 15 min. The recombinant plasmid was introduced into *E. coli* DH5α by transformation, followed by incubation (37 °C, 16–20 h). A single colony was inoculated into LB media and grown (overnight, 37 °C, shaking at 200 rpm) [16]. To confirm the successful cloning, the recombinant plasmid was sequenced by Genewiz (Suzhou, China). A recombinant plasmid encoding a variant of BFγ-CGTase (NEBFγ-CGTase) was constructed, in which the N-terminal sequence was replaced by the N-terminal sequence of Bsβ-CGTase. The primers used for constructing BFγ-CGTase-pPIC9K, Bsβ-CGTase-pPIC9K and NEBFγ-CGTase-pPIC9K are listed in Table 1. The codon-optimized version of the BFγ-CGTase gene (COBFγ-CGTase) was synthesized by Genewiz and directly cloned into the pPIC9K plasmid.

Table 1. Primers used for plasmid construction and colony PCR.

Primers	Sequences (5′-3′)
BFγ-CGT-F	GAGAGGCTGAAGCTTACGTAGAATTCGATTCTTCGACCGACTTGGAAAGTGTC
BFγ-CGT-R	CCTAGGGAATTCTTAATGGTGATGGTGATGATGGGCGCC
NEBFγ-CGT-F	GAGAGGCTGAAGCTTACGTAGAATTCGCCCCGGATACCTCGGTATCCAAC
pPIC9K-γ-F	AATTCTACGTAAGCTTCAGCCTCTCTTTTCTCGAGAGAT
pPIC9K-γ-R	CCATCACCATTAAGAATTCCCTAGGGCGGCCGCGAAT
BFγ-CGT-colony PCR-F	GTCTCTCGCTTGGATCCCAACACCATAT
BFγ-CGT-colony PCR-R	GGACCCAGATCAATCGATTGGTCCGAT
COBFγ-CGT-colony PCR-R	CCTGAATTGGGTAATTGGGATCCTGATCAATCT
NEBFγ-CGT-colony PCR-R	GGACCCAGATCAATCGATTGGTCCGAT
Bsβ-CGT-F	AGAAAAGAGAGGCTGAAGCTTACGTAATGGCCCCGGATACC
Bsβ-CGT-R	TGTCTAAGGCGAATTAATTCTCAGTGGTGGTGGTGGTGGT
pPIC9K-β-F	GAATTAATTCGCCTTAGACATGACTG
pPIC9K-β-R	AGCTTCAGCCTCTCTTTTCTCG
Bsβ-CGT-colony PCR-F	CGAGTCAGCAACAGCAACGCAGAAGC
Bsβ-CGT-colony PCR-R	GGCACGGCAACGGTGAATGTGAAC

2.4. Transformation into P. pastoris GS115 and Expression of BFγ-CGTase and Bsβ-CGTase

The recombinant plasmid was subjected to linearization using the restriction enzyme *Sal*I, and subsequently electroporated (1.5 kV, 5 ms) into *P. pastoris* GS115 competent cells. The transformation was conducted using an approach entailing the selection of transformed cells on MD plates (30 °C, 3 d). The generation of templates for PCR screening was initiated by selection and lysis of individual transformants from the MD plates [17]. These transformants were subjected to heating in 20 µL of yeast lysis buffer (95 °C, 10 min). Subsequently, 3 µL of the crude lysate was utilized for PCR amplification. The resulting amplicons were subjected to agarose gel electrophoresis. Clones that exhibited a PCR product of the expected size were identified as positive. PCR primers are listed in Table 1.

Positive transformants were inoculated into 4 mL YPD medium and grown (at 30 °C, overnight, with shaking at 250 rpm). After measuring the optical density, the cells were inoculated into 25 mL fresh BMGY medium to a starting OD_{600} of 0.1 and grown under the same conditions (30 °C, 250 rpm) for 16–18 h. Subsequently, the cells were subcultured into methanol-containing BMMY medium (30 mL) at an OD_{600} of 1.0 and induced for 72 h (30 °C, 250 rpm), with 1% methanol supplemented every 24 h. Finally, the cells were removed by centrifugation at 8000× *g* (4 °C, 30 min), and the supernatant (fermentation broth) was collected for downstream analysis [14].

2.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Fermentation supernatant (30 mL) was concentrated to approximately 1 mL by an ultrafiltration concentrator (Millipore®, Darmstadt, Germany, 4 °C, 4000 rpm). The retentate was subjected to diafiltration using a buffer (20 mM Tris-HCl, pH 8.0) and a wash volume exceeding by two-fold the original fermentation volume. After 4–5 washing cycles, the final product was concentrated to 1 mL as above. A 24-µL aliquot of the concentrated fermentation broth was subjected to SDS-PAGE analysis as previously reported [18].

2.6. Activity and Specificity Assay

The substrate was prepared by dissolving soluble starch (1.5%, *w*/*v*) in 20 mM phosphate buffer (Na_2HPO_4–NaH_2PO_4 (pH 6.0) was used for Bsβ-CGTase; Gly–NaOH (pH 10.0) was used for BFγ-CGTase). The reaction was initiated by mixing substrate and enzyme solutions in a 9:1 volume ratio, followed by incubation (50 °C, 10 min) and terminated on a boiling water bath (15 min). The activity of the BFγ-CGTase solution and the Bsβ-CGTase solution was 1 U/mL and 3 U/mL, respectively.

To determine the optimum temperature, the cyclization activity of BFγ-CGTase was assayed at 30, 40, 50, 60, 70 and 80 °C as described above. Based on the obtained optimum temperature, BFγ-CGTase was incubated at 35, 40, 45 and 50 °C for different time intervals, and the residual enzyme activity was measured to evaluate its thermal stability.

The optimum pH was determined using substrate in 20 mM buffers with pH values ranging from 3.5 to 12.0 (CH_3COOH–CH_3COONa, pH 3.5–5.0; Na_2HPO_4–NaH_2PO_4, pH 6.0–8.0; and Gly–NaOH pH 8.5–12.0). The cyclization activity of BFγ-CGTase was determined at the optimum temperature.

Quantification of γ-CD was performed by high-performance liquid chromatography (HPLC, Waters Co., Milford, MA, USA) equipped with an XBridge Amide column (4.6 mm × 200 mm, 5 µm) using 65% (*v*/*v*) acetonitrile as mobile phase. The flow rate, column temperature, and injection volume were set at 0.8 mL/min, 30 °C, and 20 µL, respectively [19].

One unit of enzyme activity (U) was defined as the amount of enzyme generating 1 mmol γ-CD per minute.

2.7. Deglycosylation Analysis

Proteins produced by *P. pastoris* often undergo glycosylation [20]. To ascertain the impact of glycosylation on enzyme activity and product specificity, 50 μg non-denatured recombinant BFγ-CGTase was initially treated with 500 U PNGase F (P2318S, Beyotime, Shanghai, China) at 37 °C for 12 h. Subsequent to the treatment, characterization was conducted by enzymatic activity, SDS-PAGE, and HPLC analyses. Samples incubated under identical conditions without addition of PNGase F served as controls [21].

2.8. Homology Modeling

The 3D structure of Bsβ-CGTase has been published (PDB: 1UKQ). Modeling of BFγ-CGTase was based on the crystal structure of γ-CGTase from *Bacillus clarkii* 7364 (PDB: 4JCM). The tertiary structures of BFγ-CGTase and NEBFγ-CGTase were constructed using the AlphaFold online website (https://AlphaFoldserver.com (accessed on 18 December 2025)). Structural analysis was performed using PyMOL™3.1 (http://pymol.org (accessed on 18 December 2025)) [9,22].

2.9. One-Factor-at-a-Time Experiments

The effects of induction temperature, initial medium pH, methanol concentration, and fermentation time on BFγ-CGTase expression were examined using a one-factor-at-a-time (OFAT) approach. During the induction phase, cultures were maintained at 30, 28, 26, or 24 °C to assess the influence of temperature on enzyme production and cell growth. Additionally, the initial pH of the medium was adjusted to 5.0, 6.0, 7.0 or 8.0 to evaluate its impact on protein production. Subsequently, methanol was supplied at final concentrations ranging from 0.5% to 4.0% (0.5% intervals) to investigate its effects on BFγ-CGTase activity and biomass accumulation. Finally, the fermentation was carried out for 48, 72, 96 or 120 h to determine the optimal induction duration. All experiments were performed in triplicate. The control condition was set as 30 °C, initial pH 6.0, 1.0% methanol, and 72 h fermentation [23].

2.10. Establishment and Optimization of an Efficient Process for γ-CD Production

Cassava starch as 30% (*w*/*v*) suspension in sodium phosphate (50 mM, pH 8.0) was used for preparation of γ-CD. Bsβ-CGTase (2.0 U/g starch) was added at 30 °C, and the temperature was raised to 100 °C, the heating process lasting at least 2 h [24]. After 60 °C, the temperature should not increase by more than 10 °C per 30 min. The resulting liquefied starch was rapidly cooled to 40 °C, followed by addition of BFγ-CGTase, BtPul, and 5% (*v*/*v*) chelating agent (cyclohexadecanone). The mixture was reacted at 160 rpm for 12 h. After the reaction, the mixture was boiled for 30 min and dried at 50 °C for later use.

A single-factor adjustment method was employed to optimize enzyme loads in the preparation process. First, dosage of BtPul was fixed at 20 U/g starch, and BFγ-CGTase was added at 1, 2, 3, 5, 7, 10 and 15 U/g starch. After determining the optimal BFγ-CGTase dosage this was applied in experiments with BtPul added of 1, 2, 3, 5, 7, 10, 15, 20 and 30 U/g starch to identify the best dual-enzyme dosage.

2.11. Statistical Analysis

All experiments were performed in three biological replicates. Data were processed and graphs were plotted using GraphPad Prism version 10.0 (GraphPad Software LLC., San Diego, CA, USA). The data were presented as the mean ± standard deviation (SD).

3. Results and Discussion

3.1. Expression and Characterization of Recombinant BFγ-CGTase

The production of BFγ-CGTase in *Escherichia coli* was relatively low [25]; therefore, *P. pastoris* was selected to obtain the amounts of BFγ-CGTase (Figure 1A) needed for the large-scale γ-CD production. The recombinant plasmids were constructed by the utilization of homologous recombination. Secretion of the recombinant protein was carefully regulated by incorporating the pan-effective α-factor signal peptide from *Saccharomyces cerevisiae*, while a 6 × His tag for the affinity purification was added to the C-terminus (Figure 1B). After linearization with *Sal*I, the plasmids were integrated into the HIS4 locus of *P. pastoris*. This integration converted the histidine auxotrophic strain into a His+ phenotype, facilitating selection on histidine-deficient MD plates. The confirmation of positive clones among His+ transformants was achieved through colony PCR. One colony PCR primer was designed to anneal to a region on chromosome 1 of the *P. pastoris* genome, and the other to a site within the coding sequence of the target gene. Consequently, a distinct amplified band was observed by integration of the target gene and successful colonies manifested a bright band of the anticipated size. Positive clones were identified for each of the three types of BFγ-CGTase (Figure 1C–E) and the Bsβ-CGTase (Figure S1) transformants. The successful transformants were selected for the fermentation process to obtain recombinant proteins.

BFγ-CGTase and Bsβ-CGTase were successfully produced in *P. pastoris* (Figure 2A,B) with cyclization activity of 0.035 U/mL and 13.3 U/mL, respectively. A histidine tag was added to the C-terminus of the recombinant protein, but nickel affinity purification was unsuccessful. Therefore, the method described in Section 2.5 was used to remove the medium and impurities as much as possible. Additionally, there were few contaminant proteins in the supernatant of the *P. pastoris* fermentation, which did not affect the expression or enzymatic properties of the recombinant protein. As shown in Figures 3 and S2, the same β-CD and γ-CD product specificity was found for the BFγ-CGTase produced in *P. pastoris* as compared to in *E. coli* BL21 (DE3) [25]. The optimal temperature and pH of BFγ-CGTase were 40 °C and 11, respectively (Figure 2D,E). Notably, optimal temperature of BFγ-CGTase from *P. pastoris* was 10 °C lower than that from *E. coli*, and it was speculated that glycosylation in the yeast affected the structure of BFγ-CGTase, leading to decreased thermal stability (Figure 2F). However, as deglycosylation was not complete for the native recombinant enzyme (Figure 4A,B), perhaps steric hindrance prevented the access of PNGase F. Subsequently, complete deglycosylation was demonstrated under denaturing conditions (Figure 4C). Glycosylation did not affect the product specificity of BFγ-CGTase; therefore, further deglycosylation treatment was not considered.

3.2. Codon Optimization and N-Terminal Substitution Had Limited Effect on Enhancing Expression Levels of BFγ-CGTase

As illustrated in Figure S3, compared with the control, no high-molecular-weight aggregates, indicative of inclusion body formation, were found in the clarified lysate and pellet fractions following cell disruption. Additionally, no protein bands were detected corresponding to BFγ-CGTase. Moreover, the clarified lysate after yeast disruption showed no activity generating γ-CD. These two experimental observations evidenced that BFγ-CGTase was obtained without important folding defects. A comparative analysis of secreted protein bands in the clarified lysate after cell disruption and the pellet indicated that the α-factor secretion pathway efficiently exported detectable amounts of the enzyme. Consequently, signal peptide optimization appeared unnecessary for enhancing the yield of secreted enzyme. The high yield of Bsβ-CGTase also informed about the promoter's suitability for this type of enzyme.

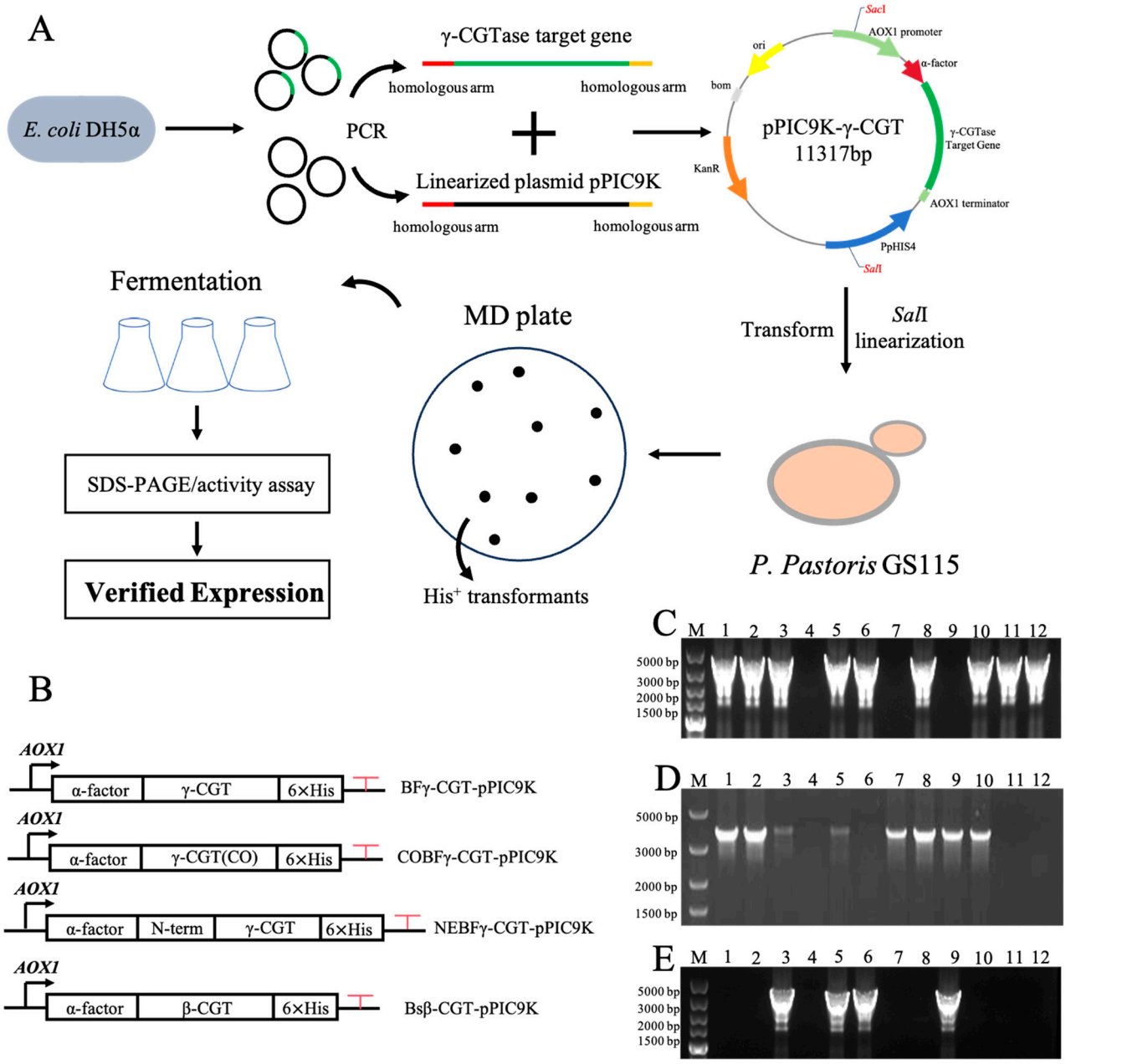

Figure 1. (**A**) Workflow for constructing and screening the recombinant *P. pastoris* GS115 strain for BFγ-CGTase expression. (**B**) Strategy for the construction of four recombinant vectors for BFγ-CGTase and Bsβ-CGTase using different optimization approaches. (**C**–**E**) Detection of positive clones by colony PCR. Line M, marker; Lines 1–12, transformants. where those with bands are positive transformants. (**C**) BFγ-CGTase; (**D**) COBFγ-CGTase; (**E**) NEBFγ-CGTase.

In the aforementioned study, Bsβ-CGTase exhibited superior expression levels and enzyme activity compared to BFγ-CGTase. Therefore, codon optimization and N-terminal substitution were employed to enhance the expression of BFγ-CGTase. According to the codon optimization evaluation tool (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0 (accessed on 10 September 2024)), the CAI value of the gene increased from 0.70 (pre-optimization) to 0.97 (post-optimization) and therefore COBFγ-CGTase was constructed and expressed in *P. pastoris*. The relative enzyme amount of COBFγ-CGTase decreased by 30% compared to the recombinant BFγ-CGTase (Figure 2C). This may be due to differences in adaptability to fermentation conditions. Further optimization of fermentation conditions

for COBFγ-CGTase was conducted, and expression levels were compared under optimal conditions in subsequent work.

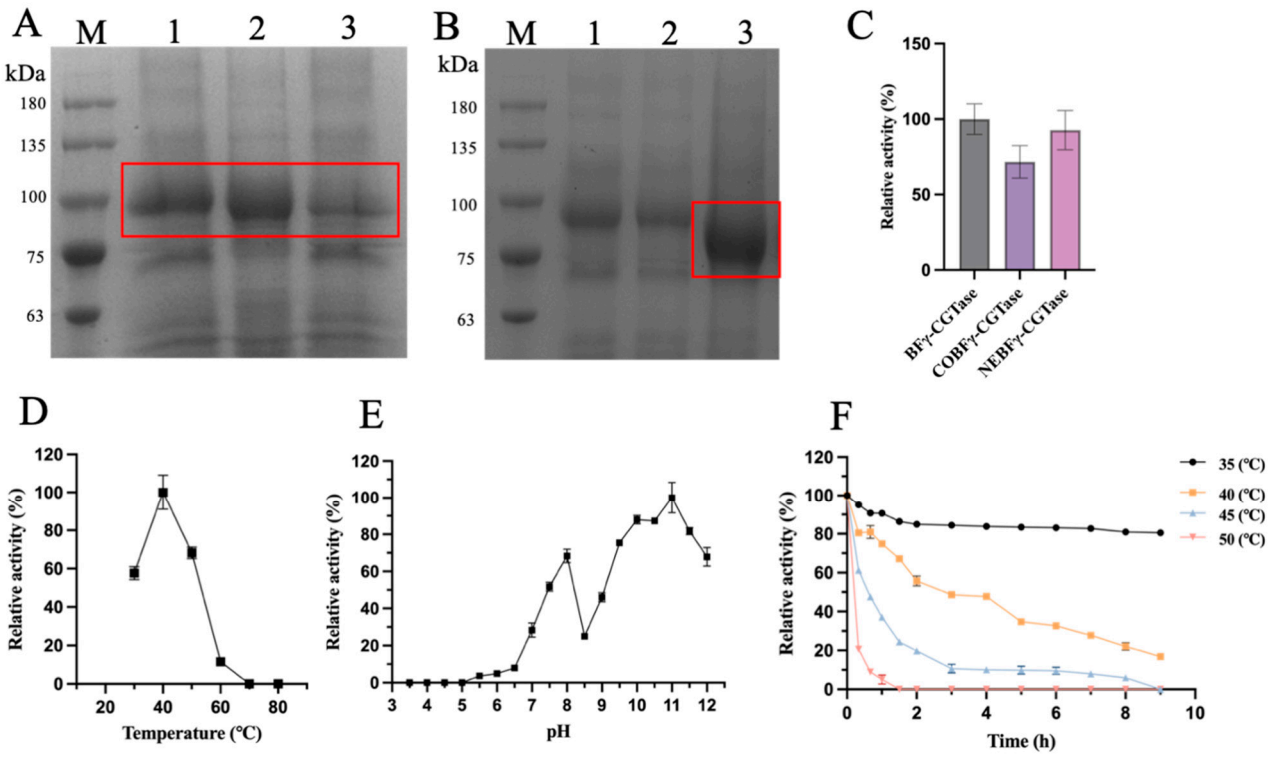

Figure 2. SDS-PAGE and relative enzyme activity of concentrated fermentation supernatants. (**A**) Concentrated fermentation supernatant. Lane M, marker; Lane 1, BFγ-CGTase; Lane 2, COBFγ-CGTase; Lane 3, NEBFγ-CGTase. Protein bands of target enzymes indicated in red boxes. (**B**) Concentrated fermentation supernatant. Lane M, marker; Lane 1 and Lane 2, BFγ-CGTase; Lane 3: Bsβ-CGTase (Lane 1 and Lane 2 were not included in the data analysis). Protein bands of target enzymes indicated in red boxes. (**C**) Relative enzyme activity analysis of BFγ-CGTase, COBFγ-CGTase and NEBFγ-CGTase. (**D**) Optimal temperature of BFγ-CGTase. The reaction was performed for 10 min on 1.5% soluble starch, pH 10.0. (**E**) Optimal pH of BFγ-CGTase. The reaction was carried out in 1.5% soluble starch at 40 °C for 10 min. (**F**) Temperature stability of BFγ-CGTase. The enzyme solution was incubated in Tris-HCI buffer at pH 7.5. The activity was determined on 1.5% soluble starch at 40 °C, pH 11.0.

Modifications of the N-terminal sequence have been shown previously to result in substantial variation in heterologous expression levels, with potential to influence the solubility, secretion efficiency, or transport efficiency of the recombinant protein [26]. For example, improved spatial accessibility to the N-terminal region increased the titer and quality resulting in 76% increase in heterologous production of SARS-CoV-2 protein in *P. pastoris* [27]. In the present study, under consistent fermentation conditions, the expression level of Bsβ-CGTase was 300 times higher than that of BFγ-CGTase, which shares sequence and structural similarity of 55.15% (https://blast.ncbi.nlm.nih.gov (accessed on 15 June 2025)) and 95.23% (https://alphafoldserver.com (accessed on 15 June 2025)), respectively. Structural alignment indicated the presence of restricting differences in the N-terminal regions of the two enzymes (Figure S4). Therefore, homologous recombination was employed to substitute the seven residues of the N-terminal segment of BFγ-CGTase with the N-terminal eight residues sequence of Bsβ-CGTase to enhance expression of BFγ-CGTase. Structural comparison revealed improved similarity between the tertiary structures for NEBFγ-CGTase, having the replaced N-terminal sequence, and Bsβ-CGTase (Figure S4). The SDS-PAGE (Figure 2A) provided evidence for successful se-

cretion of NEBFγ-CGTase by *P. pastoris* GS115. However, enzymatic activity measurements (Figure 2C) revealed no significant difference in production level between recombinant NEBFγ-CGTase and BFγ-CGTase. The above results support that N-terminal structural features were not the key factor affecting production of BFγ-CGTase by *P. pastoris* GS115.

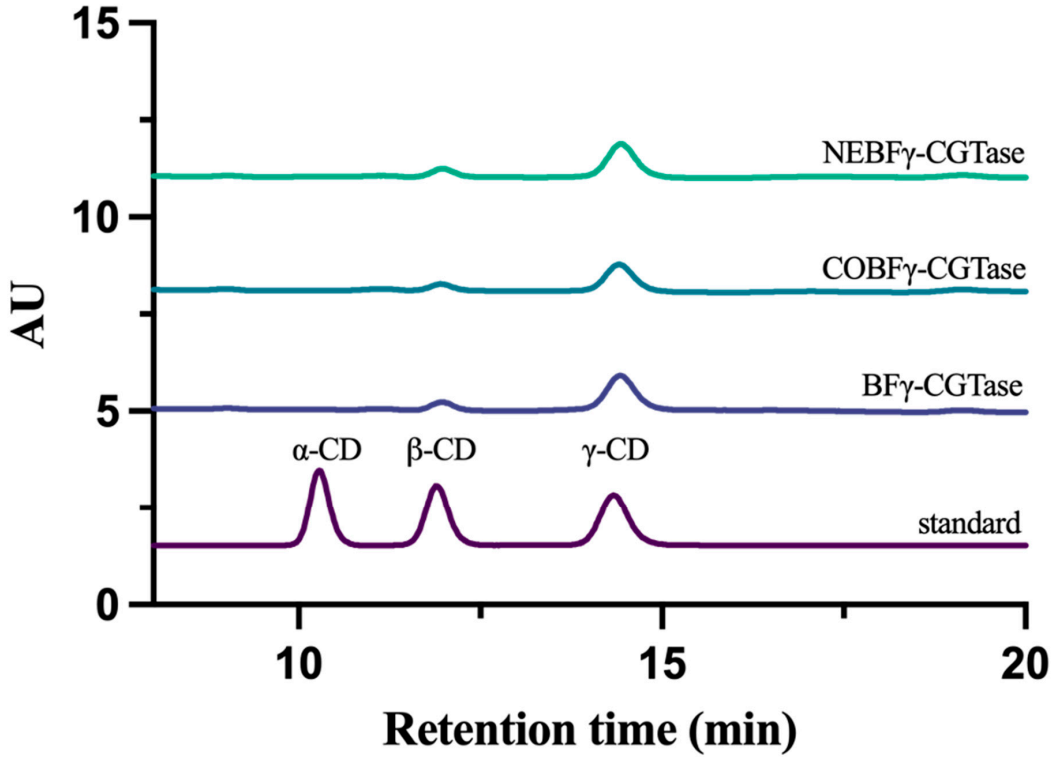

Figure 3. The product specificity of BFγ-CGTase, COBFγ-CGTase and NEBFγ-CGTase analyzed by HPLC. Retention times of cyclodextrin standards: α-CD (10.28 min), β-CD (11.89 min), γ-CD (14.33 min).

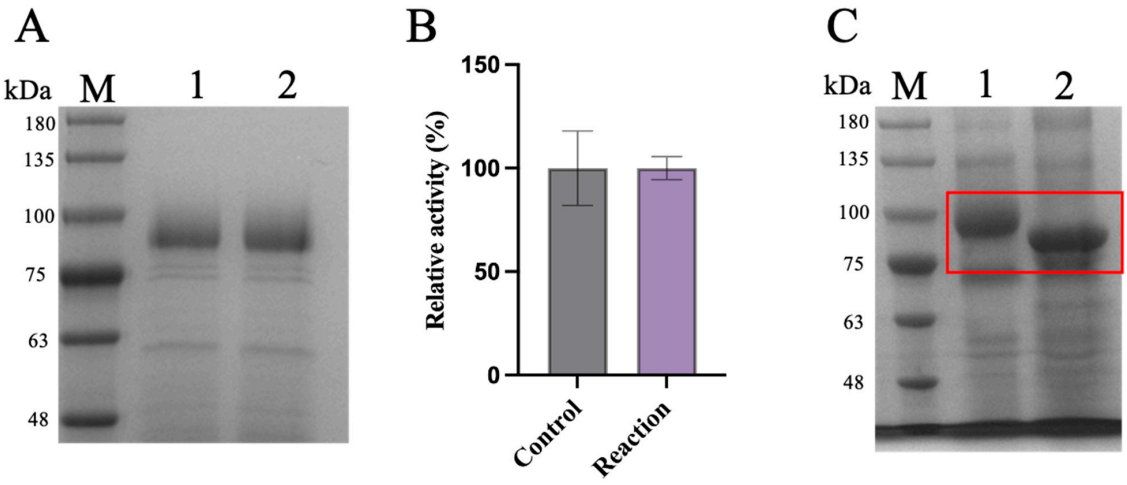

Figure 4. Deglycosylation of recombinant BFγ-CGTase and measurement of its relative activity. (**A**) Deglycosylation under non-denaturing conditions. Lane M, marker; Lane 1, control; Lane 2, recombinant BFγ-CGTase after enzymatic treatment. (**B**) Relative enzyme activity. Control, BFγ-CGTase incubated at 37 °C for 12 h without PNGase F treatment. Reaction, BFγ-CGTase was incubated at 37 °C for 12 h with PNGase F. At the end the enzyme activity was determined on 1.5% soluble starch at 40 °C and pH 11.0 for 10 min. (**C**) Deglycosylation under denaturing conditions. Denature the sample by boiling it at 100 °C for 10 min. Lane M: marker; Lane 1: control; Lane 2: recombinant BFγ-CGTase after enzymatic treatment. Protein bands of target enzymes indicated in red boxes.

3.3. Optimization of Fermentation Conditions Significantly Boosted Expression Levels of BFγ-CGTase

Fermentation parameters such as temperature, pH, and dissolved oxygen levels also impact protein yields [28]. For example, the amount of recombinant myofibril binding serine protease (rMBSP) increased 6-fold by optimizing *P. pastoris* fermentation conditions [29]. Similarly, the amount of *Ganoderma lucidum* immunomodulatory protein (FIP-glu) increased by 30% through response surface optimization of fermentation conditions [30]. Therefore, temperature, pH, amount of methanol added, and fermentation time were selected as variables subjected to single-factor optimization of fermentation conditions for BFγ-CGTase and COBFγ-CGTase. Results showed that compared to the original fermentation conditions, the expression levels of both BFγ-CGTase and COBFγ-CGTase were enhanced (Figures 5 and 6). Under optimal conditions of 26 °C, pH 7.0, 3% methanol addition, and 72 h fermentation, the yield of BFγ-CGTase increased 7.25-fold. For COBFγ-CGTase under optimal conditions, 26 °C, pH 7.0, 3% methanol addition, and 96 h fermentation, the yield increased 18.5-fold compared to the original value. Both these results demonstrated the impact of fermentation conditions and that following codon optimization, the relative effects of the four parameters changed to some degree. pH exerted the strongest influence on BFγ-CGTase production (Figure 5B), while methanol concentration most strongly affected that of COBFγ-CGTase (Figure 6C). This can explain why COBFγ-CGTase expression was initially lower than BFγ-CGTase under the original conditions and that under optimal conditions, the yield of COBFγ-CGTase was 1.83-fold higher than that of BFγ-CGTase. Notably, optimal fermentation conditions for both recombinant strains were remarkably similar, making this comparison highly relevant.

3.4. High Efficiency Preparation of γ-CD

Enhancing CD conversion rates has long been a focus for both industry and researchers in academia. Since CGTase cannot act on α(1 → 6) glucosidic bonds in starch, the initial conversion rate to cyclodextrin is limited to 30–50% [31,32]. To enhance starch utilization, it is proposed to apply debranching enzymes. However, in debranching reactions on highly concentrated starch, the debranched starch readily retrogrades [33]. Consequently, a strategy was developed combining debranching with cyclization. For instance, Jing Wu's team achieved synergistic action between isoamylase and either α-CGTase or γ-CGTase, increasing α-CD and γ-CD conversion rates by 31.2% and 22.1%, respectively [7,34]. However, given the quite low optimal temperature and lack of commercialization of isoamylase, attention was turned to pullulanase. Unfortunately, during the synergistic reaction, the produced CDs inhibited pullulanase, limiting debranching efficacy [11]. Therefore, our previously produced pullulanase mutant, having reduced sensitivity to CDs when combined with CGTase, resulting in 10% enhanced β-CD yield [35], guided the present development of γ-CD production.

A major challenge in forming γ-CD, unlike for β-CD, is the low thermal stability of the γ-CGTase, which is not able to withstand the liquefaction process of high-concentration starch. We previously proposed a γ-CGTase liquefaction process below the gelatinization temperature and achieved a yield of 21.81% γ-CD using 30% cassava starch as substrate [10]. However, for practical applications this process still suffers from the low γ-CD conversion rates due to incomplete starch utilization. Building on the prior demonstration of β-CGTase's excellent starch liquefaction capabilities, we propose efficient γ-CD production by combining β-CGTase liquefaction with simultaneous pullulanase debranching and γ-CGTase reaction (Figure 7). Since the liquefaction step lacks β-CD complexing agents and reaction equilibrium factors, minimal amounts of β-CD are generated. Moreover, β-CD can

serve as a donor in the coupling reaction to convert into γ-CD [36]. Therefore, this reaction pathway was highly feasible.

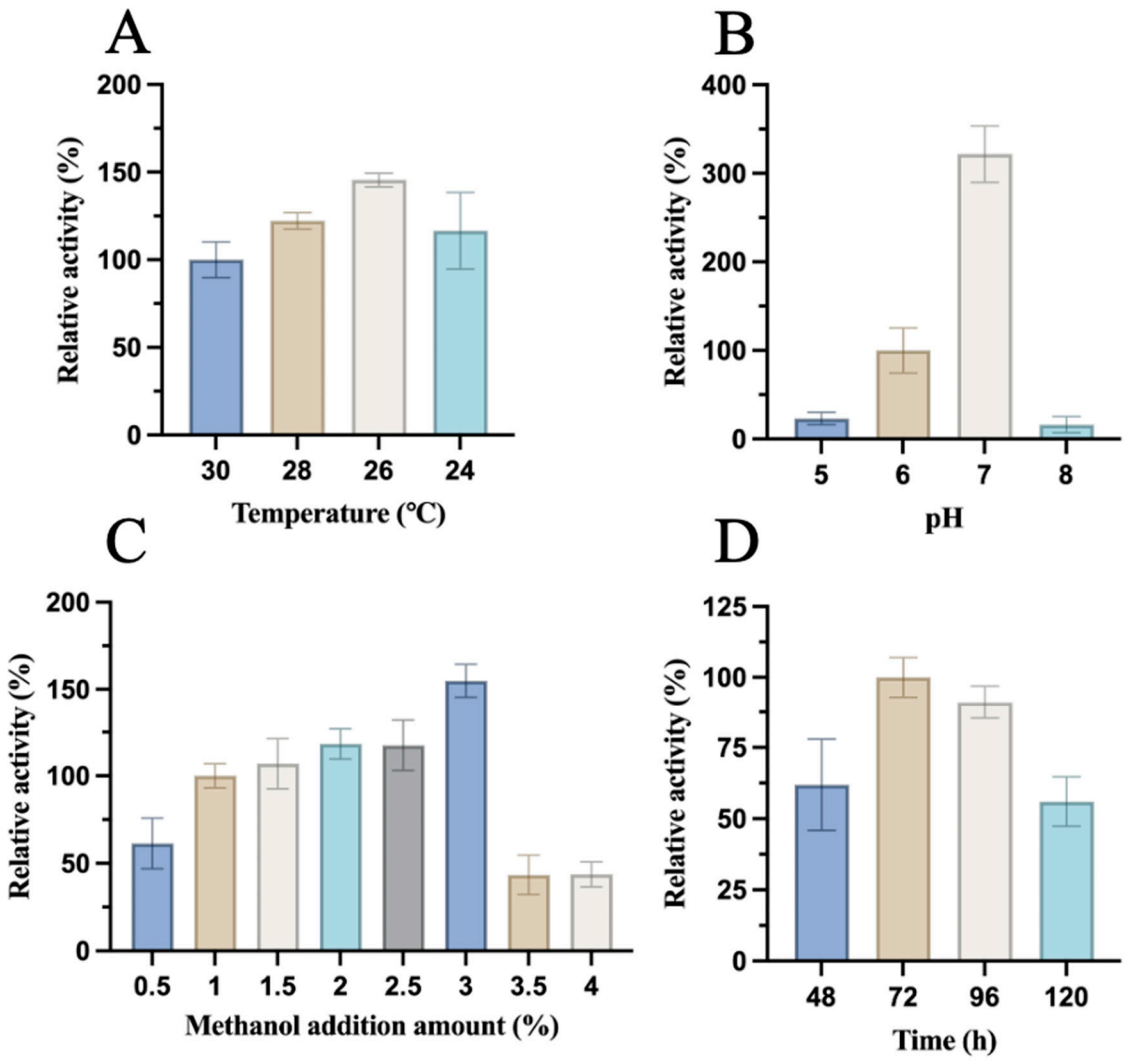

Figure 5. One-factor-at-a-time optimization of BFγ-CGTase. Expression of BFγ-CGTase at different (**A**) fermentation temperatures; (**B**) initial pH of fermentation; (**C**) amounts of methanol addition; and (**D**) fermentation times.

Considering the pH activity adaptability of Bsβ-CGTase, BFγ-CGTase, and BtPul, as well as the temperature stability of BFγ-CGTase (Figures 2D,E and S5), pH 8 and 40 °C were chosen for the preparation of γ-CD. In the conversion calculation of γ-CD, the quantification was performed using the complex of γ-CD with cyclohexadecenone, the complexing agent. HPLC results showed that its peak area was consistent with that of pure γ-CD (Figure S6), and it did not affect the quantification of the γ-CD conversion. Combined with mass spectrometry, the main product was determined to be γ-CD (Figure S7). At fixed BtPul addition of 20 U/g starch, the conversion of γ-CD exhibited a gradual increase with increasing addition of BFγ-CGTase, followed by a slight decrease (Table 2). The maximum conversion rate of 39.48% being obtained for 5 U BFγ-CGTase per gram of starch. Apparently, the substrate provided by enzymatic debranching sufficed for conversion by less than 5 U/g starch of BFγ-CGTase. As shown in Table 3, keeping BFγ-CGTase at 5 U/g starch with the augmentation of BtPul the γ-CD conversion rate exhibited an initial period of stability, followed by a gradual increase to ultimately reach highest yield at 20 U/g starch or higher amounts of BtPul. The large amount of linear substrates formed by

BtPul debranching was not utilized by BFγ-CGTase in a timely manner. The low reaction temperature causes these linear substrates to retrogradation, so γ-CD did not increase with the amount of BtPul added.

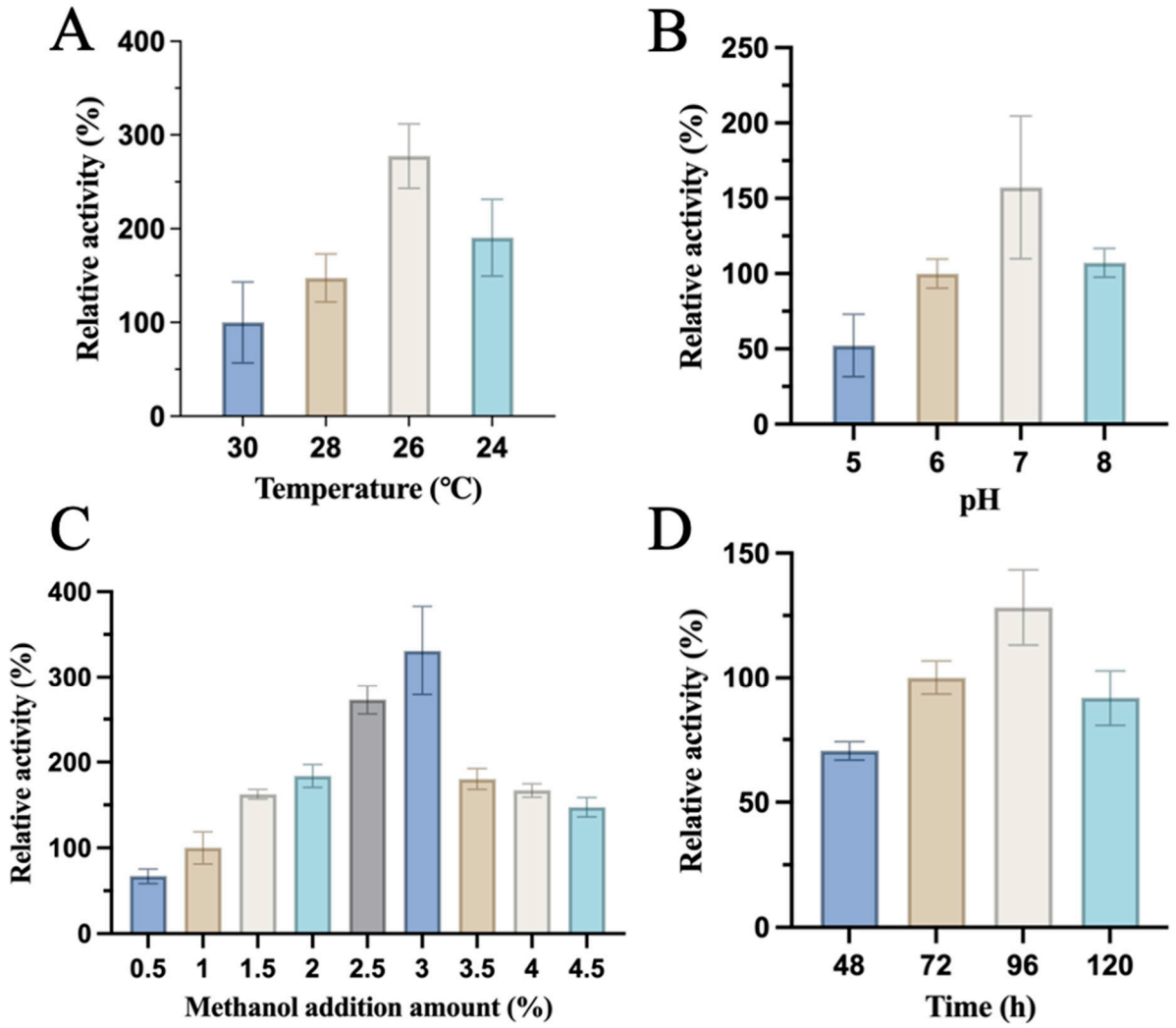

Figure 6. One-factor-at-a-time optimization of COBFγ-CGTase. Expression of COBFγ-CGTase at different (**A**) fermentation temperatures; (**B**) initial pH of fermentation; (**C**) amounts of methanol addition; and (**D**) fermentation times.

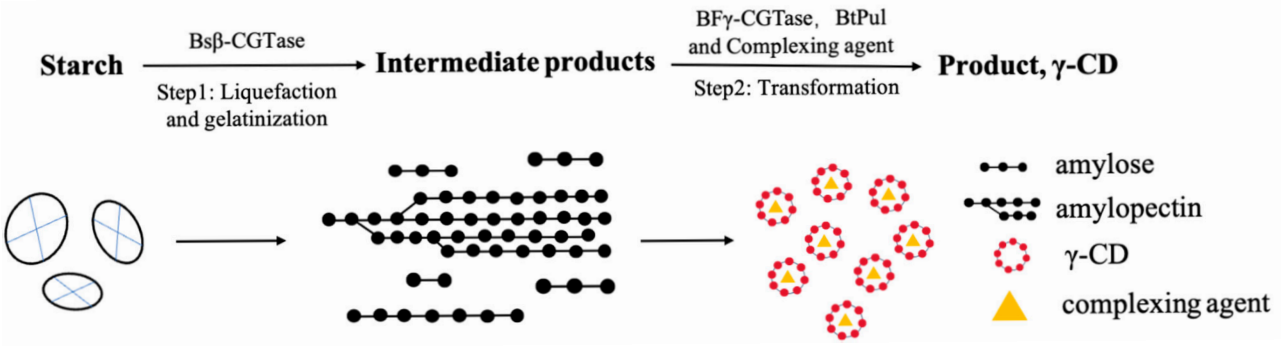

Figure 7. Schematic diagram of the efficient γ-CD production process combining liquefication by Bsβ-CGTase and simultaneous debranching by BtPul and conversion by BFγ-CGTase.

Table 2. γ-CD production under different BFγ-CGTase addition conditions [a].

Amount of BFγ-CGTase Added (U/g Starch)	Yield of γ-CD [b] (%)
1	27.95 ± 1.89 a
2	37.07 ± 0.46 bc
3	38.53 ± 0.53 bc
4	37.62 ± 0.59 bc
5	39.48 ± 0.49 c
7	36.08 ± 0.12 b
10	35.88 ± 0.14 b
15	36.29 ± 0.53 b

[a] Each value is the mean of three independent experiments. The values in the same column with lower case letters differ considerably ($p < 0.05$). [b] γ-CD is produced synchronously with BFγ-CGTase and BtPul (20 U/g starch) under the condition of 40 °C and pH 8.0 for 12 h.

Table 3. γ-CD production under different BtPul addition conditions [a].

Amount of BtPul Added (U/g Starch)	Yield of γ-CD [b] (%)
1	34.38 ± 0.23 ab
2	33.80 ± 0.02 a
3	34.52 ± 0.51 ab
4	33.90 ± 1.18 a
5	35.04 ± 0.79 ab
7	35.77 ± 1.04 abc
10	36.60 ± 2.04 abc
15	38.13 ± 0.03 bc
20	39.48 ± 0.49 c
30	39.09 ± 0.67 c

[a] Each value is the mean of three independent experiments. The values in the same column with lower case letters differ considerably ($p < 0.05$). [b] γ-CD is produced synchronously with BtPul and BFγ-CGTase (5 U/g starch) under the condition of 40 °C and pH 8.0 for 12 h.

4. Conclusions

This study proposed two strategies for the industrialization of γ-CD production based on heterologous expression to increase yield of γ-CGTase and process optimization of the starch conversion. First, BFγ-CGTase and Bsβ-CGTase were successfully secreted using *P. pastoris* as heterologous host. Through codon optimization and fermentation conditions improvement using a one-factor-at-a-time approach, 13.3-fold increase was achieved for the yield of BFγ-CGTase. The protocol established for γ-CD production involved Bsβ-CGTase liquefaction followed by simultaneous BtPul debranching and BFγ-CGTase starch conversion, obtaining a 17.67% increase in γ-CD yield through optimization. Notably, BFγ-CGTase expression in *P. pastoris* significantly reduced the reaction temperature, which was a key factor limiting further improvements in γ-CD conversion. Future research will focus on efficient expression of BFγ-CGTase with optimized reaction temperature to be applied for γ-CD production from highly concentrated starch substrates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules31040581/s1, Figure S1: Colony PCR of Bsβ-CGTase; Figure S2: HPLC plot of the reaction product of BFγ-CGTase; Figure S3: SDS-PAGE of fragmentation and precipitation; Figure S4: Comparative analysis of tertiary protein structures; Figure S5: pH activity curve of Bsβ-CGTase; Figure S6: Standard curves of γ-CD quantified by HPLC; Figure S7: Preparation of the liquid chromatography-mass spectrogram (LC-MS) of γ-CD product by double enzyme method.

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X.L.; Writing—review & editing, X.L. and B.S.; Supervision, X.L., Z.J. and Y.B.; Project administration, Y.B.; Funding acquisition, Y.B. All authors have read and agreed to the published version of the manuscript.

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References

1. Bai, Y.; Wu, Y.; Ji, H.; Jin, Z. Synthesis, separation, and purification of glucosyl-β-cyclodextrin by one-pot method. *J. Food Biochem.* **2019**, *43*, e12890. [CrossRef]
2. D'Souza, V.T.; Lipkowitz, K.B. Cyclodextrins: Introduction. *Chem. Rev.* **1998**, *98*, 1741–1742. [CrossRef] [PubMed]
3. Del Valle, E.M.M. Cyclodextrins and their uses: A review. *Process Biochem.* **2004**, *39*, 1033–1046. [CrossRef]
4. Biwer, A.; Antranikian, G.; Heinzle, E. Enzymatic production of cyclodextrins. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 609–617. [CrossRef]
5. Loftsson, T.; Duchêne, D. Cyclodextrins and their pharmaceutical applications. *Int. J. Pharm.* **2007**, *329*, 1–11. [CrossRef]
6. Kabacaoğlu, E.; Karakaş Budak, B. Heterologous expression of β-γ-type cyclodextrin glycosyltransferase of newly isolated alkaliphilic *Bacillus* sp. SD5 in *Pichia pastoris*. *Starch-Stärke* **2017**, *69*, 1600365. [CrossRef]
7. Wang, L.; Wu, D.; Chen, J.; Wu, J. Enhanced production of γ-cyclodextrin by optimization of reaction of γ-cyclodextrin glycosyltransferase as well as synchronous use of isoamylase. *Food Chem.* **2013**, *141*, 3072–3076. [CrossRef]
8. Saokham, P.; Loftsson, T. γ-Cyclodextrin. *Int. J. Pharm.* **2017**, *516*, 278–292. [CrossRef]
9. Li, X.; Zheng, D.; Wu, J.; Jin, Z.; Svensson, B.; Bai, Y. Increasing γ-CD conversion rates by improving thermostability of *Bacillus* sp. FJAT-44876 γ-CGTase. *Food Biosci.* **2023**, *51*, 102204. [CrossRef]
10. Wu, H.; Li, X.; Ji, H.; Svensson, B.; Bai, Y. Improved production of gamma-cyclodextrin from high-concentrated starch using enzyme pretreatment under swelling condition. *Carbohydr. Polym.* **2022**, *284*, 119124. [CrossRef] [PubMed]
11. Li, X.; Bai, Y.; Ji, H.; Wang, Y.; Jin, Z. Phenylalanine476 mutation of pullulanase from *Bacillus subtilis* str. 168 improves the starch substrate utilization by weakening the product β-cyclodextrin inhibition. *Int. J. Biol. Macromol.* **2020**, *155*, 490–497. [CrossRef]
12. Ding, J.; Li, X.; Jin, Z.; Abou Hachem, M.; Bai, Y. Efficient glycosylation of polyphenols via dynamic complexation of cyclodextrin and synchronous coupling reaction with cyclodextrin glycosyltransferase in water. *Int. J. Biol. Macromol.* **2024**, *280*, 136065. [CrossRef]
13. Bi, J.; Chen, S.; Zhao, X.; Nie, Y.; Xu, Y. Computation-aided engineering of starch-debranching pullulanase from *Bacillus thermoleovorans* for enhanced thermostability. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 7551–7562. [CrossRef] [PubMed]
14. Zhang, J.; Zhang, Y.; Li, M. High-level secretion and characterization of cyclodextrin glycosyltransferase in recombinant *Komagataella phaffii*. *J. Biotechnol.* **2017**, *259*, 126–134. [CrossRef] [PubMed]
15. Li, X.; Xia, W.; Bai, Y.; Ma, R.; Yang, H.; Luo, H.; Shi, P. A novel thermostable GH3 β-Glucosidase from *Talaromyce leycettanus* with broad substrate specificity and significant soybean isoflavone glycosides-hydrolyzing capability. *BioMed Res. Int.* **2018**, *2018*, 4794690. [CrossRef] [PubMed]
16. Yu, J.; Liu, X.; Guan, L.; Jiang, Z.; Yan, Q.; Yang, S. High–level expression and enzymatic properties of a novel thermostable xylanase with high arabinoxylan degradation ability from *Chaetomium* sp. suitable for beer mashing. *Int. J. Biol. Macromol.* **2021**, *168*, 223–232. [CrossRef]
17. Zhang, X.; Yang, Y.; Chen, S.; Lin, Y.; Wu, Y.; Liang, S. Engineering *Pichia pastoris* for high-level biosynthesis of squalene. *Biochem. Eng. J.* **2025**, *217*, 109677. [CrossRef]
18. Chen, Y.; Dong, J.; Li, X.; Jin, Z.; Svensson, B.; Bai, Y. Acceptor subsite mutants of *Limosilactobacillus fermentum* NCC 2970 GtfB 4, 3-α-Glucanotransferase regulate the ratio of (α1→ 3)/(α1→ 6) linkages in biosynthesized α-Glucans. *J. Agric. Food Chem.* **2024**, *72*, 19994–20004. [CrossRef]
19. Ding, J.; Li, X.; Jin, Z.; Svensson, B.; Bai, Y. Regulating the rate-limiting step of cyclodextrin glycosyltransferase glycosylation reaction for efficient production of glycosylated polyphenols. *Bioresour. Technol.* **2025**, *2025*, 132727. [CrossRef]

20. Ohara, A.; Pengyue, Z.; Cao, X.; Donini, R.; Haslam, S.M.; Polizzi, K.M. The impact of methanol concentration on recombinant protein glycosylation in *Pichia pastoris* SuperMan5. *Microb. Biotechnol.* **2025**, *18*, e70272. [CrossRef]
21. Madhavan, A.; Sukumaran, R.K. Secreted expression of an active human interferon-beta (HuIFNβ) in *Kluyveromyces lactis*. *Eng. Life Sci.* **2016**, *16*, 379–385. [CrossRef]
22. Dong, J.; Abou Hachem, M.; Wang, Y.; Li, X.; Zhang, B.; Pijning, T.; Svensson, B.; Dijkhuizen, L.; Jin, Z.; Bai, Y. Tailor-made α-glucans by engineering the processivity of α-glucanotransferases via tunnel-cleft active center interconversions. *J. Agric. Food Chem.* **2024**, *72*, 11041–11050. [CrossRef] [PubMed]
23. Batra, J.; Beri, D.; Mishra, S. Response surface methodology based optimization of β-glucosidase production from *Pichia pastoris*. *Appl. Biochem. Biotechnol.* **2014**, *172*, 380–393. [CrossRef]
24. Dai, Y.; Li, X.; Jin, Z.; Bai, Y. Improving cyclodextrin yield via retarding retrogradation of debranched starches during the preparation of cyclodextrin with cyclodextrin glycosyltransferase and pullulanase. *Food Biosci.* **2024**, *59*, 104067. [CrossRef]
25. Zheng, D.; Bai, Y.; Ji, H.; Li, X.; Wang, Y.; Jiang, T.; Jin, Z. Expression, characterization and product specificity of gamma-CGTase from *Bacillus* sp. *Food Ferment. Ind.* **2020**, *46*, 38–45.
26. Fu, Y.; Luo, Y. The N-terminal integrity is critical for the stability and biological functions of endostatin. *Biochemistry* **2010**, *49*, 6420–6429. [CrossRef]
27. Dalvie, N.C.; Naranjo, C.A.; Rodriguez-Aponte, S.A.; Johnston, R.S.; Christopher Love, J. Steric accessibility of the N-terminus improves the titer and quality of recombinant proteins secreted from *Komagataella phaffii*. *Microb. Cell Factories* **2022**, *21*, 180. [CrossRef] [PubMed]
28. Thorpe, E.D.; d'Anjou, M.C.; Daugulis, A.J. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnol. Lett.* **1999**, *21*, 669–672. [CrossRef]
29. Li, T.; Li, W.; Guo, Y.; Han, L.; Zhang, W.; Liu, R.; Feng, H. An optimization by response surface methodology for the enhanced production of rMBSP from *Pichia pastoris* and study of its application. *Prep. Biochem. Biotechnol.* **2024**, *54*, 1320–1328. [CrossRef]
30. Liu, Y.; Guo, Z.; Yang, J.; Zhou, X. Optimization of liquid fermentation conditions for N-Glycosylated ganoderma lucidum Immunomodulatory protein production by an engineered *Pichia pastoris*. *J. Pure Appl. Microbiol.* **2025**, *19*, 476. [CrossRef]
31. Liu, Z.; Wu, G.; Wu, H. Molecular cloning, and optimized production and characterization of recombinant cyclodextrin glucanotransferase from *Bacillus* sp. T1. *3 Biotech* **2022**, *12*, 58. [CrossRef] [PubMed]
32. Hao, J.-H.; Huang, L.-P.; Chen, X.-T.; Sun, J.-J.; Liu, J.-Z.; Sun, M. Identification, cloning and expression analysis of an alpha-CGTase produced by stain Y112. *Protein Expr. Purif.* **2017**, *140*, 8–15. [CrossRef] [PubMed]
33. Pohu, A.; Planchot, V.; Putaux, J.; Colonna, P.; Buléon, A. Split crystallization during debranching of maltodextrins at high concentration by isoamylase. *Biomacromolecules* **2004**, *5*, 1792–1798. [CrossRef] [PubMed]
34. Duan, X.; Chen, S.; Chen, J.; Wu, J. Enhancing the cyclodextrin production by synchronous utilization of isoamylase and α-CGTase. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 3467–3474. [CrossRef]
35. Li, X.; Ji, H.; Bai, Y.; Jin, Z. Development of pullulanase mutants to enhance starch substrate utilization for efficient production of β-CD. *Int. J. Biol. Macromol.* **2021**, *168*, 640–648. [CrossRef]
36. Qiu, C.; Wang, J.; Fan, H.; Bai, Y.; Tian, Y.; Xu, X.; Jin, Z. High-efficiency production of γ-cyclodextrin using β-cyclodextrin as the donor raw material by cyclodextrin opening reactions using recombinant cyclodextrin glycosyltransferase. *Carbohydr. Polym.* **2018**, *182*, 75–80. [CrossRef]

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