Upscale production of a recombinant cyclodextrin glycosyltransferase from *Paenibacillus macerans* in *Escherichia coli*

Yi-Nan Yang · Wen-Xin Shan · Pi-Wu Wang

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**Abstract** Cyclodextrin glucanotransferase (CGTase) is an important enzyme with multiple functions in starch utilization. In the present study, a fermentation process for the production of CGTase from *Escherichia coli* harboring the recombinant plasmid pET28b(+)−CGTase was investigated and optimized. The optimal fermentation and expression conditions were 10.0 g/L glycerol, 20.0 g/L tryptone, and 10.0 g/L yeast extract with an initial pH of 7.0, an IPTG concentration of 0.1 mM and an induction temperature of 28 °C for 10 h. The resulting CGTase activity reached up to 36.4 U/L and was 2.1-fold higher than before optimization. Under these optimal fermentation conditions, the up-scaled fermentation was carried out in a 500-L fermentor, and a CGTase activity of 45.2 U/L was achieved. This study provides a foundation for the industrial production of CGTase.

**Keywords** Fermentation · Optimization · CGTase · Production · Purification

**Introduction**

Cyclodextrin glucanotransferase (CGTase; E.C. 2.4.1.19), is a member of the α-amylase family of glycosyl hydrolases (family 13) and is an important enzyme with multiple functions in the starch utilization pathway of some bacteria, catalyzing various glucan transferring reactions with starch to produce cyclodextrins (vander Veen et al. 2000; Uitdehaag et al. 1999). CGTase is also widely applied in baking and carbohydrate glycosylation because it participates in various types of catalytic reactions. The action of CGTase begins with cleavage of an α-1,4-linkage within the glucan molecule. The newly produced reducing end is then transferred either to the nonreducing end of another molecule (disproportionation reaction) or to its own nonreducing end (cyclization reaction) (vander Veen et al. 2000). CGTase also catalyzes the reverse reaction of cyclization in which cycloamylose is opened by the enzyme, and a linear fragment is transferred to an acceptor (coupling reaction). At a certain frequency, the newly produced reducing end is transferred not to a carbohydrate acceptor but rather to a water molecule, which results in either the hydrolysis of amylose or the linearization of cycloamyloses (hydrolytic reaction). Understanding of the structure and catalytic mechanism of CGTases began in 1991 when the first structure of the CGTase from *Bacillus circulans* was obtained (Klein and Schulz 1991). Subsequently, much attention has been paid to the three-dimensional (3-D) structures of various CGTases. So far, more than fifty different CGTase crystal structures have been published, providing a basis for studies on the mechanism and engineering of CGTases. Commonly, the 3-D structure of a CGTase has a 5-domain organization: A, B, C, D, and E. Domain A, located in all enzymes of the α-amylase family and considered a catalytic area, conventionally has a (β/α)8 or triosephosphate isomerase barrel structure containing a highly symmetrical fold of 8 parallel β-strands arranged in a barrel encircled by 8 α-helices (vander Veen et al. 2000). Domain B, which consists of 44–133 amino acid residues that play central roles in substrate binding, is...
a protuberant loop between the β-strand 3 and α-helix 3 of domain A (van der Veen et al. 2000; Uitdehaag et al. 1999). It has been confirmed that replacing the amino acid residues in the B domain and introducing a salt bridge at the A/B domain interface can improve the catalytic stability and thermostability of CGTase (Wang et al. 2016; Goh et al. 2012). Domains C, D, and E comprise the C-terminal region of CGTases. Domain C has 1 maltose binding site that is responsible for substrate binding (Penninga et al. 1996). Domain E contains 2 maltose binding sites that are used as a raw starch-binding domain (Penninga et al. 1996; Dalmia et al. 1995). Domain D is an exclusive domain in CGTases, and its function is still not clear. The active site residues of CGTase are Glu257, Asp229 and Asp328. Glu257 is used as both the proton donor and acceptor, Asp229 forms a covalent intermediate with the cleaved substrate before CD formation, and Asp328 stabilizes the reaction intermediates (Uitdehaag et al. 1999). Some novel CGTases have recently been isolated from various organisms and were prepared by submerged fermentation and solid-state fermentation to meet the requirements of commercial applications (van der Veen et al. 2000). A novel CGTase was isolated from a Carboxydovella sp. via the CODEHOP strategy, and the fundamental activities were characterized and compared to two commercial CGTases (Ara et al. 2014). CGTase has gained considerable commercial importance in the last few years due to its applications in the chemical, pharmaceutical, food, beverage, clinical chemistry, biotechnology and other industries (Rather et al. 2015; Eastburn and Tao 1994; Gotsev et al. 2007; Kaupiboon and Pongsawasdi 2010). To improve its performance in industrial applications, various directed evolution techniques have been applied to modify the molecular structure of CGTase (Wang et al. 2016; Goh et al. 2012). In recent years, substantial progress has been made in the production, molecular engineering and application of CGTases (Ara et al. 2014; Rather et al. 2015). However, the production of recombinant CGTases has not yet been well studied.

Medium optimization for the expression of recombinant enzyme is an important step in industrial production processes and is definitely a pressing issue of commercial concern for biotechnology production as small modifications of process parameters, such as the composition of the production medium and induction conditions, can be crucial (Chen et al. 2009; Soni et al. 2016). Moreover, the maximum specific activity and biomass are usually achieved by the addition of complex components such as tryptone, yeast extract and metal ions, or by changes in the cultivation conditions such as pH, temperature and induction time (Romano et al. 2009). Few results have been reported regarding the effects of culture conditions on the production of CGTase in recombinant Escherichia coli.

In this current study, we attempted to optimize the medium composition and induction conditions using the one-variable-at-a-time approach for the production of recombinant CGTase from Paenibacillus macerans in E. coli, which will maximize the activity of CGTase. After optimization for the preparation of CGTase, up-scaled production of CGTase was performed, which provides a foundation for the industrial production and application of CGTase.

Materials and methods

Microorganism, medium and chemicals

Escherichia coli BL21 (DE3) (Invitrogen, Karlsruhe, Germany) and pET28b(+) (Novagen, Darmstadt, Germany) were used as host and plasmid for protein expression. Lysogeny broth (LB) (5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl) was used to culture E. coli and recombinant E. coli strains, which were grown aerobically at 37 °C. To maintain plasmids, the corresponding antibiotics were added to the media used to culture E. coli strains harboring plasmids. All chemicals were of analytical grade and commercially available.

The inoculum was produced in LB medium that was adjusted to pH 7.0 before sterilization. The composition of the initial fermentation medium was the same as the inoculum medium unless specifically noted otherwise. To maintain plasmids, 50 µg/mL of kanamycin (Kan) was added to the media.

All chemicals and solvents used in this study were purchased from Sigma (Missouri, St. Louis, USA) or Shanghai Chemical Co. (Shanghai, China) and were of analytical grade and commercially available.

Construction of an expression plasmid for the CGTase gene

A 2142-bp nucleotide sequence of a CGTase with 714 amino acids from P. macerans (GenBank Accession No. AAC04359) was synthesized using a PCR assembly method after optimization of the codons using gene designer software with E. coli as host (Villalobos et al. 2006; Liu et al. 2014, 2017; Rydzanicz et al. 2005). The synthesized gene with a 6× His-tag was inserted into the expression vector pET28b(+) between the NcoI and XhoI
 restriction endonuclease sites. The ligated plasmid pET28b(+)–CGTase was transformed into *E. coli* BL21 (DE3) using the heat shock method (Chung et al. 1989). For the selection of *E. coli* transformants, Kan was added to the medium at an appropriate concentration.

Expression and purification of the recombinant CGTase

*Escherichia coli* BL21 (DE3) harboring the recombinant plasmid was grown at 37 °C in 50 mL of LB media containing 50 µg/mL of Kan until the optical density at 600 nm (OD<sub>600</sub>) of the culture reached 0.6–0.8. The expression of recombinant CGTase was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG), and the culture was then incubated at 28 °C for 10 h. The bacterial cells were harvested by centrifugation at 10,000×g for 10 min at 4 °C and were washed twice with a physiological saline solution (NaCl, 0.9%). The cells were then resuspended in 30 mL of 100 mM Trís–HCl (pH 7.6) and treated by sonication with a Vibra-Cell VC 505 ultrasonic processor (Sonics and Materials Inc., Newtown, USA) at 300 W for 30 min. Cell debris was removed by centrifugation at 12,000×g for 20 min at 4 °C. The supernatant was used as the crude extract.

The recombinant proteins were purified under native conditions using a Nickel-NTA resin (Bio-Rad, Hercules, CA, USA), and enzyme purification was carried out according to a previously reported procedure (Liu et al. 2014). The purity of the preparations was determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Different amounts of protein from the purification steps were determined by SDS-PAGE using a Mini-gel system (Bio-Rad). The gels were cast with 0.75 mm spacers (Bio-Rad). Polyacrylamide gels contained a 5% acrylamide stacking gel (pH 6.8) and a 12% separating gel (pH 8.8). The proteins in the gel were stained with Coomassie brilliant blue R-250.

Cultivation conditions

Seed cultures were prepared by inoculating 50 mL of LB medium in a 250-mL flask with cells transferred from an agar slant, which were subsequently incubated for 10–12 h at 37 °C and 150 rpm. Two percent (v/v) of the seed culture was inoculated into 50 mL of LB medium in a 250-mL flask and incubated at 37 °C until the OD<sub>600</sub> of the culture reached 0.6–0.8. The expression of recombinant CGTase was induced by the addition of 0.1 mM IPTG at 28 °C for 10 h.

Biomass determination

Biomass from 10 mL of culture samples was determined after removal of the medium by centrifugation at 10,000×g for 10 min. The cell pellets that resulted from the centrifugation were washed with distilled water and dried at 80 °C to a constant weight. Measurements were calibrated to g dry cell weight (DCW)/L. All the experiments were performed in triplicate.

Medium optimization for recombinant CGTase production

Eleven different types of media were tested to investigate their effects on recombinant CGTase production and biomass (Table S1). The components of the media were varied using the one-variable-at-a-time approach, and the optimal medium was selected for further experiments. To improve the biomass and the production of recombinant protein, the effects of different concentrations of glycerol (2, 5, 8, 10 and 30 g/L), the available nitrogen sources (tryptone, yeast, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub> and urea), the presence of metal ions (Ni<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>3+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, Ba<sup>2+</sup>, Li<sup>+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>), and the initial pH of the medium (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) were investigated.

Optimization of induction conditions

To enhance the expression of recombinant CGTase, the influences of different concentrations of IPTG (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 mM) as well as the induction temperature (25, 28, 30, 32 and 37 °C) and the induction time (2, 4, 6, 8, 10 and 12 h) were studied.

Fermentation of recombinant *E. coli* harboring the CGTase gene

Recombinant *E. coli* BL21(DE3) harboring the *CGTase* gene was maintained on LB agar slants and inoculated into 500-mL flasks containing optimal medium and 50 μg/mL Kan. After 4 h cultivation, the cultures were then inoculated into a 50-L bioreactor (Shanghai BaoXing Bio-engineering Equipment Co., LTD., Shanghai, China) containing 30 L of optimal medium and were cultured at 37 °C and 500 rpm for 5 h. The airflow was 1.1vvm and the pH was adjusted to 7.0. Then, the cultures were inoculated into a 500-L bioreactor (Shanghai BaoXing Bio-engineering Equipment Co., LTD.) at 37 °C and 240 rpm. The airflow was 1.1 vvm and the pH was adjusted to 7.0. When the OD<sub>600</sub> reached approximately 10, 0.1 mM IPTG was added at 28 °C to induce enzyme expression.
Enzymatic activity assay

Bacterial cells were harvested from 10 mL of fermentation broth by centrifugation at 10,000 x g for 10 min at 4 °C and were washed twice with physiological saline solution (NaCl, 0.9%). Then, the cells were resuspended in 10 mL of a 50 mM phosphate buffer solution (pH 6.0) containing 3% (w/v) soluble starch. After incubation at 40 °C for 10 min, 10 mL of 1.0 M hydrochloric acid was added to stop the reaction. Subsequently, 1 mL of the reaction mixture was combined with the same volume of 0.1 mM methyl orange solution and kept at 20 °C for 20 min, followed by measurement at 505 nm. The α-cyclodextrin concentration was calculated corresponding to the α-cyclodextrin standard curve. Enzyme activity (U) was defined as the amount of enzyme required to produce 1 μmol α-cyclodextrin in 1 min at 20 °C and pH 6.0. As the CGTase studied in this work was fermentation broth-bound, the enzyme activity was calibrated as units per liter of fermentation broth.

Statistical analysis

In this study, if not specifically noted, all experiments were performed in triplicate. Analysis of variance was carried out using the SAS program version 8.1 (SAS Institute Inc., Cary, NC, USA). Least significant difference was computed at p < 0.05. All the Figures in this study were drawn using the origin software version 8.0 (OriginLab Corp., Northampton, MA).

Results and discussion

Expression of the CGTase gene

After codon optimization, the synthesized DNA fragment was subcloned into the expression vector pET28b(+) to construct the recombinant plasmid pET28b(++)-CGTase. Subsequently, the recombinant plasmid was introduced into E. coli BL21 (DE3) competent cells and several clones were isolated. A positive transformant containing recombinant pET28b(++)-CGTase was identified using colony PCR and double enzymatic digestion. In the recombinant E. coli harboring pET28b(++)-CGTase, recombinant protein expression was induced by the addition of 0.1 mM of IPTG at 28 °C for 10 h when the OD_{600} reached 0.6. SDS-PAGE showed protein bands that were visualized in gels by staining with Coomassie brilliant blue. The SDS-PAGE analysis (Fig. 1) shows a band with a molecular mass of approximately 74 kDa in Lane 6, which is in agreement with the predicted value based on the amino acid sequence of the CGTase. The purified protein was observed as a single band on the SDS-PAGE gel, indicating that the protein was quite pure.

Optimization of the medium composition

Determination of the initial medium

The carbon source used in the fermentation medium is one of the major factors that affects enzyme production, and therefore, selection of the carbon source is important for optimization (Fuchs et al. 2002). For large-scale production of CGTase for industrial uses, the cultivation conditions for the production of CGTase from recombinant E. coli need to be investigated and improved. In this study, our approach to improve E. coli enzyme activity and cell growth was first based on the screening of various medium components and induction conditions at the flask level. To optimize the medium components for cultivation of recombinant E. coli, we investigated the influence of 11 different types of media on enzyme activity and cell growth. The results showed that the highest enzyme activity (23.5 U/L) and biomass (2.5 g DCW/L) were achieved in LB-glycerol medium (Fig. 2). Due to the simple composition and high effectiveness of the LB-glycerol medium, it was selected as the initial medium for subsequent experiments. The slightly higher enzyme activity obtained in glycerol medium indicated that the presence of glycerol enhanced cell build-up, leading to quick and efficient expression of the recombinant CGTase. Furthermore, the accumulation of acetic
Acid is concomitant with low levels of recombinant protein production and is harmful on the growth of *E. coli*, and less acetic acid was formed in the LB-glycerol medium than in the LB-glucose medium.

**Effect of the glycerol concentration on enzyme activity and biomass**

Determination of the carbon source (glycerol) concentration is critical to the optimization of enzyme production, and it exerted a significant effect on the expression of CGTase (Seyis and Aksoz 2005). Figure 3 shows the effects of different concentrations of glycerol on cell concentration and CGTase activity in the recombinant *E. coli* fermentation process. The results indicate that the biomass increased with increasing concentrations of glycerol, and a maximum enzyme activity of 27.5 U/L was obtained at a glycerol concentration of 10 g/L, but no significant improvements in growth rate were observed with higher concentrations of glycerol. However, the enzyme activity decreased when concentrations of glycerol exceeded 10 g/L, indicating that high concentrations of glycerol decrease enzyme production. The LB-glycerol medium consists mainly of complex nitrogen-containing compounds, and the cells are forced to use these as energy sources, which affects the amount of biomass produced and the expression of recombinant enzyme obtained. The use of glycerol as a carbon source in this study yielded better growth rates and enzyme activity, similar to a previous report (Berger et al. 2011). The results also suggested that exceeding the optimal concentrations of glycerol leads to inhibition of cell growth and a consequent decrease in recombinant protein production (Shiloach and Fass 2005).

**Effects of the tryptone and yeast extract concentrations on enzyme activity and biomass**

Nitrogen sources, including organic and inorganic nitrogen, play an important role in the production of enzymes (Chen et al. 2009). Organic nitrogen sources can not only supply cell growth factors and amino acids for cell metabolism and enzyme synthesis, they also supply vitamins and trace metals, thereby affecting the growth of the organism and thus increasing CGTase production (Basar et al. 2010; Choosri et al. 2011). Yeast extract in combination with tryptone has been widely used for enzyme production in a large number of bacteria (Seyis and Aksoz 2005). Different concentrations of tryptone (Table S2) and yeast extract (Table S3) were tested. As indicated in Fig. 4, both the biomass and enzyme activity improved with increases in
the tryptone concentration. However, the enzyme activity did not increase further when the concentration of tryptone in the medium exceeded 20 g/L; tryptone contributed to cell growth, but caused lower enzyme activity at higher concentrations (≥30 g/L). At a constant yeast extract concentration, an increase in the tryptone concentration resulted in growth of biomass (2–3.275 g DCW/L). In addition, the enzyme activity increased from 14.9 to 31.3 U/L when the tryptone concentration was increased from 5 to 20 g/L. Moreover, a slight increase in biomass was found to lead to a decrease in enzyme activity, which suggests that adding higher concentrations (exceeding the optimal concentrations) of the nutrients would lead to a decrease in recombinant protein production. For these reasons mentioned above, we chose 20 g/L as the optimal concentration of tryptone for further optimization.

When concentration of yeast extract was 10 g/L, the highest enzyme activity of 32.5 U/L was reached (Fig. 5). An increase in the yeast extract concentration from 10 to 30 g/L led to a significant decrease in the enzyme activity, but had no significant effect on cell growth. Therefore, we selected 10 g/L as the optimal yeast extract concentration for further fermentation. It has been reported that yeast extract is helpful for the expression and stability of recombinant proteins when compared with other complex sources (Kweon et al. 2001; Lee et al. 1997; Yoon et al. 1996). Higher yields of cells but low enzyme activity were obtained with high concentrations of yeast extract. One possible reason may be that yeast extract is a complex nitrogen source, and therefore, the cells must secrete more protease for its enzymatic degradation before utilization, which would result in less CGTase production.

The LB-glycerol medium consists mainly of complex nitrogen-containing complex compounds, and the cells are forced to use them as energy sources. However, when the concentrations of tryptone and yeast extract were high or low, the ratio of carbon to nitrogen (ratio of C/N) is affected. The ratio of C/N in the medium also regulates the growth of E. coli and the fermentation process for CGTase production. When the ratio was too high, it led to the slow growth of cells and was not beneficial for accumulation of the recombinant protein. However, when the ratio was too low, the cells grew well at the early stage, but deteriorated earlier, which affected expression of the recombinant enzyme.

Effects of inorganic nitrogen sources on enzyme activity and biomass

In this study, NH₄Cl, (NH₄)₂SO₄, NH₄NO₃ and urea were used to evaluate the effects of inorganic nitrogen sources on enzyme activity and biomass. The results are listed in Table 1. Inorganic nitrogen sources in the form of NH₄⁺, NO₃⁻ or urea showed a slight inhibitory effect on enzyme activity and cell growth. Thus, it was not necessary to add inorganic nitrogen sources to the fermentation medium.

Effects of metal ions on enzyme activity and biomass

The effects of metal ions on enzyme activity and biomass were determined by the addition of different metal ions, including CuCl₂, NiCl₂, MnCl₂, CaCl₂, LiCl, MgCl₂, BaCl₂, ZnSO₄, FeCl₃ and FeSO₄, to the medium at a final concentration of 0.1 g/L. The results in Table 2 revealed that the positive influences of the additional metal ions were not notable compared with the control. Only Mg²⁺ slightly enhanced the enzyme activity and cell growth. Moreover, the results showed that low biomass and enzyme activities were obtained when the medium was supplemented with Fe³⁺, Fe²⁺ and Mn²⁺. However, no enzyme activity inhibition was observed in the presence of chelating agent EDTA, which illustrated that the CGTase obtained in this study does not require metal ions for catalysis.
Effects of the initial pH on enzyme activity and biomass

In the LB-glycerol medium, neutral pH led to high, soluble expression of the recombinant protein as reported in many other studies (Shiloach and Fass 2005). The initial pH of the medium was adjusted to 4.0–9.0 using 1 M HCl or NaOH, and then the effects on enzyme activity and biomass were examined. The results are shown in Fig. 6. The maximum enzyme activity and biomass obtained were 33.8 U/L and 3.1 g/L, respectively, when the initial pH of the medium was kept at 7.0. The enzyme activity decreased to 22.1 and 25.9 U/L when pH values were adjusted to 5.0 and 9.0, respectively, indicating that a slightly basic pH condition is preferred for cell growth and enzyme production. One possible reason for this finding is that a weakly basic environment reduces the inhibition on cell growth caused by acetate accumulation during E. coli cultivation, which enhances cell growth and improves the production of recombinant protein (Eiteman and Altman 2006). Furthermore, at pH 7.0, plasmid copy numbers were higher compared to other pH conditions, which led to high, soluble expression of the recombinant protein (Yang et al. 2012). Thus, the initial pH of medium was kept at 7.0 for further fermentations.

### Table 1 Effects of inorganic nitrogen sources on enzyme activity and biomass

| No. | Nitrogen source | Concentration (g/L) | Biomass (g DCW/L) | Enzyme activity (U/L) |
|-----|-----------------|---------------------|-------------------|-----------------------|
| 1   | Control         | 0                   | 3.08 ± 0.03       | 32 ± 1.13             |
| 2   | NH₄Cl           | 2                   | 2.97 ± 0.08       | 29.3 ± 0.61           |
| 3   | (NH₄)₂SO₄       | 2                   | 3.02 ± 0.11       | 28.9 ± 0.63           |
| 4   | NH₄NO₃         | 2                   | 2.89 ± 0.09       | 22.9 ± 0.45           |
| 5   | Urea            | 2                   | 3.05 ± 0.05       | 20.3 ± 1.15           |

### Table 2 Effects of metal ions on enzyme activity and biomass

| Metal ions | Biomass (g DCW/L) | Specific activity (%) |
|-----------|-------------------|-----------------------|
| Control   | 3.02 ± 0.09       | 100 ± 1               |
| Ni⁺       | 2.65 ± 0.04       | 81 ± 2                |
| Zn²⁺      | 3.05 ± 0.02       | 100 ± 2               |
| Ca²⁺      | 1.98 ± 0.05       | 56 ± 5                |
| Fe³⁺      | 1.80 ± 0.03       | 54 ± 10               |
| Fe²⁺      | 1.92 ± 0.08       | 58 ± 6                |
| Cu²⁺      | 2.45 ± 0.05       | 78 ± 8                |
| Ba²⁺      | 2.85 ± 0.10       | 89 ± 6                |
| Li⁺       | 2.25 ± 0.07       | 75 ± 7                |
| Mg²⁺      | 3.08 ± 0.05       | 115 ± 7               |
| Mn²⁺      | 2.05 ± 0.11       | 64 ± 10               |
| EDTA      | 3.01 ± 0.03       | 97 ± 5                |

#### Optimization of induction conditions

**Effects of the IPTG concentration on enzyme activity and cell growth**

It is reported that the IPTG concentration is important for soluble enzyme expression (Durany et al. 2004). The effects of the IPTG concentration on enzyme activity and cell growth have been studied (Fig. 7). The highest enzyme activity and biomass were obtained by induction with 0.1 mM IPTG and reached 37.5 U/L and 2.99 g DCW/L, respectively. However, enzyme activity and biomass decreased when the concentration of IPTG exceeded 0.1 mM. Furthermore, high concentrations of IPTG inhibited cell growth and led to a decrease in the production of recombinant protein. This finding was similar to that observed in previous studies (Choi et al. 2000). For upscale production of CGTase, the concentration of IPTG must be minimized according to the expected amount of biomass and recombinant enzyme to ensure economic feasibility (Kweon et al. 2001).

**Effects of the induction temperature on enzyme activity and cell growth**

The induction temperature also had a significant effect on the biomass and recombinant protein expression. Lower
induction temperatures enhanced expression of the recombinant CGTase; conversely, higher temperatures reduced the protein expression. Usually, the induction temperature for obtaining soluble enzyme with high activity is set at 28 °C (Lo et al. 2007). Experiments at different temperatures were carried out to evaluate the influence of the induction temperature (25–37 °C) on target protein expression in E. coli. As shown in Fig. 8, a proper temperature led to a high level of protein expression. The maximum enzyme activity and biomass were obtained using an induction temperature of 28 °C, reaching 42.4 U/L and 2.995 g DCW/L, respectively. The results also showed that higher temperatures had a remarkable effect on the induction of recombinant protein because the higher bacterial growth rate conferred a much greater metabolic burden on the cells, leading to the formation of inclusion bodies and increased probability of plasmid loss and recombinant protein expression failure. However, temperatures that are too low will decrease cell growth, which leads to a reduction in expression levels (Murby et al. 1996).

Effects of the induction time on enzyme activity and cell growth

Compared to the induction temperature and IPTG concentration, the induction time did not significantly affect production of the enzyme, though the longer durations did not result in lower recombinant enzyme expression, and a shorter induction time is economical for upscale production (Lee et al. 1997). The effects of induction time on enzyme activity were investigated from 2 to 12 h at 28 °C with an IPTG addition of 0.1 mM (Fig. 9). The results indicated that the recombinant enzyme could be efficiently induced at 10 h, reaching an enzyme activity of 36.3 U/L. At longer induction times, there was a slight decrease in enzyme activity. However, the biomass increased with elongation of induction time. It was concluded that the highest level of recombinant protein expression indicated the optimal induction time, and longer durations led to a lower yield of recombinant protein expression, likely due to cell lysis and proteolytic degradation (Murby et al. 1996). In addition, from a production efficiency perspective, a longer induction time is not economical (Lo et al. 2007). Therefore, based on Fig. 9, an induction time of 10 h was selected as the optimal induction time.

Up-scaled fermentation for producing CGTase

To increase the productivity of recombinant E. coli harboring CGTase, fermentations in a 500-L fermenter were carried out. After culturing for 10 h, a 500-mL culture was inoculated into a 50-L fermenter to continue to cultivate as seed for the up-scaled fermentation. The pH was
adjusted to 7.0 using 25% (v/v) ammonia water and 50% (v/v) phosphoric acid. A 10% (v/v) inoculum was used, the initial cultivation temperature was 37 °C, the agitation rate was kept at 200 rpm, and air was supplied continuously at 1.1 vvm. When the OD_{600} reached 10, the cultivation temperature was reduced from 37 to 28 °C, and IPTG was added to a final concentration of 0.1 mM to induce expression of the CGTase in the recombinant E. coli. The details of the up-scaled fermentation are shown in Fig. 10. After induction at 28 °C for 10 h, recombinant cells harboring the CGTase were harvested. After the seeds were inoculated into fermentation broth, the cells grew quickly, reaching logarithmic phase within 2 h, which greatly shortened the fermentation period. During this phase, the recombinant E. coli grew quickly and most of the nutrition was consumed. In this phase, dissolved oxygen showed a sharp decline. After approximately 5.0 h of cultivation, the OD_{600} reached above 10, and IPTG was added for induction. The CGTase activity then increased rapidly, and relatively high activity could be detected after 1 h induction. After cultivation for 15 h, the cell biomass and the activity of CGTase simultaneously reached their maxima. Ultimately, the cell biomass and activity of CGTase achieved were 45.2 U/L and 3.39 g DCW/L, respectively. To the best of our knowledge, this is the first report on the optimization of culture conditions for recombinant E. coli producing CGTase, and significant improvement of enzyme activity was achieved. Enhanced production of the CGTase protein will facilitate the production and application of this enzyme, especially for the industrial preparation of CGTase.

Conclusions
In this work, after optimization of the medium components and culture conditions, the highest amount of CGTase was achieved in a medium containing 10 g/L glycerol, 20 g/L tryptone, and 10 g/L yeast extract with an initial pH of 7.0. The optimal induction conditions for recombinant CGTase from E. coli consisted of an IPTG concentration of 0.1 mM, an induction temperature of 28 °C, and an induction time of 10 h. After up-scaled in 500 L fermentor, the final enzyme activity and biomass reached were 45.2 U/L and 3.39 g DCW/L. This study paved a foundation for up-scaled production of CGTase.

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Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest in the publication.

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