JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

High-Level Extracellular Production of α-Cyclodextrin Glycosyltransferase with recombinant *Escherichia coli* BL21 (DE3)

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ABSTRACT: High-level production of α-cyclodextrin glycosyltransferase (CGTase) is one of the key factors in α-cyclodextrin (CD) preparation. In the present study, a fed-batch fermentation strategy for high-cell-density cultivation of *Escherichia coli* and the extracellular production of recombinant α-CGTase from *Paenibacillus macerans* JFB05-01 was established. A combined feeding strategy based on both specific growth rate before induction and the amount of glycerol residues after induction was used to control cell growth, acetate production, and glycerol consumption. When induced by lactose, a feeding solution with complex nitrogen was found beneficial for α-CGTase production. In addition, different induction temperatures and induction points were investigated, and the results indicated that these factors played an important role in α-CGTase production. When induced at 25 °C and at a dry cell weight of 30 g L^{-1}, the extracellular activity of α-CGTase could reach 275.3 U mL^{-1}.

KEYWORDS: *Escherichia coli*, *Paenibacillus macerans*, α-cyclodextrin glycosyltransferase, extracellular production, induction strategy

INTRODUCTION

Cyclodextrins (CDs), which possess a hydrophilic outside and a hydrophobic central cavity, are cyclic α-1,4-glucans composed of six to more than 100 glucose units.[1] The most common forms of CDs are α-, *β*-, and *γ*-CD with six, seven, and eight glucose units, respectively.[2] The ability of CDs to form inclusion complexes with various hydrophobic compounds has led to their application in the food, cosmetic, and pharmaceutical industries.[3]

Cyclodextrins are produced by converting starch or starch derivates through an intramolecular transglycosylation reaction of cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19).[1] Cyclodextrin glycosyltransferase is a member of the α-amylase family (family 13) of glycosyl hydrolases,[3] and it has been found in many bacterial species, mainly *Bacillus*.[4] However, due to the limited expression level, CGTase is one of the rate-limiting factors in CD preparation. According to the major CD products, CGTase has been further classified into α-, *β*-, and *γ*-CGTase. Owing to the wider applications of α-CD in molecular recognition, nanomaterials, and food industries for its better solubility and smaller internal cavity, α-CGTase is attracting more interest in scientific research nowadays.

Escherichia coli is the most commonly used host microorganism for heterologous protein expression. CGTase has been successfully produced in the cytoplasm by *E. coli*.[5] However, targeting the protein to the culture medium has several advantages over intracellular production, such as facilitating downstream processing and achieving higher-level expression.[6] Previously, our laboratory performed the extracellular production of α-CGTase from *Paenibacillus macerans* JFB05-01 in recombinant *E. coli* BL21(DE3) with OmpA signal peptide. A strategy of increasing secretion of α-CGTase to the liquid medium by *E. coli* in shake flasks was developed, and α-CGTase activity in the medium reached 48.0 U mL^{-1} after 48 h of cultivation.[7−9] In addition, a preliminary investigation on a 3-L fermentation scale indicated that synthetic medium was an optimal culture to achieve high cell density for *E. coli*.[10] In the present study we have attempted to investigate the α-CGTase activity using synthetic medium with lactose induction. Additionally, different cultivation conditions, such as feeding strategies and induction methods, were investigated on a 3-L fermentation scale to further promote α-CGTase secretion into the culture medium. The results showed that a two-stage feeding strategy with complex nitrogen sources (peptone and yeast extract) was suitable for high-cell-density culture and protein production. When induced at 25 °C and intermediate cell density (dry cell weight (DCW) of 30 g L^{-1}) by lactose, the yield of α-CGTase in culture media could reach 275.3 U mL^{-1}, which represents the highest extracellular yield and productivity of α-CGTase reported so far.

MATERIALS AND METHODS

Bacterial Strain and Plasmid. The recombinant *E. coli* BL21-(DE3) carrying α-*cgt* gene of *P. macerans* JFB05-01 (constructed in our laboratory) was used for all fermentation experiments. The plasmid with OmpA signal peptide instead of pelB was derived from the vector pET-20b(+).[8]

Media and Feeding Solutions. Luria−Bertani (LB) medium, containing NaCl 10 g L^{-1}, peptone 10 g L^{-1}, yeast extract 5 g L^{-1}, pH 7.0, was used for seed culture. The medium for α-CGTase production was modified Riesenberg medium,[11] which contains glycerol 8.0 g L^{-1}, $(NH_4)_2HPO_4$ 6 g L^{-1}, KH_2PO_4 10.5 g L^{-1}, citric acid 1.7 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 3.4 g L^{-1}, and trace metal solution, 10 mL L^{-1}, pH 7.0. The trace metal solution contains $FeSO_4 \cdot 7H_2O$ 10.0 g L^{-1}, $ZnSO_4 \cdot 7H_2O$ 5.25 g L^{-1}, $CuSO_4 \cdot 5H_2O$ 3.0 g L^{-1}, $MnSO_4 \cdot 4H_2O$ 0.5 g L^{-1}, $Na_2B_4O_7 \cdot 10H_2O$ 0.23 g L^{-1}, $CaCl_2$ 2.0 g L^{-1}, and $(NH_4)_6Mo_7O_{24}$ 0.1 g L^{-1}. The feeding solutions were classified into three kinds according to

Received: January 4, 2011
Accepted: March 18, 2011
Revised: March 14, 2011
Published: March 18, 2011

the nitrogen addition: (1) control without extra nitrogen: glycerol 500 g L^{-1}, and $MgSO_4 \cdot 7H_2O$ 30 g L^{-1}; (2) FS1 with inorganic nitrogen: glycerol 500 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 30 g L^{-1}, and NH_4Cl 18 g L^{-1}; (3) FS2 with complex nitrogen: glycerol 500 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 30 g L^{-1}, peptone 15 g L^{-1}, and yeast extract 30 g L^{-1}. FS2 and FS3 were designed in accordance with the principle of equal mass of nitrogen. The induction medium contained 200 g L^{-1} lactose.

Cultivation Conditions. The seed culture was started by inoculating 100 μL of frozen glycerol stock (kept at −75 °C) and cultured for 8 h in 50 mL of LB medium (containing 100 μg mL^{-1} ampicillin) in a 250 mL shake flask at 37 °C and 200 rpm. A 10% (v/v) concentration of inoculum was inoculated into modified Riesenberg medium (containing 100 μg mL^{-1} ampicillin) for fed-batch cultivation. Fed-batch cultivation was performed in a 3-L fermentor (BioFlo 110, New Brunswick Scientific Co., Edison, NJ) which consisted of three phases. The first one was characterized by batch phase with an initial glycerol concentration of 8 g L^{-1} and temperature of 37 °C. After inoculation, the DO value decreased suddenly with a concomitant decrease in pH and glycerol. The end of glycerol consumption was detected by a sudden increase in both DO and pH value, and then the preinduction phase of fed-batch cultivation started. During the second phase, 2% (w/w) glycine was added at a dry cell weight (DCW) of 9 g L^{-1}. When a DCW of 30 g L^{-1} was reached, the inducer was fed at 0.8 g L^{-1} h^{-1} and temperature decreased to 30 °C (unless otherwise stated) for α-CGTase production, and then the postinduction phase began. During the whole process, the pH was kept at 7.0 by 25% (v/v) ammonia solution. Antifoam was added manually only when it was necessary. The dissolved oxygen (DO) level was kept at 30% of air saturation by cascading impeller speed and supplementation of air with pure oxygen. Dissolved oxygen concentration, pH, temperature, and impeller speed were recorded using Advanced Fermentation Software (AFS) from New Brunswick Scientific Co. Inc.

Determination of Bacteria Biomass. Cell growth was monitored during cultivation by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (BioPhotometer plus, Eppendorf Co., Hamburg, Germany). Samples were approximately diluted with 0.9% (w/v) NaCl at an OD value exceeding 0.8. To determine the DCW, 5 mL of culture broth was centrifuged at 13 800*g* for 10 min. The pellet was washed with 0.9% (w/v) NaCl, recentrifuged, and then dried to a constant weight at 105 °C.

Cell Fractionation. Culture supernatants were obtained by centrifugation at 13 800 g for 5 min at 4 °C, and the supernatant was used as an extracellular fraction. To obtain the periplasmic fraction, equal amounts of bacteria were harvested and washed twice by 50 mM phosphate buffer (pH 6.0) and then completely resuspended to the original volume by adding 30 mM Tris−HCl solution (pH 8.0) containing 25% (w/v) sucrose and 1 mM EDTA. The cell suspension was incubated on ice. The supernatant was collected by periplasmic fractionation. The total α-CGTase activity was the sum of periplasmic and extracellular enzyme activity.

SDS-PAGE Gel Electrophoresis. The SDS-PAGE was performed using 5% stacking gel and 12% separating gel (Bio-Rad Laboratories, Hercules, CA).

Assay of α-CGTase Activity. The α-CGTase activity was determined in terms of cyclizing activity by the methyl orange (MO) method as described previously.[9,12]

Determination of Glycerol and Acetate. Fermentation supernatant was analyzed for glycerol and acetate by using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) with a differential refractive index detector (RID). The analytical conditions used were as follows: Shodex SUGAR SH1011 column (Showa Denko, Japan), 8.0 mm ID × 300 mm; column temperature, 50 °C; mobile phase, 5 mM H_2SO_4; flow rate, 0.8 mL min^{-1}; injection volume, 5 μL; detector temperature, 30 °C.

RESULTS AND DISCUSSION

Feeding Strategy for High-Cell-Density Culture. The yield of recombinant protein production depends on both the biomass concentration and the specific cellular productivity.[13] To achieve a high biomass concentration in fed-batch cultivation, high-cell-density cultivation (HCDC), using appropriate feeding strategies, is preferable.[14] The feeding strategies include indirect feedback methods (such as pH-stat and DO-stat), predetermined feeding methods (such as exponential feeding), and feeding according to substrate uptake or demand.[15] Exponential feeding is a simple method that allows cells to grow at a constant growth rate below the critical value for acetate formation.[16] Acetate is an extracellular byproduct of aerobic fermentation, which usually inhibits growth and recombinant protein formation at a concentration higher than 2.0 g L^{-1}.[17] Usually, accumulation of acetate can be prevented by a limited supply of carbon source,[18] and exponential feeding was a suitable strategy to achieve this. However, the induction process of engineered cells without feedback could lead to the accumulation of substrate in the medium. This can be due to the change in cellular physiology and metabolism caused by the induction of recombinant proteins. Previously, it has been reported that the changes in cellular physiology and metabolism were dependent on temperature, methods of induction, and nature of recombinant proteins, all of which led to difficulties in the evaluation of nutrition needs by cells.[19,20] Thus, feeding strategies based on the substrate residues after induction should be applied.

Based on the above analysis and the specific expression system of the recombinant cell utilized, in the present study, a two-stage feeding strategy was applied. During the preinduction phase, the glycerol feeding rate was increased exponentially according to exponential feeding method,[21] and the cell growth was controlled at a specific growth rate (μ_{set}) of 0.25 h^{-1}. When the DCW of the cell reached a certain value, the postinduction phase began and the feeding rate was shifted to a gradient-decreasing method (Figure 1A). Under this feeding strategy, the *E. coli* cells were cultivated for 26 h from 0.14 g L^{-1} to 94.4 g L^{-1}, and the biomass was maintained without reduction for another 4 h (Figure 1A). Moreover, considerable accumulation of glycerol or acetate (below 1.0 g L^{-1}) in the whole process was not observed (Figure 1B).

Effect of Nitrogen Supplementation in Feeding Solution on Cell Growth and α-CGTase Production. Nitrogen is a critical medium component for bacteria growth and producing performance. In above high-cell-density cultivation, ammonia served as the sole nitrogen source as well as base to control pH. With the aim to improve α-CGTase production by recombinant *E. coli*, the effect of nitrogen supplementation in the feeding solution on cell growth and enzyme production was investigated. Two kinds of nitrogen sources were chosen: inorganic nitrogen (FS1) and complex nitrogen (FS2). For the control, no extra nitrogen was added.

The results showed that the cell concentration obviously increased when the feeding solution was supplemented with nitrogen sources. The highest DCWs obtained in FS1 and FS2 were 82.1 and 94.4 g L^{-1}, respectively, which were 1.2- and 1.3-fold that of control (Figure 2A). The extracellular activity obtained in FS2 was 105.1 U mL^{-1}, which was 2.3-fold that of control (Figure 2B). However, the extracellular activity obtained in FS1 was 8.8 U mL^{-1}, which was only 19.0% of that of control (Figure 2B).

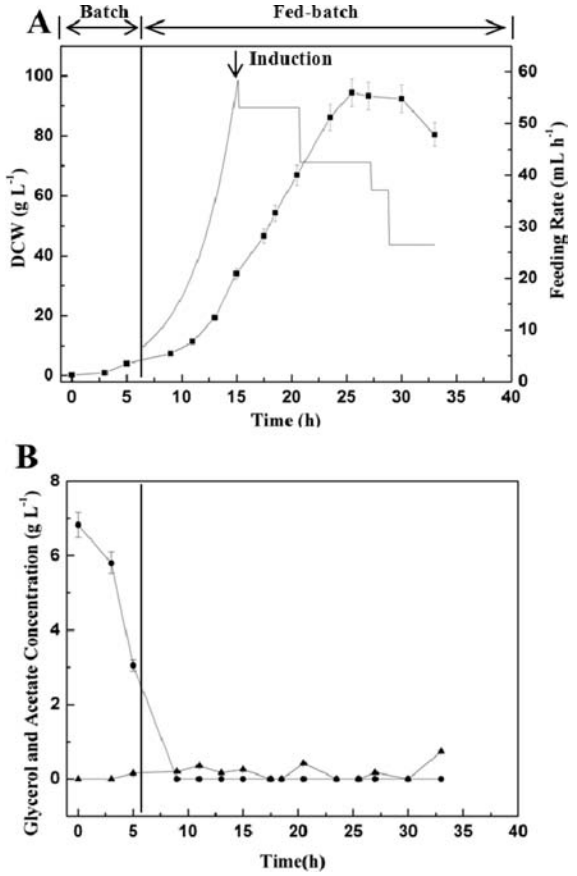

Figure 1. Fed-batch culture of recombinant *E. coli* BL21 (DE3). A: cell concentration (■) and glycerol feeding rate (solid line). B: glycerol (●) and acetate (▲). Arrows indicated the starting point of induction.

In our engineered *E. coli* cells, α-CGTase was first synthesized as a precursor with OmpA signal peptide, which has a feature essential for the secretion of proteins into the periplasm of *E. coli*. During transport of pre-enzyme across the inner membrane, the signal sequence of OmpA was cleaved by signal peptidase to yield the mature α-CGTase. The mature enzyme was then transported into the medium by nonspecific periplasmic leakage. Thus, the extracellular activity of α-CGTase was dependent on both periplasmic transport and nonspecific periplasmic leakage.

In order to analyze the effect of nitrogen supplementation on extracellular enzyme production, the activity of α-CGTase in the periplasm of our engineered *E. coli* cells was measured, and the total activity of the expression was calculated as the sum of activities from the culture medium and periplasmic space. As seen from Figure 2C, the total activity obtained in FS2 reached 177.2 U mL^{-1}, which was 2.1-fold that of control. It is known that complex nitrogen, such as peptone and yeast extract, can provide not only a nitrogen source but also special factors, such as amino acids, trace metals, cofactors, and vitamins.[22−24] In the present study, these factors may promote recombinant protein synthesis and translocation, which lead to an enhanced total activity of α-CGTase in FS2. However, the total activity in FS1 was quite low, which accounted for 16.5% (14.1 U mL^{-1}) of that in control. The phenomenon of ammonium chloride suppression of α-CGTase production has not been reported previously and requires further investigation.

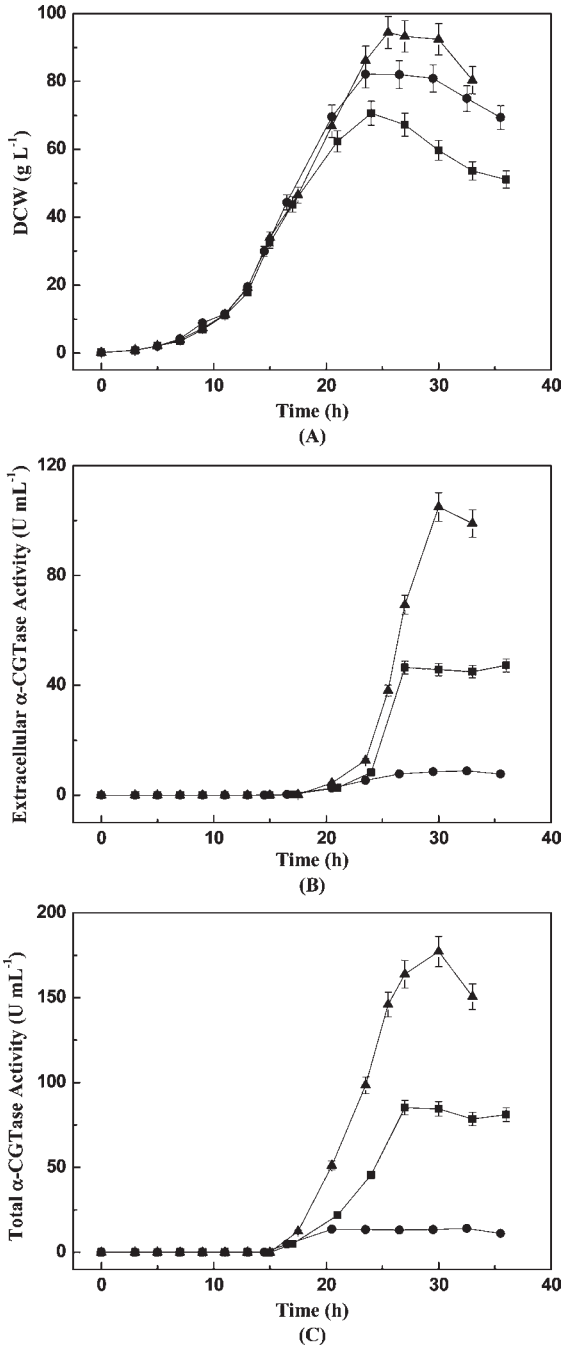

Figure 2. Comparison of the time profiles for cell concentration (A), extracellular α-CGTase activity (B), and total α-CGTase activity (C) obtained from cultivations with different nitrogen supplementation to the feeding solution. ■ Control; ● FS1; ▲ FS2.

Despite its considerable influence on total α-CGTase activity, nitrogen supplementation in the feeding solution showed no significant influence on extracellular secretion. The capability of extracellular secretion was calculated as extracellular activity divided by total activity. On the basis of the above calculation, the capability of extracellular secretion in control, FS1, and FS2 was 54.4%, 62.4%, and 59.3%, respectively.

Effect of Induction Temperature on Cell Growth and α-CGTase Production. Induction temperature is an important parameter for recombinant protein production in *E. coli*.[25,26]

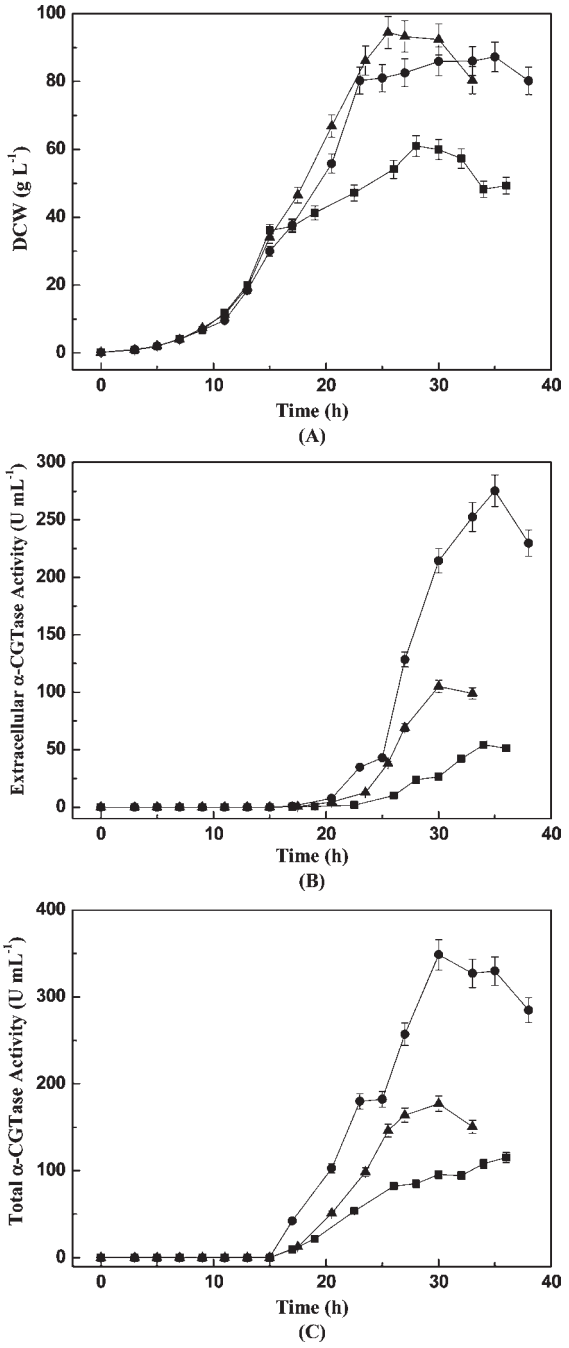

Figure 3. Comparison of the time profiles for cell concentration (A), total α-CGTase activity (B), and extracellular α-CGTase activity (C) obtained from cultivations at different induction temperatures. ■ 20 °C; ● 25 °C; ▲ 30 °C.

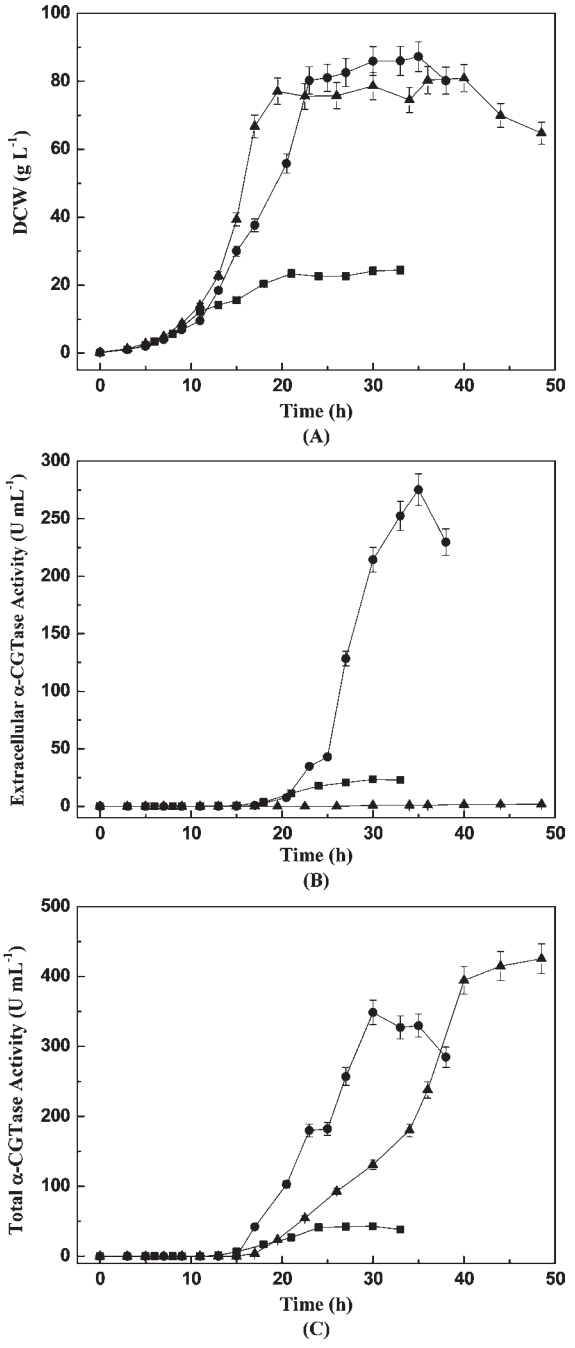

Figure 4. Comparison of the time profiles for cell concentration (A), total α-CGTase activity (B), and extracellular α-CGTase activity (C) obtained from cultivations induced at low cell density (■ 15 g L^{-1}), intermediate cell density (● 30 g L^{-1}), and high cell density (▲ 60 g L^{-1}).

Previously, flask experiments indicated that induction at 37 °C had a negative impact on α-CGTase production. High temperature may cause excessive speed of α-CGTase synthesis, leading to large amounts of inactive protein aggregates, i.e., inclusion bodies (data not shown). On the basis of this observation, we developed a two-stage temperature fermentation strategy, in which three different induction temperatures (20 °C, 25 °C, and 30 °C) were investigated.

As depicted in Figure 3A, the final biomass reached 94.4 g L^{-1} at an induction temperature of 30 °C, which was 1.6- and 1.1-fold that of 20 °C and 25 °C, respectively. However, a high level of biomass did not lead to a high protein yield. The extracellular α-CGTase activity obtained at 30 °C was lower than that at 25 °C but higher than that at 20 °C. Thus, induction at 25 °C was optimal for α-CGTase extracellular production, at which the total and extracellular α-CGTase activities reached 348.6 U mL^{-1} and 275.3 U mL^{-1}, respectively (Figure 3B,C). Besides, the capability of extracellular secretion at 25 °C was also the highest, reaching 79.0%.

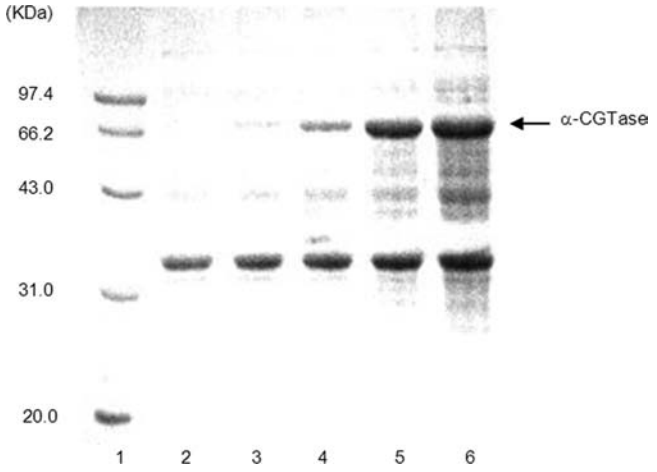

Figure 5. SDS-PAGE analysis of extracellular fraction under the optimal fermentation condition. Lane 1: marker; lanes 2−6: culture time of 15, 20, 25, 30, and 35 h.

Lower temperature is believed to reduce protein synthesis and decrease fluidity of the cytoplasmic membrane, which influences membrane-associated cellular functions such as periplasmic transport and nonspecific periplasmic leakage.[27] Thus, induction at 20 °C resulted in the lowest total and extracellular enzyme activity. Although high temperature enhances the fluidity of the cell membrane and the rate of protein synthesis, overexpression of proteins often results in the intracellular accumulation of inclusion bodies, which may affect the cell physiology and protein transport. According to our results, the induction at 25 °C led to a better coordination between protein synthesis and translocation and attained the highest yield of α-CGTase in the culture medium.

Effect of Induction Point on Cell Growth and α-CGTase Production. Recombinant protein expression generally imposes certain metabolic burden on the cell growth, which may reduce the cell growth rate, cell yield, protein expression, and plasmid stability.[28] In order to lower the extent of metabolic burden imposed on the cell, the optimal point for α-CGTase induction was investigated in the present study, in which lactose was fed at a DCW of 15, 30, and 60 g L^{-1}, respectively. As a result, cell growth was seriously inhibited when induced at low cell density, under which the maximum DCW was only 23.4 g L^{-1} (Figure 4A). As a consequence, this serious inhibition of cell growth led to reduced extracellular α-CGTase activity of 23.3 U mL^{-1} (Figure 4B). Although no significant inhibition was observed at high-cell-density induction, the extracellular α-CGTase activity was also quite low, only 1.9 U mL^{-1}. Thus, induction at intermediate cell density was the optimal condition, and the maximum α-CGTase activity in the medium reached 275.3 U mL^{-1} (Figure 4B). As seen from the SDS-PAGE analysis of extracellular fraction (Figure 5), the concentration of recombinant proteins in the medium increased along with time after induction.

The above results showed that the extracellular production of recombinant α-CGTase was seriously affected by the time of induction, similar to that observed in the production of human leptin[29] and glycine oxidase.[20] In order to analyze the effect of induction points on extracellular production of α-CGTase, the total activities under three conditions were compared. Induction at high cell density rendered the highest total activity of 425.7 U mL^{-1}, while the capability of extracellular secretion was only 0.5%, leaving 99.5% of the total activity in the periplasm. In addition, under the above induction condition, most of the enzymes were produced during the stationary phase (Figure 4A, C). Previously, it was reported that when the stationary phase is approached, the cell membrane becomes more rigid.[26] Increased rigidity of the cell membrane is believed to not be suitable for nonspecific periplasmic leakage, which possibly leads to the decreased secretion of periplasmic proteins into the culture medium induced at high cell density.

So far, to our knowledge, there are only a few reports on the extracellular α-CGTase production with a maximum yield of 113.0 U mL^{-1} in synthetic medium.[7−10] Scientific investigations have been focusing on extracellular β-CGTase production. Lee applied a pH-stat cultivation of recombinant *E. coli* to achieve β-CGTase secretion, and the production reached 21.6 U mL^{-1}.[30] Kuo reported the highest β-CGTase activity obtained by fed-batch in alkalophilic *Bacillus* sp. was 56.0 U mL^{-1}.[31] In the present study, a fed-batch fermentation strategy for the extracellular production of α-CGTase by recombinant *E. coli* BL21 (DE3) has been established. Under the optimum condition, the enzyme activity in the culture media reached 275.3 U mL^{-1}, which represents the highest extracellular yield of α-CGTase ever reported. In addition, due to its simplicity, the fermentation strategy might be used for the extracellular production of other heterogonous proteins expressed in *E. coli*.

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Funding Sources

This work was financially supported by the National Natural Science Foundation of China (30970057), the Key Program of National Natural Science Foundation of China (20836003), Jiangnan University Youth Foundation (2009LQN24), the Program of Innovation Team of Jiangnan University (2008CXTD01), the Fundamental Research Funds for the Central Universities (JUSRP20917), and the open program for key laboratory of industrial biotechnology ministry of education (KLIB-KF200902).

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