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High-level production of γ-cyclodextrin glycosyltransferase in recombinant *Escherichia coli* BL21 (DE3): culture medium optimization, enzymatic properties characterization, and product specificity analysis

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Abstract

Purpose: γ-Cyclodextrin glycosyltransferase (γ-CGTase) catalyzes the biotransformation of low-cost starch into valuable γ-cyclodextrin (γ-CD), which is widely applied in biotechnology, food, and pharmaceutical industries. However, the low specificity and activity of soluble γ-CGTase increase the production cost of γ-CD, thereby limiting its applications. Therefore, the present study aimed at optimizing an economical medium for high production of γ-CGTase by the recombinant *Escherichia coli* (*E. coli*) BL21 (DE3) and evaluating its enzymatic properties and product specificity.

Methods: The γ-CGTase production was optimized using the combination of Plackett-Burman experimental design (PBD) and Box-Behnken design-response surface methodology (BBD-RSM). The hydrolysis and cyclization properties of γ-CGTase were detected under the standard assay conditions with buffers of various pHs and different reaction temperatures. The product specificity of γ-CGTase was investigated by high-performance liquid chromatography (HPLC) analysis of three CDs (α-, β-, γ-CD) in the biotransformation product of cassava starch.

Results: The γ-CGTase activity achieved 53992.10 U mL^{-1} under the optimum conditions with the significant factors (yeast extract 38.51 g L^{-1}, $MgSO_4$ 4.19 mmol L^{-1}, $NiSO_4$ 0.90 mmol L^{-1}) optimized by the combination of PBD and BBD-RSM. The recombinant γ-CGTase exhibited favorable stability in a wide pH and temperature range and maintained both the hydrolysis and cyclization activity under the pH 9.0 and 50 °C. Further analysis of the products from cassava starch catalyzed by the γ-CGTase reported that the majority (90.44%) of product CDs was the γ form, which was nearly 11% higher than the wild enzyme. Cyclododecanone added to the transformation system could enhance the γ-CD purity to 98.72%, which is the highest purity value during the transformation process reported so far.

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Conclusion: The yield of γ-CGTase activity obtained from the optimized medium was 2.83-fold greater than the unoptimized medium, and the recombinant γ-CGTase exhibited a favorable thermal and pH stability, and higher γ-cyclization specificity. These results will provide a fundamental basis for the high productivity and purity of γ-CD in the industrial scale.

Keywords: γ-Cyclodextrin glycosyltransferase, Enzyme activity, Product specificity, Enzymatic property

Introduction

Cyclodextrin glucosyltransferase (CGTase; EC2.4.1.19), a member of the α-amylase family, is an important enzyme that catalyzes the biotransformation of starch and related carbohydrates to cyclic-oligosaccharides, called cyclodextrins (CDs) through glycosyltransferase reaction (Terada et al. 1997; Li et al. 2007). These oligosaccharides are comprised of 6, 7, and 8 glucose residues, known as α-CD, β-CD, and γ-CD, respectively (Alves-Prado et al. 2008). CDs exhibit a hydrophilic outer surface and a non-polar/hydrophilic cavity that encapsulates the hydrophobic guest molecules and forms various inclusion complexes to alter the chemical and physical properties of organic compounds. Compared with α- and β-CD, γ-CD is widely acknowledged for its wider application in numerous industries, primarily in the pharmaceutical and food industries due to its larger cavity size, higher water solubility, more bioavailability, and less toxicity (Szejtli 1998; Munro et al. 2004; Challa et al. 2005). However, the potential ability of γ-CD has not been explored to date due to its low yield, high cost, and most importantly, the lack of high-specific and high-activity γ-CGTase. Accumulating researches have proved that bacterial CGTases catalyzes CD formation mostly comprising of α-, β-, and γ-CD, but yet the separation of γ-CD is expensive and time consuming (van der Veen et al. 2000). Therefore, further research on developing a γ-CGTase that primarily produces the γ form and improving its enzyme activity is warranted (Wang et al. 2013).

So far, several attempts have been made to screen high-specific γ-CGTase-producing bacterial strains. Hirano et al. reported a γ-CGTase derived from *Bacillus* sp. g-825-6 that principally produced γ-CD under any pH with no α-CD, but the enzymatic activity in the fermentation medium was only 277 U mL^{-1} (Hirano et al. 2006). Takada et al. discovered a highly specific γ-CGTase from *Bacillus clarkii* 7364 that converted pregelatinized potato starch into CDs accounting for 79% γ, but its enzymatic activity only reached 0.17 U mg^{-1} of proteins (Takada et al. 2003). However, the extremely low expression of γ-CGTases proved the aforementioned wild strains to be unsuitable for the industrial production of γ-CD. Later, *E. coli* was used as the host bacteria to improve the CGTase activity, but the production only reached 22 U mL^{-1} due to the formation of non-bioactive inclusion bodies (Jemli et al. 2008). Wang et al. added β-CD as a chemical chaperone to the culture medium and observed the yield of soluble γ-CGTase cyclization activity to be 50.29 U mL^{-1}(Wang et al. 2018a). However, the addition of β-CD developed impurity in the subsequent starch transformation reaction, which might reduce the purity and extraction of γ-CD. Wang et al. (2017) optimized the γ-CGTase gene from *Bacillus clarkii* 7364 according to the biased codons of *E. coli* BL21 (DE3), and developed its prokaryotic expression strain. Soluble expression of the recombinant γ-CGTase was achieved by reducing the induction temperature of *E. coli* BL21 (DE3) to 28 °C (Wang et al. 2017). In addition, optimization of the medium essential components has also been an essential strategy for enhancing the final output of the desired product (Long et al. 2018). However, the existence of several factors in the fermentation medium and the interaction between them are still unclear (Cui and Zhao 2012; Wang et al. 2014; Wu et al. 2018). Therefore, a valid and reliable analytical method lowering the reagent consumption and laboratory work would be desirable to obtain the maximum soluble γ-CGTase expression.

The yield of CDs from starch differs depending on the properties of CGTases and the environmental conditions (Mora et al. 2012). The production and purity of CDs could be maximized under the optimum conditions of hydrolysis and cyclization reaction because the CGTases exhibited different properties at various temperatures and pH. Zhang et al. discovered a recombinant β-CGTase which showed high specific activity at 80 °C without any γ-CGTase activity (Zhang et al. 2017). Takada et al. reported that the γ-CGTase produced by *Bacillus clarkii* 7364 showed the maximum cyclization activity at pH 10.5–11.0, and at 60 °C (Takada et al. 2003). Hence, the recombinant γ-CGTase properties, optimized according to the codons of *E. coli* BL21 (DE3), should be further studied to improve the transformation efficiency.

In the present study, the γ-CGTase expression was significantly enhanced in *E. coli* BL21 (DE3) strain by optimizing the culture medium for the first time using the Box-Behnken design-response surface methodology (BBD-RSM), which is a valid optimization method with

short cycle and high precision of regression equation. Furthermore, the enzymatic properties and specificity of the recombinant γ-CGTase were studied to elevate the purity and yield of γ-CD during the enzyme-catalyzed starch transformation, indicating the potential of this enzyme for improving the production of γ-CD with high productivity and purity.

Materials and methods

Bacterial strains and plasmids

The *E. coli* BL21(DE3) expression strain harboring the recombinant plasmid cgt/pET22b (+), which contains the γ-CGTase gene sequence from *Bacillus clarkii* 7364 (Genbank Accession No.: AB082929), was designed and synthesized according to the biased codons of *E. coli* BL21 (DE3) reported by Wang et al. (2017). Briefly, the γ-CGTase (Accession number: AB082929) sequence was *E. coli* codon optimized and synthesizd by Generay Biotech Company (Shanghai, China) as NcoI/BamHI fragment. This DNA fragment was firstly cloned into the pMD18-T (TAKARA, Japan) vector. After hydrolyzation with the NcoI and BamHI restriction enzymes, the γ-CGTase fragment was inserted into the pET22b (+) plasmid (Cat. No. 69744-3), generating plasmid pET22b (+)-γCGTase. To confirm the constructed plasmid pET22b (+)-γCGTase, the T7 (5′-TAATACGACT CACTATAGGG-3′) and T7 Ter (5′-GCTAGTTATT GCTCAGCGG-3′) primers were also used to test the existence of γ-CGTase gene. The plasmid was further confirmed by sequencing (Sangon Biotech Company, Shanghai, China). Then the verified plasmid pET22b (+)-γCGTase was transformed into *E. coli* BL21 (DE3) competent cells (TAKARA, Japan) to construct the recombinant *E. coli* BL21/pET22b (+)-γCGTase strain. Other general DNA manipulations were performed based on standard protocols. T4 DNA ligase and the restriction enzymes used in this study were purchased from TAKARA.

Medium and cultivation

The glycerol stock of pET22b (+)-γ-CGTase/*E. coli* BL21 (DE3) strain was inoculated in a 10-mL LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.0) containing 100 μg mL^{-1} ampicillin, and then cultivated overnight at 37 °C and incubated on a shaking incubator (Zhicheng Company, Shanghai, China) with a rotational radius of 10 cm at 200 rpm. The tryptone and yeast extract were purchased from Aobox Company (Beijing, China). The seed culture was then diluted (1%, v/v) with 50 mL TB medium (12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 5 g L^{-1} glycerol, 2.31 g L^{-1} KH_2PO_4, 12.54 g L^{-1} K_2HPO_4) containing ampicillin and incubated in a rotary shaker (Zhicheng Company, Shanghai, China) at 37 °C and 200 rpm until the optical density at 600 nm (OD_{600}) reached 1.0-1.5. Isopropyl-β-d-thiogalactoside (IPTG) at a final concentration of 1 mmol•L^{-1} was added to induce the expression of γ-CGTase. IPTG was purchased from the Generay Biotech Company (Shanghai, China). After 5 h induction, the bacteria were collected by centrifugation at 8000×*g* for 15 min.

Crude enzyme preparation

Around 10 mL of lysis buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), pH 8.5) containing 5 μg mL^{-1} lysozymes (Generay Biotech Company, Shanghai, China) was added per gram of bacteria and then resuspended. After the cell lysis at 4 °C for 20 min, again 10 mg of deoxycholic sodium, followed by 10 μmol of $MgCl_2$, and 25 μmol of DNase (Generay Biotech Company, Shanghai, China) was added per gram of bacteria. The lysate was thoroughly stirred and incubated for 2 h at 4 °C. The supernatant was isolated through centrifugation at 12000×*g* for 15 min at 4 °C to obtain the crude enzyme extraction of γ-CGTase. The centrifuge was purchased from Eppendorf Company (Hamburg, Germany).

Assay of γ-CGTase activity

The hydrolysis activity of γ-CGTase was measured as dextrinizing power by Kaneko's method (Kaneko et al. 1990) with some modifications. Firstly, the obtained diluted crude enzyme extraction (0.01 mL) was added into the mixture of 0.2 mL Tris-HCl buffer (50 mmol L^{-1}, pH 9.0) and freshly prepared 0.2 mL of potato starch solution (0.25%, w·v^{-1}). Then, 0.5 mL of 3% (v·v^{-1}) glacial acetic acid was immediately added to terminate the enzymatic reaction, after incubation in the water bath for 10 min at 55 °C. Later, 3 mL of 0.05 g L^{-1} iodine solution was added into the system and diluted with distilled water to the final volume achieved 10 mL. Afterward, the absorbance, i.e., OD_2 was measured at 700 nm using a spectrophotometer (Jingmi Instrument Company, Shanghai, China), taking distilled water as blank and the absorbance value of the control group as OD_1. One unit of enzyme activity was defined as a ten percent decrease in OD_1. Enzyme activity was calculated according to the equation:

$$U \cdot mL^{-1} = (OD_1 - OD_2) \cdot OD_1^{-1} \cdot 1000 \cdot \text{dilution ratio} \quad (1)$$

The cyclization activity of γ-CGTase was detected by the bromocresol green (BCG) method of Kato's (Kato and Horikoshi 1984) with some modifications. A reaction mixture comprising 1 mL of 1% (w·v^{-1}) soluble starch in Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) and 0.8 mL of the diluted enzyme was incubated at 55 °C for 10 min.

Then, the enzymatic reaction was terminated by the addition of 4 mL of citric acid/sodium citric buffer (pH 4.2), followed by adding 0.2 mL 5 mM BCG in 20% (v/v) ethanol and incubated at room temperature for 15 min. After the reaction, the reaction mixture's absorbance at 630 nm was measured and the amount of γ-CD was calculated according to the standard curve. One unit of γ-CD-forming activity was defined as the amount of enzyme-producing 1 μmol of γ-CD per minute.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The total protein of the samples was quantified using a BCA assay kit (TAKARA, Japan). Later, the same amount of protein was electrophoresed on 12% SDS-PAGE gels, prepared according to the instructions of the kit (Beyotime Biotechnology, Shanghai, China). After electrophoresis, the gel was stained with 0.1% Coomassie bright Blue R-250 (Solarbio, Beijing, China) for 1 h, and then discolored by oscillating in the shaker (Zhicheng Company, Shanghai, China) overnight. The signals were detected using the Bio-rad gel imaging system (California, USA). Signal intensities from each band were quantified using the IMAGE J software.

Single-factor analysis

Several important variables, such as carbon, nitrogen, metal ions, and phosphate sources, were selected for the single factor analysis, where the bacterial growth and production of enzymatic activity were considered as the screening criteria. The glycerol in the basal TB medium was replaced with seven common carbon sources, soluble starch, sucrose, maltose, lactose, glucose, galactose, and arabinose. The total carbon contents of each carbon source were the same as that of the glycerol in TB. The addition of each nitrogen source was also consistent with the total nitrogen contents of the basal TB medium. Similarly, nine metal ions, including K^+, Zn^{2+}, Fe^{3+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+}, were added to the basal medium at the same concentration of 2 mmol L^{-1}, respectively. PO_4^{3-} was maintained at the level of 0.10 mol L^{-1}. Notably, in each experiment, the screening factor was changed by keeping the other factors constant.

Plackett-Burman experimental design

The Plackett-Burman experimental design was employed to select significant factors from the multi-factor experiment, which does not consider the interaction between the factors. The single-factor analysis resulted in selecting the high and low levels of each variable from the maximum response interval and these are listed in Supplementary Table 1. The PBD was designed using the Design-expert 10.0 software. The effects of sucrose, yeast extract, K_2SO_4, $MgSO_4$, $NiSO_4{\cdot}7H_2O$, KH_2PO_4, and K_2HPO_4 on the production of γ-CGTase were also investigated. The design matrix with response values for screening variables is shown in Supplementary Table 2. In this study, 12 experiments were conducted and the most optimal variables were selected for further evaluation. Based on regression analysis of the variables, significant levels at 95% level ($p < 0.05$) were considered to significantly affect the hydrolysis activity of γ-CGTase (Table 1).

The steepest ascent path

The steepest ascent path was employed to detect the proper direction for altering the levels of these three key variables. The step size was determined according to the effect of each factor. The variables, experimental design, and response values are depicted in Table 2. The maximum level achieved by the γ-CGTase activity was considered proximate to the center point for the experimental optimization design.

Box-Behnken design

Since the three variables (yeast extract, $MgSO_4$, and $NiSO_4$) play a vital role in the production of γ-CGTase by *E. coli* BL21 (DE3), the BBD-RSM was employed to further examine and refine the optimal levels of these significant variables. A three-level, three-factorial BBD system was designed to investigate and validate the fermentation parameters affecting the γ-CGTase activity. The inflection point in the experiment of the steepest ascent path was taken as the central point (Supplementary Table 3), while γ-CGTase activity was taken as the response value of BBD (Table 3). A total of 17 experiments with five center points were conducted based on the output of the Design-expert 10.0 software. In consideration of the linear terms, square terms, and linear interaction items, the second-order equation response was designed by Eq. (2) through the least squares method:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response variable; β_0, β_i, β_{ij}, and β_{ii} are constants and regression coefficients of the model, and x_i and x_j represent the independent variables of the coded values. The optimum values of the selected variables were determined by solving the regression equation and analyzing the three-dimensional surface plots.

Purification of γ-CGTase

The purification process of γ-CGTase from the crude enzyme solution was carried out according to Wang's

Table 1 The estimated parameters of variables in Plackett-Burman factorial design

Code	Terms	Stdized effects	% Contribution	*F* value	*p* value
	Model			15.52	0.0093
A	Sucrose	−6869.62	4.73	5.33	0.0082
B	Yeast extract	25998.41	67.74	76.32	0.0009
C	K_2SO_4	1014.94	0.10	0.12	0.7502
D	$MgSO_4$	8952.89	8.03	9.05	0.0396
E	$NiSO_4$	−12073.70	14.61	16.46	0.0154
F	KH_2PO_4	−3497.02	1.23	1.38	0.3051
G	K_2HPO_4	−272.78	0.0075	0.0084	0.9314

method (Wang et al. 2017). Briefly, the lysis supernatant containing γ-CGTase was deposited overnight with a 50% saturation of $(NH_4)_2SO_4$ solution. Later, it was centrifuged at 4 °C and 12000×*g* in a refrigerated centrifuge (Eppendorf Company, Hamburg, Germany); the precipitate was collected and dissolved with acetic acid buffer (pH 5.5). After dialysis in the acetic acid buffer (pH 5.5) for three times, the protein solution was filtered by a 0.45-μm membrane and then purified by AKTA protein purifier (GE Company, American). The elution peaks were collected by eluting the target protein with acetic acid buffer containing 10 g L^{-1} α-CD via an α-CD-Sepharose 6B affinity column. The elution buffer containing γ-CGTase was dialyzed in distilled water, and then lyophilized powder was prepared.

HPLC analysis of the conversion products by γ-CGTase

A total of 10% (w·v^{-1}) cassava starch was prepared with Tris-HCl buffer at pH 8.0, followed by the addition of γ-CGTase for pre-gelatinization of the starch solution at 700 U per gram starch for 15 h at 55 °C. The supernatant was then centrifuged at 5000×g for 10 min in a centrifuge (Eppendorf Company, Hamburg, Germany) and filtered through a 0.45-μm membrane for HPLC detection. For HPLC detection, the conditions were maintained as follows: the chromatographic column was Thermo phenyl-2 HYPERSIL (4.6 mm × 150 mm), the mobile phase was 100% aqueous solution, the flow rate was 1.0 mL min^{-1}, the detector was differential refraction k-2301 (KNAUER, Germany), the column temperature was 40 °C, and the injection volume was 10 μL.

Results

Screening procedure: single-factor analysis of basal culture medium

Carbon is the primary source of energy for microbial growth (Ebadipour et al. 2016). To evaluate the effects of different carbon sources on γ-CGTase production, the glycerol in the terrific broth (TB) medium was replaced with seven common carbon sources, such as soluble starch, sucrose, maltose, lactose, glucose, galactose, and arabinose. As depicted in Fig. 1a, sucrose was found to be most advantageous for the soluble expression of γ-CGTase by SDS-PAGE analysis. Figure 1b illustrates that the use of sucrose as a carbon source exhibited the highest hydrolysis activity of the recombinant γ-CGTase (35051.26 U mL^{-1}) by 1.84-fold greater than that of the controls (19035.63 U mL^{-1}). Further evaluation indicated that the cell density of the *E. coli* BL21 (DE3) strain in the control group (TB medium) was the highest among all the carbon sources, and the sucrose group took the second place with OD_{600} value reaching 96% of the control group. Therefore, sucrose was considered as the optimal carbon source to achieve significant bacterial growth and enzymatic activity.

Table 2 Steepest ascent experiment

Run	Variable			CGTase activity (U mL^{-1})
	Yeast extract (g L^{-1})	$MgSO_4$ (mmol L^{-1})	$NiSO_4$ (mmol L^{-1})	
1	18	2.67	1.46	28094.42
2	27	3.33	1.23	32509.23
3	36	4	1	50778.34
4	45	4.67	0.77	42607.18
5	54	5.33	0.54	43044.62
6	63	6	0.31	40944.88

Table 3 The BBD matrix for coded variables along with actual and predicted responses

Run	A	B	C	CGTase activity (U mL^{-1})		
				Actual response	Predicted response	Residual
1	0	0	0	52591.46	53615.57	−1024.11
2	0	1	1	48695.64	48236.91	458.72
3	0	0	0	54168.75	53615.57	553.18
4	1	−1	0	47940.07	48009.29	−69.21
5	−1	0	−1	43689.00	43365.18	323.82
6	0	0	0	52820.12	53615.57	−795.46
7	−1	1	0	46464.77	46395.55	69.21
8	0	0	0	54618.22	53615.57	1002.65
9	0	1	−1	48267.38	48660.41	−393.04
10	−1	−1	0	47786.02	47651.12	134.90
11	0	−1	1	50046.18	49653.15	393.04
12	0	0	0	53879.31	53615.57	263.74
13	−1	0	1	44883.23	45411.17	−1007.58
14	0	−1	−1	43933.89	50002.88	−527.94
15	1	0	−1	46560.11	46032.17	1007.58
16	1	1	0	51981.52	61103.53	527.94
17	1	0	1	48499.38	48823.21	−323.82

To detect an alternative nitrogen source for the production of γ-CGTase, several nitrogen sources, such as $(NH_4)_2SO_4$, diammonium citrate, NH_4Cl, tryptone, yeast extract, and compound organic nitrogen sources (tryptone, yeast extract = 1:1) were added to the TB medium. The experimental results indicated that the use of yeast extract and compound nitrogen source in the TB medium increased the soluble expression of γ-CGTase (Fig. 1c). It was also observed in Fig. 1d that the addition of tryptone, yeast extract, and compound nitrogen source into the culture medium obtained a higher hydrolysis activity of γ-CGTase than the control group, respectively. In contrast, the utilization of yeast extract medium as a nitrogen source maximized the cell density of *E. coli* BL21 (DE3) with an OD_{600} value of 5.077 (Fig. 1d). Accumulating these results, yeast extract was chosen as the optimal nitrogen source for the production of γ-CGTase.

Metal ions play an important role in enhancing the enzymatic activity and stabilizing the microbial cell structures and regulating bacterial growth conditions, such as osmotic pressure and redox potential (Ihssen et al. 2010). To determine the effect of different metal ions on the production of γ-CGTase, nine metal ions, such as K^+, Zn^{2+}, Fe^{3+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+} prepared with deionized water were added to the basal medium at a final concentration of 2 mmol/L, respectively. As illustrated in Fig. 1e, the addition of $MgSO_4$ and $NiSO_4$ significantly promoted the soluble expression of γ-CGTase compared to other metal ions. Similarly, the addition of K_2SO_4, $MgSO_4$, and $NiSO_4$ significantly increased the γ-CGTase activity and the bacteria growth compared to the control group (Fig. 1f). The effect of PO_4^{3-} in TB medium containing 0.10 mol L^{-1} of Na_3PO_4 was assessed in this study. As depicted in Fig. 1e and f, the soluble expression, the enzyme activity, and the OD_{600} value of γ-CGTase decreased with the addition of PO_4^{3-} to the culture medium. Therefore, K_2SO_4, $MgSO_4$, and $NiSO_4$ were selected to be the potential metal ions for addition into the medium, instead of Na_3PO_4.

Screening of key variables influencing the activity of recombinant γ-CGTase: Plackett-Burman design

PBD is a proficient statistical approach to detect the most important factor involved in an experiment (Han et al. 2017). As summarized in Supplementary Table 1, seven variables were selected to detect the key factors affecting the hydrolysis activity of recombinant γ-CGTase by PBD, based on the results of single-factor experiment results. Supplementary Table 2 showed that the design matrix and response value of the 12 experimental sets varied from 1270.65 to 56452.99 U mL^{-1}. According to the estimated parameters of PBD depicted in Table 1, the fitting model was significant for the γ-CGTase activity as the p value was 0.0093 ($p < 0.05$). The results also demonstrated the three most significant variables, i.e., yeast extract ($p = 0.0009$), $MgSO_4$ ($p = 0.0396$), and $NiSO_4$ ($p = 0.0154$), contributing 67.74%, 8.03%, and 14.61%, respectively, to the enzymatic activity. Therefore, the yeast extract, $MgSO_4$, and $NiSO_4$ were selected to carry out the steepest ascent experiment.

Confirming stage: the steepest ascent path

Based on the analysis of the screening design, yeast extract, and $MgSO_4$ exerted positive effects on the recombinant γ-CGTase production, whereas $NiSO_4$ exerted a negative impact. To achieve the optimal experimental region with a maximum response, the concentration of yeast extract and $MgSO_4$ should be increased, and simultaneously, the concentration of $NiSO_4$ should be reduced. The non-significant factors in the PBD were chosen at the maximum or minimum concentrations according to their positive or negative effects, namely, sucrose 1.5 g L^{-1}, K_2SO_4 0.36 g L^{-1}, KH_2PO_4 0.86 g L^{-1}, K_2HPO_4 6.14 g L^{-1}. The experimental design and corresponding results of the steepest ascent path are depicted in Table 2. The hydrolysis activity of γ-CGTase reached the peak (51706.04 U mL^{-1}) when the levels of yeast extract, $MgSO_4$, and $NiSO_4$ were of 7.2 g L^{-1}, 4 mmol L^{-1}, and 1 mmol L^{-1}, respectively, suggesting the value of the γ-CGTase activity to be proximal to the region of maximum response.

Superior optimization stage: Box-Behnken design

The BBD-RSM was employed to refine the optimal levels of yeast extract, $MgSO_4$, and $NiSO_4$.

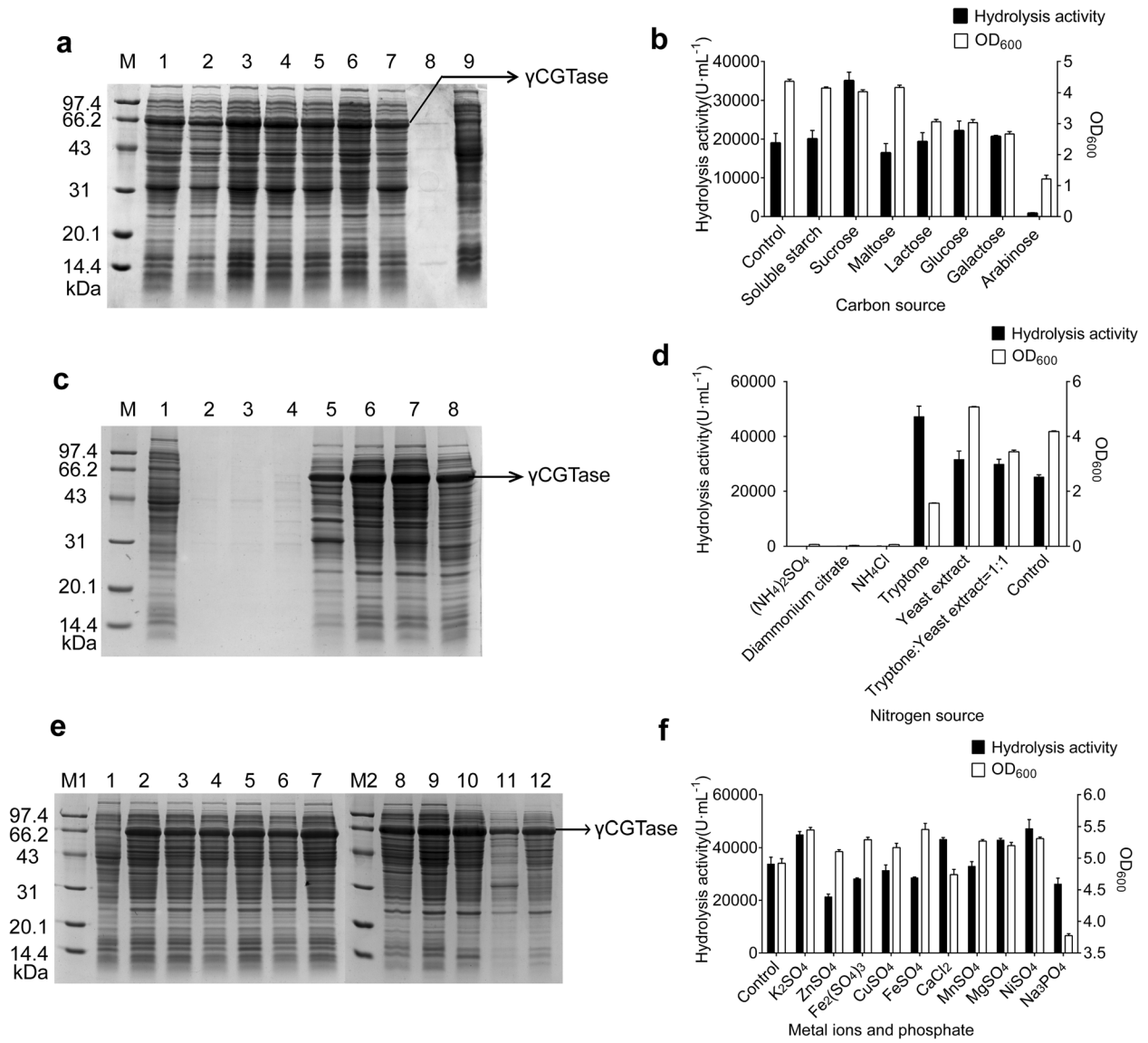

Fig. 1 The effects of different single factors on the production of recombinant γ-CGTase from *E. coli* BL21 (DE3). **a** Soluble γ-CGTase expression in *E. coli* BL21 (DE3) cultured by different carbon sources. Lane M, protein ladder; lane 1, control (glycerol); lane 2, soluble starch; lane 3, sucrose; lane 4, maltose; lane 5, lactose; lane 6, glucose; lane 7, galactose; lane 8, arabinose; and lane 9, non-induced strain. **b** γ-CGTase activity in lytic supernatant and OD_{600} in shake-flask culture with different carbon sources. **c** Soluble γ-CGTase expression in *E. coli* BL21 (DE3) cultured by different nitrogen sources. Lane M, protein ladder; lane 1, non-induced strain; lane 2, $(NH_4)_2SO_4$; lane 3, diammonium citrate; lane 4, NH_4Cl; lane 5, tryptone; lane 6, yeast extract; lane 7, tryptone: yeast extract = 1:1; and lane 8, control (tryptone, yeast extract = 1:2). **d** γ-CGTase activity and OD_{600} in shake-flask cultures with different nitrogen sources. **e** Soluble γ-CGTase expression in *E. coli* BL21 (DE3) cultured by phosphate and different metal ions. lane M1, protein ladder; lane 1, non-induced strain; lane 2, K^+; lane 3, Zn^{2+}; lane 4, Fe^{3+}; lane 5, Cu^{2+}; lane 6, Fe^{2+}; lane 7, Ca^{2+}; lane M2, protein ladder; lane 8, Mn^{2+}; lane 9, Mg^{2+}; lane 10, Ni^{2+}; lane 11, $PO4^{3-}$; and lane 12, control (no metal ions or $PO4^{3-}$ added). **f** γ-CGTase activity and OD_{600} in shake-flask cultures with $PO4^{3-}$ and different metal ions. The data were presented from three independent experiments

Supplementary Table 3 depicts the variables and levels in BBD. The design matrix for coded variables, along with actual and predicted responses, is depicted in Table 3. The final regression equation in terms of coded factors was evaluated by the Design-expert 10.0 software, and the obtained Eq. (3) is mentioned below:

$$Y = 53615.57 + \mathbf{1519.76A} + \mathbf{712.89B} + \mathbf{1209.26C} + \mathbf{1340.67AB} + 186.26AC - \mathbf{1421.01BC} - \mathbf{3450.16A^2} - \mathbf{1622.32B^2} - \mathbf{4257.48C^2} \quad (3)$$

where Y is the predicted response of γ-CGTase activity. A, B, and C are the coded values of the test variables,

yeast extract, $MgSO_4$, and $NiSO_4$, respectively. Table 4 shows the statistical significance of Eq. (3) fitting analyzed by ANOVA. The p value of the quadratic regression model was less than 0.0001, suggesting the model to be significant and adequate. The values of the regression coefficient R^2 (0.9776) established an excellent correlation between the experimental and predicted response values. The adjusted determination coefficient (adj R^2) value was found to be 0.9487, indicating that the total variation of 94.87% for the production of γ-CGTase was attributed to the independent variables.

The three-dimensional (3D) response surface plots and the respective contour plots (Fig. 2) obtained from the BBD experiments are the graphical representations of the regression equation. Figure 2a depicts that γ-CGTase activity varied with the concentrations of yeast extract (A) and $MgSO_4$ (B) and reached the peak when the concentrations were 38.51 g L^{-1} and 4.19 mmol L^{-1}, respectively, while $NiSO_4$ (C) was maintained at zero level. As illustrated in Fig. 2b and c, the response value showed an upward movement at first and then downregulated, indicating the existence of the maximum predicted value of the γ-CGTase activity. Furthermore, the interaction between AB and BC was significant since the p values (0.0130, 0.0099) were less than 0.05 (Table 4) and the shape of the contour plot was elliptical (Fig. 2). The maximum predicted γ-CGTase activity by Design-expert was 53992.10 U mL^{-1} under the optimum conditions with yeast extract 38.51 g L^{-1}, $MgSO_4$ 4.19 mmol L^{-1}, and $NiSO_4$ 0.90 mmol L^{-1}. To validate the optimization results, three repeated experiments were conducted at optimal parallel conditions, and the average enzymatic activity value of soluble γ-CGTase was 52680.86 ± 329.72 U mL^{-1} (n = 3), which was within the 95% confidence interval of the maximum predicted value. This suggested that the predicted model was satisfactory and accurate for optimizing the culture compositions for γ-CGTase production from the recombinant *E. coli* BL21 (DE3).

Enzymatic properties of recombinant γ-CGTase

The enzymatic properties of γ-CGTase were then analyzed to obtain the optimum reaction conditions for starch hydrolysis and γ-CD cyclization, which were the two most important reactions in the γ-CD production process. The γ-CGTase purification from the lysed supernatant of recombinant *E. coli* BL21 (DE3) was performed by affinity column chromatography using α-CD-immobilized Sepharose 4B as the matrix. The effects of pH and temperature on the hydrolysis and cyclization activity of the purified γ-CGTase were studied under the standard assay conditions with buffers of various pHs and different reaction temperatures. The effect of pH on the hydrolysis activity of γ-CGTase is illustrated in Fig. 3a. The enzyme obtained a maximum hydrolysis activity at pH 10.0 and 73% activity at pH 9.0. After 24 h treatment at 4 °C, γ-CGTase retained more than 85% of its initial activity over the pH range of 7.0-10.0 (Fig. 3b). The profiles of γ-CGTase hydrolysis activity at different temperatures are illustrated in Fig. 3c. The optimum temperature of hydrolysis activity was determined to be 55 °C (pH 10.0), and γ-CGTase showed 80% activity at 50 °C. However, the enzyme retained its original hydrolysis activity upon heating to a temperature below 45 °C for 20 min, and the hydrolysis activity decreased to 87% after 20 min of treatment at 50 °C; upon increasing this temperature, a drastic reduction in enzyme activity was observed (Fig. 3d).

Table 4 ANOVA results of the quadratic model for CGTase activity (R^2 = 0.9776; Adj R^2 = 0.9487)

Source	SS	*df*	MS	*F*-Value	*p* value	
Model	2.008×10^8	9	2.231×10^7	33.91	< 0.0001	Significant
A	1.848×10^7	1	1.848×10^7	28.08	0.0011	
B	4.066×10^6	1	4.066×10^6	6.18	0.0418	
C	1.170×10^7	1	1.170×10^7	17.78	0.0040	
AB	7.190×10^6	1	7.190×10^6	10.93	0.0130	
AC	1.388×10^5	1	1.388×10^5	0.21	0.6600	
BC	8.077×10^6	1	8.077×10^6	12.28	0.0099	
A^2	5.012×10^7	1	5.012×10^7	76.18	< 0.0001	
B^2	1.108×10^7	1	1.108×10^7	16.84	0.0045	
C^2	7.632×10^7	1	7.632×10^7	116.00	< 0.0001	
Residual	4.605×10^6	7	6.579×10^5			
Lack of fit	1.543×10^6	3	5.143×10^5	0.67	0.6126	Not significant
Pure error	3.062×10^6	4	7.656×10^5			
Cor total	2.054×10^8	16				

SS sum of squares, *df* degrees of freedom, *MS* mean square

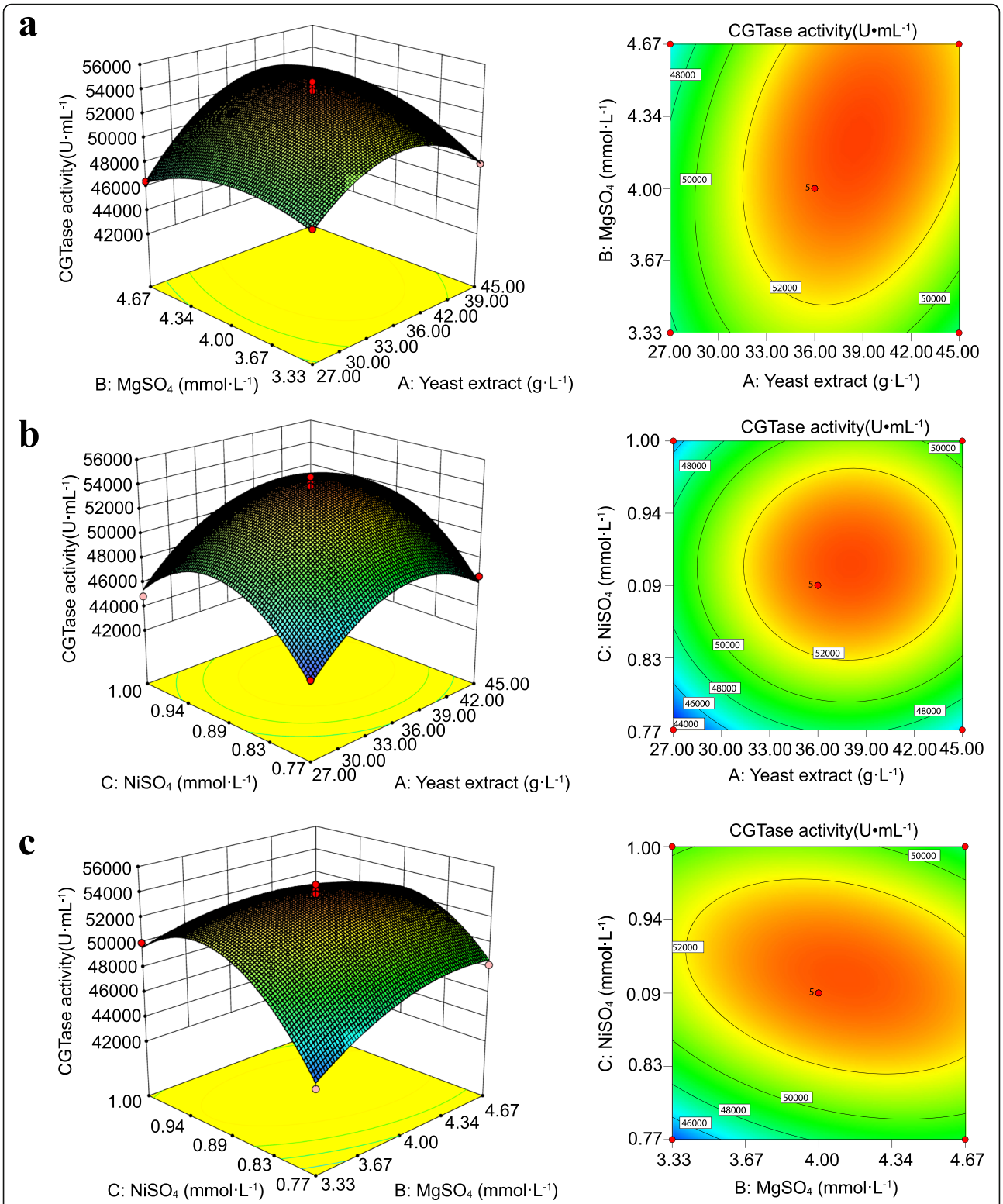

Fig. 2 Response surface and contour plots for the production of γ-CGTase by the recombinant *E. coli* BL21 (DE3) strain. **a** Mutual effect of yeast extract and $MgSO_4$ concentration on recombinant γ-CGTase activity. **b** Mutual effect of yeast extract and $NiSO_4$ concentration on recombinant γ-CGTase activity. **c** Mutual effect of $MgSO_4$ and $NiSO_4$ concentration on recombinant γ-CGTase activity

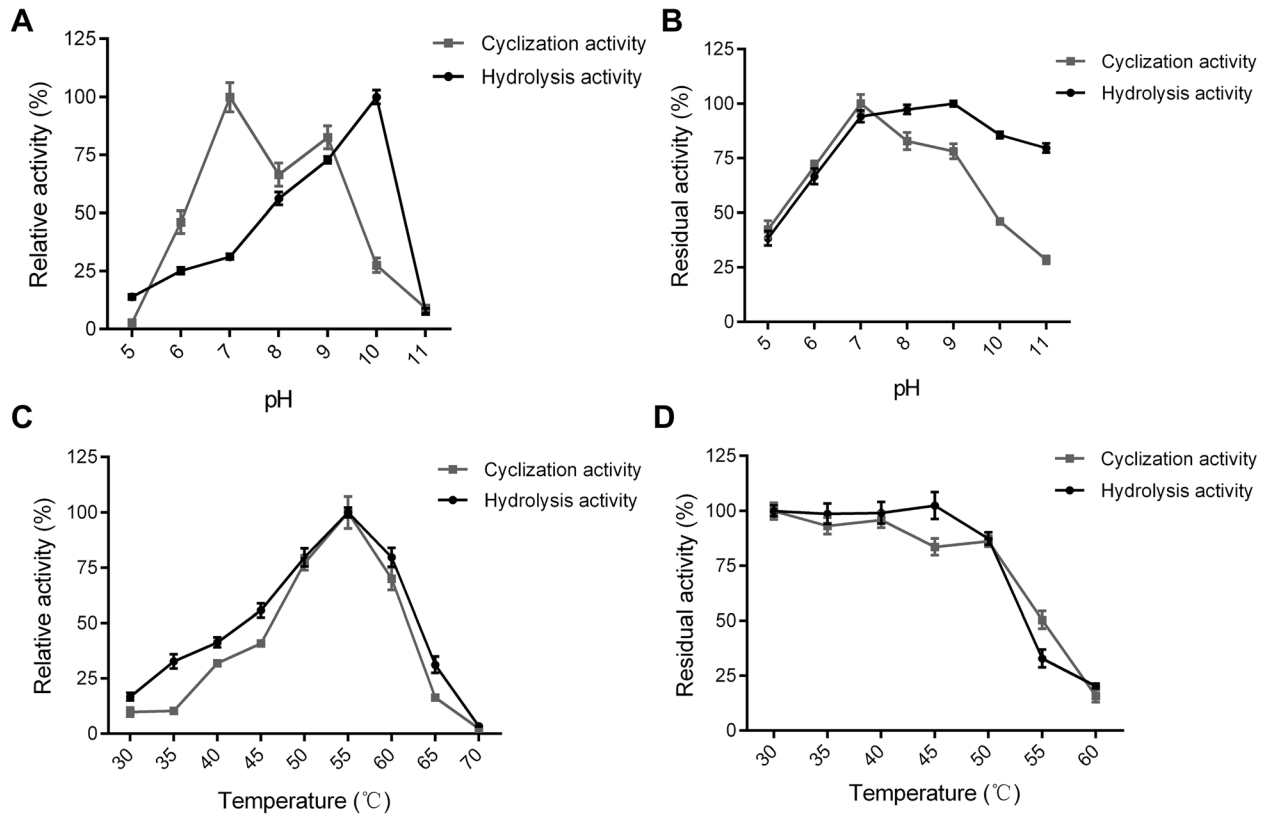

Fig. 3 Effects of pH and temperature on the activity and stability of purified γ-CGTase. **a** Optimum pH of γ-CGTase activity. **b** pH stability of γ-CGTase activity. 0.1 M citrate buffer was used at pH 5-6; 0.1 M NaH_2PO_4-Na_2HPO_4 was used at pH 7; 0.05 M Tris-HCl buffer was used at pH 8-9; 0.1 M Na_2CO_3- $NaHCO_3$ buffer was used at pH 10-11. **c** Optimum temperature of γ-CGTase activity. **d** Thermal stability of γ-CGTase activity. The data were presented from three independent experiments

The effects of pH and temperature on the cyclization activity of the purified γ-CGTase were also presented in Fig. 3. The cyclization activity of γ-CGTase reached the maximum at pH 7.0 and the second at pH 9.0, and retained more than 78% of its initial activity over a pH range of 7.0-9.0 after incubated at 4 °C for 24 h (Fig. 3a and b). The cyclization activity was optimally active at 55 °C (pH 7.0) and slightly decreased after 20 min of treatment at 35 to 50 °C. With the increase in the temperature from 55 to 70 °C, the γ-CGTase cyclization activity reduced significantly from 50 to 98% after 20 min of treatment (Fig. 3c and d).

The product specificity of recombinant γ-CGTase

The synthesis of γ-CD from cassava starch biotransformation catalyzed by γ-CGTase without organic solvent added, known as the solvent-free method, was executed under the condition of pH 9.0 and 50 °C for 12 h. The concentrations of three CDs (α-, β-, γ-CD) in the products were investigated by HPLC (Fig. 4a-c). The γ-CGTase exhibited a higher γ-cyclization specificity than the native one because the proportion of γ-CD (90.44%) in the product CDs from cassava starch biotransformation was about 11% higher than that of the wild enzyme (79%) [10], and no α-CD was detected (Fig. 4d).

The organic solvent has been widely added during the industrial production of CDs to obtain high purity and yield. Cyclododecone was found to be an efficient organic solvent that could promote γ-CD production (Wang et al. 2013). In this research, the role of cyclododecone in the synthesis of γ-CD from starch bioconversion catalyzed by the recombinant enzyme was studied. Cyclododecone was added to the reaction system at a final concentration of 5% (w/v). Meanwhile, the other conditions for the reaction were the same as the solvent-free method reported above. As depicted in Fig. 4d and e, the mass ratio of γ: β-CD in the conversion product was calculated to be 98.72:1.28, and the yield of γ-CD reached 42.21% (w/w). Compared with the solvent-free method, the ratio of γ-CD increased by 8.29%, and its yield increased by 4.46-fold.

Discussion

The hydrolysis of starch and cyclization of cyclodextrin occur simultaneously during the industrial-scale production of cyclodextrin. However, CGTase is usually added

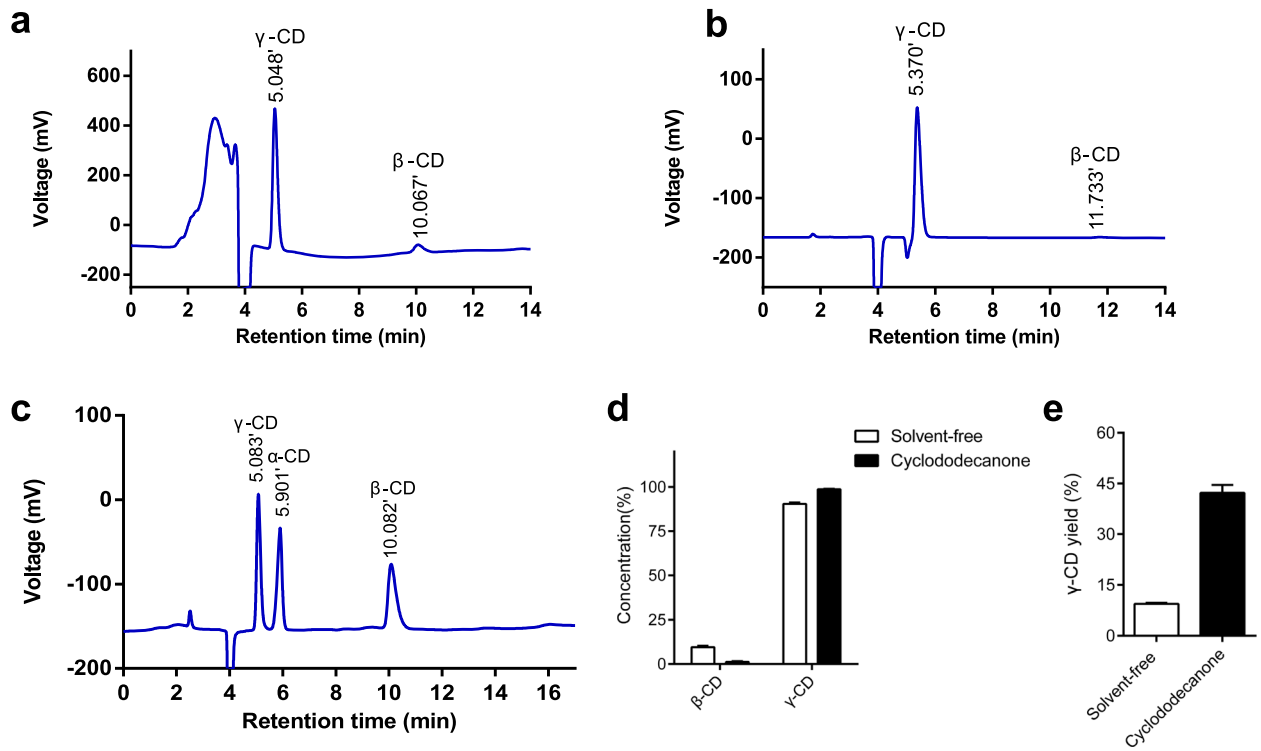

Fig. 4 The product specificity analysis of γ-CGTase. The concentrations of α-, β-, and γ-CD in the products of **a** non-solvent group, **b** the standard substance, and **c** the cyclododecanone group were determined by HPLC. **d** The purity and **e** the yield of γ-CD was calculated according to the result of HPLC. The data were presented from three independent experiments

as the only enzyme in this process without other amylases. This may attribute to the fact that the hydrolysis of starch by amylases could not be well-controlled, but might increase the cost (Cami and Majou 1991; Yang et al. 2007; Gen 2015a; Gen 2015b). Hence, the hydrolysis activity of γ-CGTase plays an essential role in the manufacturing of γ-CD, even though the enzyme has been reported to primarily catalyze the transglycosylation reactions (cyclization, coupling, and disproportionation). The improved hydrolysis activity of CGTases depends on the significant expression vectors or the host cells, as well as the optimum culture media (Wang et al. 2018b; Mahmud et al. 2019; Upadhyay et al. 2019). This is the first study to optimize the essential culture medium compositions of γ-CGTase to enhance the soluble expression and activity in *E. coli* BL21 (DE3) strain and explore its enzymatic properties to enhance the productivity and purity of γ-CD.

Considering the various factors and their interactions in the culture medium of engineered *E. coli* BL21 (DE3), the development of reasonable and valid optimization methodologies is necessary to avoid the unreliable results of conventional one-dimensional research (Wang et al. 2014; Ebadipour et al. 2016; Wu and Ahn 2018; Zhang et al. 2019). In this research, the single-factor analysis elucidated that sucrose, yeast extracts, and metal irons (K^+, Mg^{2+}, and Ni^{2+}) could increase bacterial growth and production of soluble γ-CGTase. Later, PBD was applied to analyze the most three significant variables (yeast extract, $MgSO_4$, and $NiSO_4$). Finally, the RSM was employed to further optimize the levels of yeast extract, $MgSO_4$, and $NiSO_4$ by BBD after confirmation of the center point through the steepest ascent path. Under the optimum conditions, the maximum predicted γ-CGTase activity was found to be 53992.10 U mL^{-1}, which was 2.83-fold greater than the observed value in the culture medium before optimization. To the best of our knowledge, most of the known γ-CGTases produced by the wild microbial strains have exhibited extremely low hydrolysis activity or cyclization activity (Takada et al. 2003; Hirano et al. 2006; Goo et al. 2014; Ji et al. 2011; Wang et al. 2018a, 2018b). In the current study, the experimental γ-CGTase hydrolysis activity (52680.86 ± 329.72 U mL^{-1}) was significantly higher than the wild enzyme and the previously reported studies.

The properties and specificity of γ-CGTase were found to be the essential elements that influenced the production of γ-CD. The hydrolysis and cyclization activity of recombinant γ-CGTase exhibited favorable thermal stability below 50 °C and pH stability at a range of pH 7.0-9.0. Most importantly, the γ-CGTase maintained both the hydrolysis and cyclization activity under the same

condition (pH 9.0 and 50 °C), which could make the starch transformation process simpler, reliable, and easy to be controlled. The γ-CGTase also exhibited a higher γ-cyclization specificity because the proportion of γ-CD (90.44%) in the product CDs from cassava starch biotransformation was about 11% more than that of the wild enzyme (79%), with no α-CD detected (Takada et al. 2003), and the yield of γ-CD was 9.47%. When cyclododecone was added to the conversion reaction system, the yield of γ-CD reached 42.21% (w/w), 4.46-fold higher than the non-solvent method. Meanwhile, the γ-CD purity (98.72%) achieved the highest value during the transformation process from starch reported so far. Therefore, our findings suggest that the recombinant γ-CGTase would provide a theoretical basis for the industrial-scale production of γ-CD.

Irrespective of the significant results, several limitations accompany our study that could not be neglected. Firstly, the optimization results should be further verified on the fermenter level, pilot-scale tests, and industrial-scale production since the current data are restricted to the shake-flask level only. Secondly, the conditions of γ-CD converted by γ-CGTase, such as substrate concentration and additive amount of cyclododecone, and the amount of γ-CGTase, need to be further optimized. However, further research to overcome these issues is currently being investigated in our laboratory.

Conclusions

In this study, the soluble γ-CGTase activity from the recombinant *E. coli* BL21 (DE3) obtained through culture components optimization by BBD-RSM achieved 2.83-fold greater than the unoptimized medium. The recombinant γ-CGTase showed favorable thermal and pH stability, and high γ-cyclization specificity. It was concluded that the addition of cyclododecanone to the transformation system could enhance the γ-CD purity to 98.72%, which is the highest purity value during the transformation process reported so far. Therefore, it could provide an insight to the industrial-scale production of γ-CD with high purity.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-020-01610-8.

Additional file 1: Supplementary Table 1. Factors and experimental domain for the screening with Plackett-Burman design. **Supplementary Table 2.** Plackett-Burman design matrix with response value for screening variables (in coded level) affecting the yield of CGTase activity. **Supplementary Table 3.** Experimental ranges and levels in BBD.

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Authors' contributions

MD designed the study, carried out the experiments, and drafted the manuscript, YW (Yan Wang) and GY designed the study and revised the manuscript. JL and YW (Yi Wan) supervised the project and revised the manuscript. YD and YM analyzed the experimental data. All authors read and approved the final manuscript.

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Availability of data and materials

The data set supporting the results of this article are included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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