

## Enhanced secretion of recombinant $\alpha$ -cyclodextrin glucosyltransferase from *E. coli* by medium additives

Runrong Ding<sup>a,b</sup>, Zhaofeng Li<sup>a,b</sup>, Sheng Chen<sup>a,b</sup>, Dan Wu<sup>a,b</sup>, Jing Wu<sup>a,b,\*</sup>, Jian Chen<sup>a,b,\*\*</sup>

<sup>a</sup> State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, People's Republic of China

<sup>b</sup> School of Biotechnology and the Key Laboratory of Industrial Biotechnology, Education of Ministry, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, People's Republic of China

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### ABSTRACT

The purpose of this study was to investigate the effect of medium additives on the secretion of recombinant  $\alpha$ -cyclodextrin glucosyltransferase ( $\alpha$ -CGTase) into the culture media of *Escherichia coli*. It is found that supplementation of the *E. coli* culture with SDS, glycine,  $\text{Ca}^{2+}$  or  $\text{Na}^+$ , individually, facilitated the secretion of  $\alpha$ -CGTase. Orthogonal experiment showed that the optimal condition to achieve maximal secretion of  $\alpha$ -CGTase was the supplementation with 0.03% SDS, 400 mM  $\text{Na}^+$ , 0.3% glycine and 10 mM  $\text{Ca}^{2+}$  together. Under this condition, extracellular enzyme activity reached 12.89 U/ml, which is 15 times higher than that of the culture without any additives. Further analysis showed that the permeability, fluidity and phosphatidylglycerol content of the *E. coli* cell membrane under the optimized condition were significantly increased in comparison to those under the control condition. These might be the potential mechanisms for the increased secretion of  $\alpha$ -CGTase from the periplasmic compartment into the culture medium.

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### 1. Introduction

Cyclodextrin glucosyltransferase (CGTase, EC2.4.1.19), an important member of glycosyl hydrolase family, catalyses the formation of cyclodextrins (CDs) [1] from starch and related carbohydrates. CDs have a cylindrical shape, which is hydrophobic inside and hydrophilic outside. The most common types of CDs are  $\alpha$ -,  $\beta$ - and  $\gamma$ -CDs consisting of 6, 7 and 8 glucose units linked by  $\alpha$ -1,4-glycosidic, respectively. CDs are able to form inclusion bodies with many hydrophobic molecules and change their physical and chemical properties, which makes CDs attractive for various applications in many different fields, e.g. food, medicine, agriculture, cosmetics, chemical and environmental protection [2,3]. However, due to the high cost of production, application of CDs has been restricted. In order to reduce the cost, significantly improving the low yields of CGTase during the process of production has been expected [4].

\* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, People's Republic of China. Tel.: +86 510 85327802; fax: +86 510 85327802.

\*\* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, People's Republic of China. Tel.: +86 510 85327802; fax: +86 510 85327802.

E-mail addresses: [jingwu@jiangnan.edu.cn](mailto:jingwu@jiangnan.edu.cn), [jingwu80@hotmail.com](mailto:jingwu80@hotmail.com) (J. Wu), [jchen@jiangnan.edu.cn](mailto:jchen@jiangnan.edu.cn) (J. Chen).

CGTase is an extracellular enzyme identified in a number of bacteria, mainly *Bacillus* [4]. To overcome the low yield of CGTase produced by wild strains, studies have been carried out to genetically engineer a CGTase gene in *Escherichia coli* for its overexpression [5]. Previously, our laboratory cloned CGTase gene from *Paenibacillus macerans* strain JFB05-01 and expressed it in *E. coli* [6]. The expression vector, pET-20b(+)/cgt, contains a periplasmic PelB secretory signal peptide. During the process of bacterial cultivation, it is observed that most of the PelB-fused CGTase was accumulated in the periplasmic compartment and small amount of CGTase was non-specifically secreted to the culture medium. This “leaky” phenomenon was also reported in studies on the expression and secretion of other recombinant proteins [7].

The microbial cell membrane is highly selective for the translocation of intracellular and extracellular materials. Alterations of cell membrane permeability normally change the rates of absorbing raw materials and exporting the metabolites, which is one of the important approaches for artificial control of microbial metabolisms. Alteration of the membrane permeability can be achieved by multiple strategies, e.g. genetic engineering [8] or utilization of media additives [9]. Chemical additives are currently being used for multiple purposes. For example, Liu et al. [10] found that 0.05–0.1% (v/v) Tween-80 could improve extracellular thermophilic protease secretion by *Bacillus stearothermophilus* and a maximal 12.7% enhancement was achieved by addition of 0.1% Tween-80.

**Table 1**  
Results and analysis of orthogonal experiment.

No.	SDS (%)	Na <sup>+</sup> (mM)	Glycine (%)	Ca <sup>2+</sup> (mM)	α-CGTase activity (U/ml)
1	0.01	400	0.3	2.5	9.37
2	0.01	500	0.75	5	6.03
3	0.01	600	0.1	10	6.03
4	0.02	400	0.75	10	10.6
5	0.02	500	0.1	2.5	6.64
6	0.02	600	0.3	5	9.38
7	0.03	400	0.1	5	9.08
8	0.03	500	0.3	10	12.12
9	0.03	600	0.75	2.5	8.47
K <sub>1</sub>	21.43	29.05	30.87	24.48	
K <sub>2</sub>	26.62	24.79	25.1	23.88	
K <sub>3</sub>	29.67	23.88	21.75	28.75	
k <sub>1</sub>	7.14	9.68	10.29	8.16	
k <sub>2</sub>	8.87	8.26	8.37	7.96	
k <sub>3</sub>	9.89	7.96	7.25	9.53	
Error R	2.75	1.72	3.04	1.62	

Na<sup>+</sup> and Ca<sup>2+</sup> were added at the beginning of cultivation, glycine and SDS were added after cultivation of 16 and 20 h, respectively.

To achieve high production of recombinant α-CGTase from *P. macerans*, in our laboratory, the effect of SDS, Triton X-100, Tween-80, Tween-20, cetyltrimethyl ammonium bromide (CTAB), pronine, glycine, sucrose, sorbitol, Na<sup>+</sup>, Mg<sup>2+</sup> or Ca<sup>2+</sup> on the secretion of CGTase was investigated. It was found that, among all these chemicals, SDS, Na<sup>+</sup>, glycine and Ca<sup>2+</sup> could improve extracellular production of the enzyme. In the present study, the effect of these four chemical additives as well as their interaction on the extracellular secretion of α-CGTase was investigated in detail. In addition, the underlying mechanisms for the enhanced enzyme secretion were exploited.

## 2. Materials and methods

### 2.1. Materials

The recombinant plasmid *cgt/pET-20b(+)* carrying the mature *cgt* gene of *P. macerans* JFB05-01 and *E. coli* BL21(DE3) harboring plasmid *cgt/pET-20b(+)* were constructed previously by our laboratory. Peptone and yeast powder were obtained from Oxoid Ltd. Glycine (AR) and SDS were purchased from Shanghai Chemical Reagent Ltd. attached to China Medicine Group (Shanghai, China). o-Nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Beyotime Institute of Biotechnology (Nantong, China). N-phenyl-1-naphthylamine (NPN) was purchased from Aladdin-reagent Ltd. (Shanghai, China). Glucose-6-phosphate, NADP, NADPH, phosphatidylethanolamine and phosphatidylglycerol were purchased from Sigma-Aldrich (Milwaukee, WI, USA). All inorganic compounds were of reagent grade or higher quality.

### 2.2. Cultivation condition

A single colony of *E. coli* BL21 (DE3) cells harboring plasmid *cgt/pET-20b(+)* was inoculated into 10 ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin in a 25 ml flask and grown at 37 °C. The overnight culture was diluted (1:25) in 100 ml of terrific broth (TB) medium in a 500 ml flask and then incubated on a rotary shaker (200 r/min) at 25 °C. To investigate the effect of chemical additives on the growth of *E. coli* or extracellular secretion of recombinant α-CGTase, chemical additives were supplemented to the culture medium in the beginning of culture unless otherwise stated. At certain time intervals, samples were collected and analyzed for OD<sub>600</sub> and enzyme activities. Each value represents the mean of three independent measurements and varied from the mean by not more than 10%.

### 2.3. Orthogonal experiment

Based on the individual experiment of SDS, Na<sup>+</sup>, glycine and Ca<sup>2+</sup>, an L<sub>9</sub>(3<sup>4</sup>) orthogonal experiment was designed by using these four chemicals as parameters. The level of experimental factors and orthogonal experimental designs were summarized in Table 1.

### 2.4. Cell fractionation

Culture supernatants were obtained by centrifugation at 10,000 × g for 5 min at 4 °C and the supernatant was used as an extracellular fraction. To separate the periplasmic fraction, the bacterial pellets from 1 ml broth culture were washed twice by 30 mM Tris-HCl buffer (pH 7.0) and then completely resuspended in the same buffer containing 25% (w/v) sucrose and 1 mM EDTA. After incubation on ice for

2 h, the bacterial suspension was centrifuged at 10,000 × g for 5 min at 4 °C and the supernatant was used as a periplasmic fraction.

### 2.5. Assay of α-CGTase

The α-cyclodextrin forming activity was determined by the methyl orange (MO) method as described previously [11] with slight modifications. Briefly, 0.1 ml of the culture supernatant (appropriately diluted in 50 mM phosphate buffer) was incubated with 0.9 ml of 3% (w/v) soluble starch in 50 mM phosphate buffer (pH 6.0) at 40 °C for 10 min. The reaction was terminated by 1.0 M HCl (1.0 ml) followed by addition of 1.0 ml MO (0.1 mM) in 50 mM phosphate buffer (pH 6.0). After the reaction mixture was incubated at 16 °C for 20 min, the amount of α-cyclodextrin in the mixture was spectrophotometrically determined by measuring the absorbance at 505 nm. One unit of α-CGTase activity was defined as the amount of enzyme that was able to produce 1 mol of α-cyclodextrin in every minute under the test conditions described above.

### 2.6. Assay of glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was assayed as described previously [12] with some modifications. The total volume of the reaction mixture was 1.0 ml containing 1 mM glucose-6-phosphate, 0.075 mM NADP, 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl (pH 8.0) and appropriate culture supernatant. The production of NADPH was determined by measuring absorbance at 340 nm using a spectrophotometer at 25 °C. One unit of glucose-6-phosphate dehydrogenase (G6PDH) activity was defined as the amount of enzyme that was able to produce 1 μmol NADPH per minute under the test conditions described above.

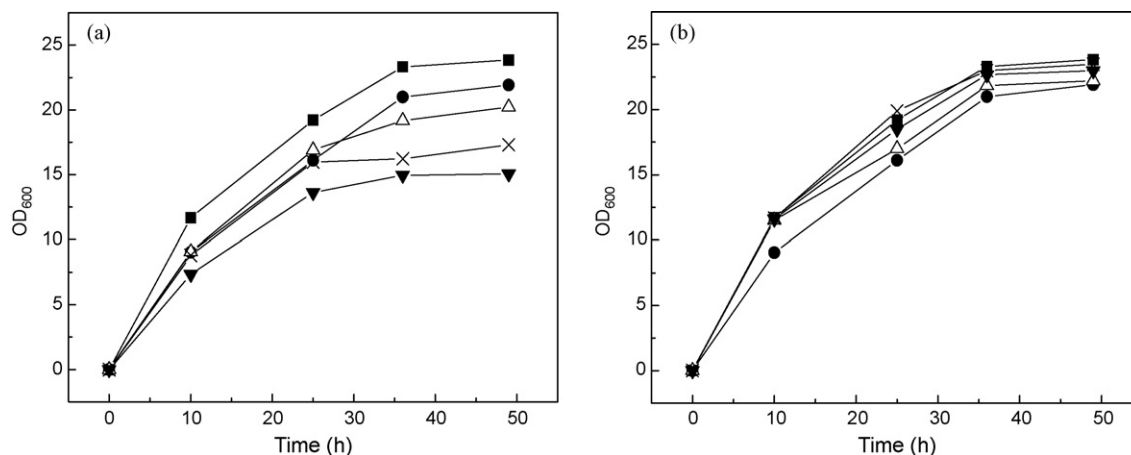
### 2.7. Assay of outer and inner membrane permeability

Outer membrane permeability was determined by using N-phenyl-1-naphthylamine access assay as described previously [13]. Briefly, *E. coli* cells were collected at different time points, centrifuged at 3000 × g and suspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD<sub>600</sub> of 0.5. NPN was added with a concentration of 10 μM into quartz cuvettes containing 2 ml of cell suspension. The sample was mixed by inverting the cuvette immediately prior to fluorescence monitoring. Fluorescence was measured using a 650-60 spectrofluorometer with slit widths set to 5 mm and excitation and emission wavelengths set to 350 and 428 nm, respectively.

Permeability of the inner membrane was assessed by measuring the access of o-nitrophenyl-β-D-galactoside to the cytoplasm as described previously [14]. Briefly, ONPG was added with a concentration of 100 μg/ml to the *E. coli* BL21 (DE3) suspension as described above. Substrate cleavage by β-galactosidase was determined by measuring OD<sub>420</sub> using a spectrophotometer.

### 2.8. Analysis of phospholipids in the cell membrane

The phospholipids were extracted as described previously [15] with slight modifications. Briefly, 30 ml of broth culture was centrifuged at 10,000 × g for 5 min and pellets were resuspended in 30 ml of 50 mM sodium phosphate buffer (pH 6.0). The sample was disrupted by ultrasonication with a SONFER. Two volumes of chloroform/methanol (2/1) were added and vortexed for 30 min. Two phases were separated by low-speed centrifugation (2500 × g). The lower phase was collected and mixed with 1/4 volume of methanol/water (1/1) solution. After centrifugation, the chloroform phase containing phospholipids was evaporated under vacuum in a rotary evaporator till the volume was below 2–3 ml. The remaining solution was dried under a nitrogen stream and redissolved in 1 ml chloroform. Phospholipids are



**Fig. 1.** The influence of SDS upon growth of *E. coli*. (a) The effect of SDS concentration on cell growth. *E. coli* cells were cultured in TB medium with 0.02% (●), 0.05% (△), 0.1% (×) or 0.15% (▼) SDS, respectively. The culture without SDS was used as control (■). (b) The effect of SDS addition time on cell growth. *E. coli* cells were cultured in TB medium with addition of 0.02% SDS at 0 h (●), 12 h (△), 20 h (▼) or 30 h (×) of cultivation. The culture without SDS was used as control (■).

separated by HPLC with the ELSP detector with the following operating conditions: the parameter of the column was Lichrosorb Si-60, 30 cm × 0.46 cm i.d., 5 μm; the mobile phase was chloroform/methanol/2% ammonium (80:16:4, v/v/v) with a flow speed of 1 ml/min; the column temperature was 30 °C.

### 2.9. Analysis fatty acids in the cell membrane

The fatty acids methyl esters were determined as described previously [16] with modifications. Briefly, 0.04 g phospholipids prepared above were dissolved in 0.5 ml benzene/petroleum ether (1:1, v/v), mixed with 2 ml 0.4 M KOH–methanol solution and heated at 50 °C for 15 min. After cooling at room temperature, the esterified fatty acids were extracted with 2 ml hexane. The organic phase containing esterified fatty acids was analyzed by Finnigan GC–MS 4610B on a 122-7032 DB-WAX 30M capillary column. Hydrogen was used as the carrier gas with a split ratio of 10:1. The temperature parameters were 180 °C for 0.5 min, 6 °C/min to 215 °C, 3 °C/min to 230 °C, and 230 °C for 12.5 min. The samples were analyzed by using an electron ionization mass selective detector with a 200 °C injector.

## 3. Results and discussion

### 3.1. Effect of SDS on the extracellular production of α-CGTase by *E. coli*

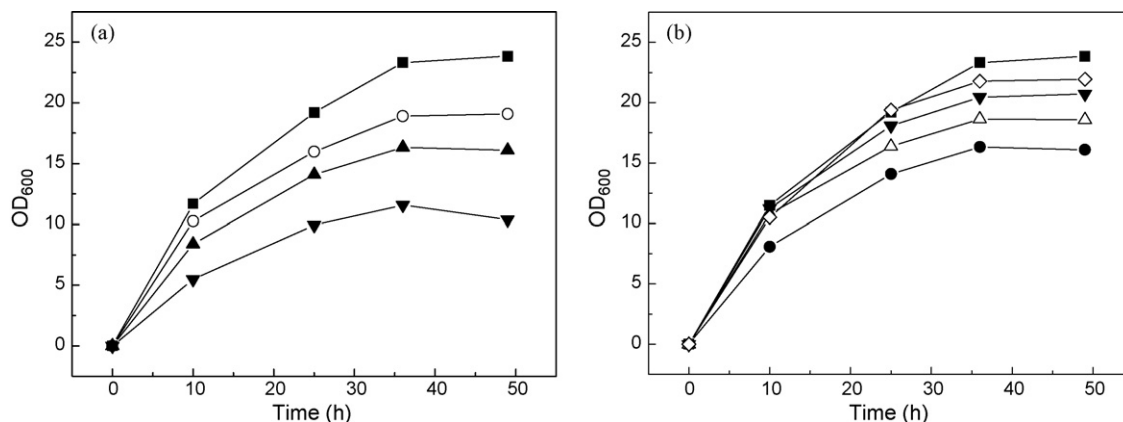
It has been reported that SDS, an anionic surfactant, is able to inhibit the biosynthesis of fatty acid, leading to the inadequate synthesis of membrane phospholipids and subsequent enhanced cell membrane permeability of *E. coli* [17]. In the present study, the effect of SDS at different concentrations on the growth of *E. coli* and

extracellular α-CGTase production was investigated. The results showed that SDS inhibited the growth of *E. coli* in a dose dependent manner (Fig. 1a). For example, the OD<sub>600</sub> of 36 h *E. coli* culture supplemented with 0.15% SDS was 15.2, which was significantly lower than that of the control without additives. However, extracellular enzymatic activity of α-CGTase for the *E. coli* culture with SDS additive was significantly higher than that of the control. The optimal concentration of SDS was determined to be 0.02% in which the extracellular α-CGTase activity reached as high as 3.93 U/ml, 4.62 times of that in the control culture.

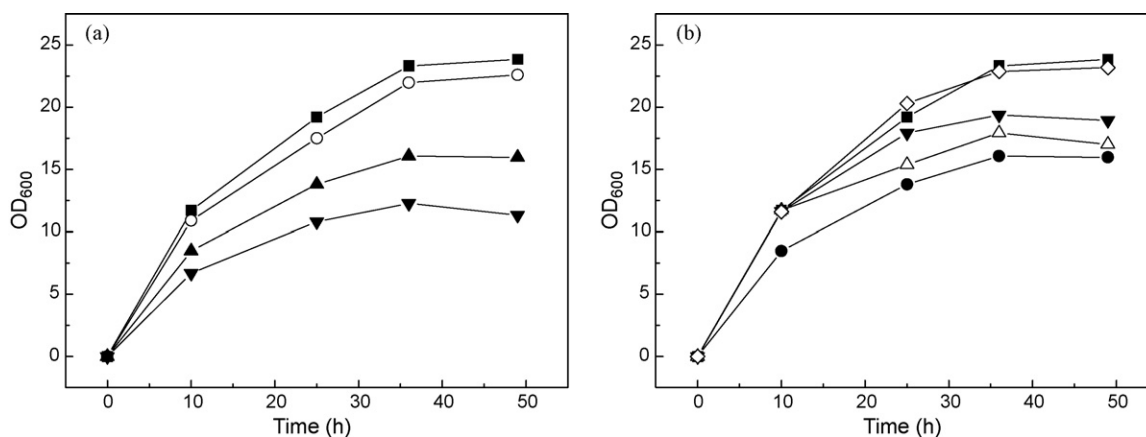
In addition, the effect of 0.02% SDS supplementation at different time points on the growth of *E. coli* and extracellular α-CGTase activity was determined (Fig. 1b). The inhibitory role of SDS supplementation on cell growth at later time points was decreased compared to that at earlier time points. For example, the OD<sub>600</sub> for the culture with SDS addition at 30 h of cultivation was similar to that for the control culture without SDS additive. The highest extracellular α-CGTase activity of 5.34 U/ml was obtained at the condition when SDS was supplemented at 20 h of cultivation.

### 3.2. Effect of glycine on the extracellular production of α-CGTase by *E. coli*

Although the effect of glycine on extracellular production of α-CGTase by *E. coli* has been previously studied by our laboratory [18], the similar experiment was also performed in order to have



**Fig. 2.** The influence of glycine upon growth of *E. coli*. (a) The effect of glycine concentration on cell growth. *E. coli* cells were cultured in TB medium with 0.3% (○), 0.75% (▲) or 1% (▼) glycine, respectively. The culture without glycine was used as control (■). (b) The effect of glycine addition time on cell growth. *E. coli* cells were cultured in TB medium with addition of 0.75% glycine at 0 h (●), 10 h (△), 16 h (▼) or 30 h (◇) of cultivation. The culture without glycine was used as control (■).



**Fig. 3.** The influence of Na<sup>+</sup> upon growth of *E. coli*. (a) The effect of Na<sup>+</sup> concentration on cell growth. *E. coli* cells were cultured in TB medium with 100 mM (○), 500 mM (▲) or 700 mM (▼) Na<sup>+</sup>, respectively. The culture without Na<sup>+</sup> was used as control (■). (b) The effect of Na<sup>+</sup> addition time on cell growth. *E. coli* cells were cultured in TB medium with addition of 500 mM Na<sup>+</sup> at 0 h (●), 10 h (△), 20 h (▼) or 30 h (◇) of cultivation. The culture without Na<sup>+</sup> was used as control (■).

a better comparison among all the chemical additives utilized in the present study. Consistent with previous study, supplementation of the culture with glycine inhibited bacterial growth (Fig. 2a). The OD<sub>600</sub> for the 40 h culture was decreased approximately 19%, 30% and 50%, respectively, when 0.3%, 0.75% and 1% glycine was supplied. However, extracellular enzymatic activity of  $\alpha$ -CGTase for the *E. coli* culture supplemented with glycine was significantly higher than that for the control without glycine addition. The enzymatic activity in the culture supplemented with 1% glycine reached 6.87 U/ml, which was 7.08 times higher than that in the control without glycine. The enhanced secretion of recombinant enzymes from *E. coli* by glycine has also been reported by other investigators [19]. The potential mechanism was glycine-induced modification of peptidoglycan structure in the cell wall [12], which resulted in a marked increase of cell membrane permeability.

Although maximal extracellular  $\alpha$ -CGTase activity was achieved when 1% glycine was supplied, the largest inhibition of bacterial growth was also obtained; therefore, 0.75% glycine was selected as optimal concentration to study the glycine addition timing on the effect of cell growth and extracellular enzyme secretion. The results showed that supplementation with glycine at later time points had less inhibition on the cell growth (Fig. 2b). The extracellular  $\alpha$ -CGTase activity in 40 h culture supplemented with glycine after 16 h of cultivation reached 8.2 U/ml, which was 9.65 times higher than that in the control sample without addition of glycine.

### 3.3. Effect of Ca<sup>2+</sup> on the extracellular production of $\alpha$ -CGTase by *E. coli*

Metal ions are the essential factor for cell growth and enzymatic activity. Previously, it was found that high concentration of Ca<sup>2+</sup> (20 mM) could promote the growth and survival of *E. coli* cells grown in the culture medium rich in glycine, leading to a further increase in the glycine-induced extracellular secretion of the recombinant  $\alpha$ -CGTase [20]. In the present study, the effect of Ca<sup>2+</sup> alone on *E. coli* growth and extracellular  $\alpha$ -CGTase activity was determined in detail. The results showed that the OD<sub>600</sub> of the culture at stationary phase was 23.5, 24, 27, 24.7, and 23.9, respectively, when 0, 1, 2.5, 3 and 5 mM Ca<sup>2+</sup> was supplied, indicating that Ca<sup>2+</sup> (1–5 mM) slightly increased the growth of *E. coli*. The reason for this promotion has been speculated to be that Ca<sup>2+</sup> might stabilize the lipopolysaccharide in the outer membrane [21] and help to maintain the orderly nature of the cell membrane [12]. In addition, Ca<sup>2+</sup> can reduce the hydrolysis of peptidoglycan by peptidoglycan hydrolase and has been often used to control the process of cell autolysis [12].

Beside the promotion of cell growth, addition of Ca<sup>2+</sup> in the medium slightly enhanced the extracellular  $\alpha$ -CGTase activity. When the cultivation was supplemented with 2.5 mM Ca<sup>2+</sup>, the extracellular activity of  $\alpha$ -CGTase reached 1.12 U/ml, which was 1.3 times of that in the control culture without addition of Ca<sup>2+</sup>.

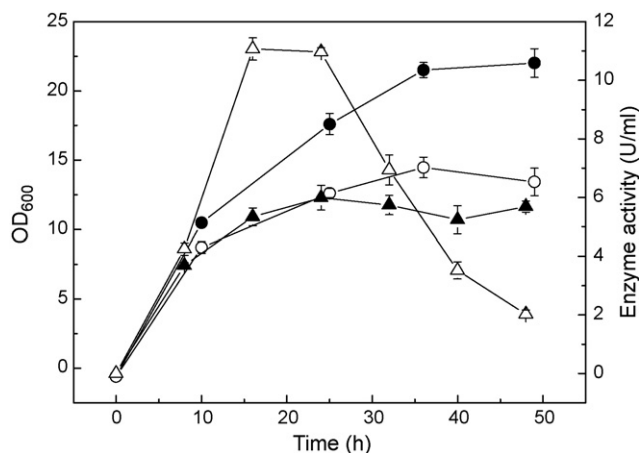
### 3.4. Effect of Na<sup>+</sup> on the extracellular production of $\alpha$ -CGTase by *E. coli*

Since osmoregulation is an important biological processes by which it can prevent the cells from dehydration, studies on the osmotic regulation is significant for the industrial production of enzymes [22]. Na<sup>+</sup> is a commonly used osmoticum. In the present study, the effect of Na<sup>+</sup> on *E. coli* growth and extracellular production of  $\alpha$ -CGTase were investigated. The results showed that supplementation of low concentration (100 mM) of Na<sup>+</sup> had almost no effect on bacterial growth (Fig. 3a); however, high concentration (500 mM or more) of Na<sup>+</sup> significantly inhibited the cell growth. When 500 or 700 mM Na<sup>+</sup> was supplied, the OD<sub>600</sub> of bacteria culture at the stationary phase was 16.08 and 12.26, respectively, which accounted for 68.92% and 52.55% of that in control culture without addition of Na<sup>+</sup>. On the contrast, addition of high concentration of Na<sup>+</sup> markedly increased the production of extracellular  $\alpha$ -CGTase. The optimal concentration of Na<sup>+</sup> was determined to be 500 mM. In this condition, after 40 h of cultivation, the activity of  $\alpha$ -CGTase reached 3.6 U/ml, which was 4.2 times of that in control culture without addition of Na<sup>+</sup>.

Since addition of 500 mM Na<sup>+</sup> into the culture medium significantly inhibited *E. coli* growth, the effect of its addition timing on the bacterial growth and extracellular enzyme production was investigated. The results showed that, when 500 mM Na<sup>+</sup> was supplied after 0, 10, 20 and 30 h of bacterial cultivation, the OD<sub>600</sub> of the culture at stationary phase was 16.08, 17.2, 18 and 22.8, and the extracellular  $\alpha$ -CGTase activity was 3.6, 2.8, 2.13, and 1.9 U/ml, respectively (Fig. 3b). These results suggested that addition of Na<sup>+</sup> at later time points relieved the inhibition of bacterial growth, but reduced the extracellular production of  $\alpha$ -CGTase. Therefore, it was suggested to supplement the culture medium with 500 mM Na<sup>+</sup> in the beginning of the cultivation process to achieve maximal  $\alpha$ -CGTase secretion.

### 3.5. Orthogonal experiments among SDS, Na<sup>+</sup>, glycine and Ca<sup>2+</sup>

In order to investigate the potential interaction effect among SDS, Na<sup>+</sup>, glycine and Ca<sup>2+</sup> on enzyme secretion, an orthogonal experiment L<sub>9</sub>(3<sup>4</sup>) was designed based on the results of



**Fig. 4.** Cell growth and  $\alpha$ -CGTase production by recombinant *E. coli* cultured in the optimized media supplementation condition. The culture without additives was used as control. Growth curve: (●) the culture without additives, (○) the culture under the optimized conditions. Periplasmic  $\alpha$ -CGTase activity: (▲) the culture without additives, (△) the culture under the optimized conditions.

above single-factor analysis. The level of experimental factors, orthogonal experimental designs and results were summarized in Table 1.

As shown from Table 1, the major factor affecting the extracellular production of  $\alpha$ -CGTase in *E. coli* was glycine, followed by SDS, while  $\text{Ca}^{2+}$  had a minimal effect. Based on the results from orthogonal experiments, the optimal condition to achieve maximal extracellular production of  $\alpha$ -CGTase was supplementation the culture with 0.03% SDS, 400 mM  $\text{Na}^+$ , 0.3% glycine and 10 mM  $\text{Ca}^+$  together.

This optimum condition was further experimentally confirmed. The OD<sub>600</sub> in the culture of this condition was significantly lower than that in the control without additives (Fig. 4), while the extracellular  $\alpha$ -CGTase activity in the culture media was significantly improved. After 40 h of cultivation under optimal condition, the extracellular  $\alpha$ -CGTase activity reached 12.89 U/ml, while the extracellular  $\alpha$ -CGTase activity under the control condition was only 0.81 U/ml.

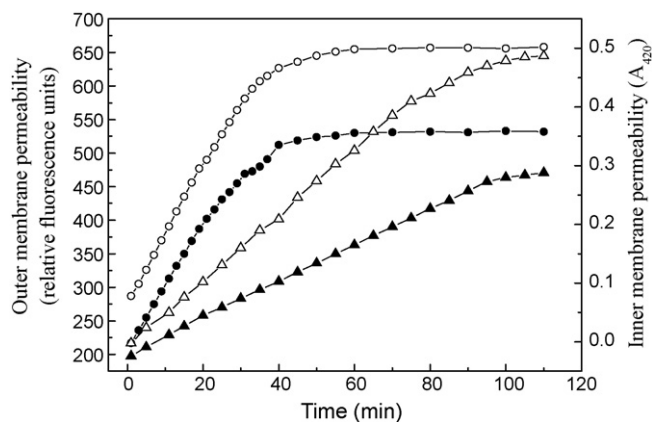
In addition, it was observed that, during the first 30 h of cultivation, the periplasmic  $\alpha$ -CGTase activity of bacteria grown under the optimal condition was higher than that under control condition (Fig. 4), while after 30 h of cultivation, it was turned to be lower than the control. These results suggested that periplasmically accumulated  $\alpha$ -CGTase in the optimized condition was secreted into the culture medium more quickly than that in the control condition.

It has been reported that the level of glucose-6-phosphate dehydrogenase, an intracellular protein, in the culture medium reflected the degree of cell autolysis [12]. In order to exclude the possibility that the increased extracellular activity of  $\alpha$ -CGTase was due to the bacterial autolysis, the activity of G6PDH in the culture medium was examined. The result showed that, after 40 h of cultivation, G6PDH activity in culture medium of the optimal condition and control condition was very low, both were around 0.009 U/ml. Thus, it seems like that there was no significant cell autolysis after supplementation with additives.

### 3.6. Potential mechanisms of the enhanced extracellular $\alpha$ -CGTase activity by additives

#### 3.6.1. Effects of additives on cell membrane permeability

In order to elucidate the mechanism of the increased extracellular production of  $\alpha$ -CGTase by supplementation with additives in recombinant *E. coli* cells, we quantitatively determined the perme-



**Fig. 5.** Concurrent measurements of *E. coli* outer and inner cell membrane permeability. Outer cell membrane permeability: (●) the culture without additives, (○) the culture under the optimized conditions. Inner cell membrane permeability: (▲) the culture without additives, (△) the culture under the optimized conditions.

ability of the inner and outer membrane of recombinant *E. coli* cells cultured under an optimal and control conditions.

Hydrophobic fluorescent probe NPN was used to monitor the integrity of the outer membrane, while access of ONPG to the bacterial cells was used as a probe to detect the permeability of the inner membrane. The results showed that both the outer and inner membrane permeability of bacterial cells grown under optimal condition was higher than that under control condition without additives (Fig. 5).

#### 3.6.2. Effects of additives on the phospholipid composition in the cell membrane

Phospholipids are the main component of cell membrane. As the structure and functional unit of cell membrane, a small variation of its content and ratio may change the permeability and fluidity of cell membrane [23,24]. *E. coli* membrane phospholipids are mainly composed of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and a small amount of phosphatidylcholine (PC). In the present study, in order to have a better comparison, the PG content under the optimal and control condition were measured in parallel for several times. The results showed that, in each measurement, the amount of PG under the optimal condition was higher than that in control condition, the mean value of 20.3% under optimal condition and 18.2% under control condition were obtained. In addition, the PE content of bacterial cells grown under optimal condition was 75.8%, while it was 71% under control condition (Table 2). It has been reported that anionic phospholipids are in favor of the translocation of proteins into extracellular milieu, which may be related with SecA, an important translocation chaperone for the secretion of proteins [25]. Breukink et al. [26] found that SecA has stronger binding ability with the monolayer membrane composed of the anionic phospholipids than that composed of dual ionic phospholipids. Ulbrandt et al. [27] also found that the amount of SecA bond to the membrane increased significantly when anionic phospholipids exist. In the cell membrane, PE has dual ionic characteristics and does not have any net charge in the physiological pH condition, while PG carries negative charges. In the present study, the

**Table 2**  
Analysis of phospholipids composition of cell membrane lipid of recombinant *E. coli*.

	Phospholipids (%)		
	PE	PG	Other
Control	75.8	18.2	6
Sample	71	20.3	8.2

**Table 3**  
Analysis of fatty acids composition of cell membrane lipid of recombinant *E. coli*.

Type of fatty acids	Control		Sample	
	Peak time (min)	Content of FA (%)	Peak time (min)	Content of FA (%)
C12:0	3.04	0.36	3.04	0.32
C14:0	4.25	4.94	4.24	1.93
C14:1	5.28	0.29	5.28	0.11
C15:0	5.05	0.17	5.05	0.23
C16:0	5.98	28.89	5.99	23.44
C16:1	6.28	1.3	6.28	2.34
C17:0	7.44	27.45	7.44	26.11
C18:0	8.26	0.92	8.26	1.4
C18:1	8.67	13.41	8.67	20.85
C19:1	10.18	22.28	10.19	23.27
Unsaturation ratio	0.37		0.47	

The number after C represents carbon numbers of fatty acyl, the number after colon represents the unsaturated fatty acids.

increase of negative charged PG in cell membrane of *E. coli* cultivated under optimal condition may favor the binding of SecA with cell membrane, which resulted in the enhanced secretion of enzyme.

On the other hand, it has also been reported that there is an interaction between PG and the target protein precursor, especially its signal peptide, PelB [25]. Signal peptide contains a positively charged N-terminus, a hydrophobic core consisting of 7–15 residues, which can pass through the membrane hydrophobic center, and a polar C-terminus. Demel et al. [28] replaced the positively charged N-terminal domain with negatively charged N-terminus in the signal peptide and found the trans-membrane efficiency was greatly reduced. These results indicated the importance of the interaction between positively charged N-terminus in the signal peptide of targeted protein and cell membrane for the secretion of target proteins. In the present study, the increase of negatively charged PG in the cell membrane of *E. coli* cultivated under optimal condition may favor the interaction between precursor of  $\alpha$ -CGTase and the cell membrane, thereby enhancing the translocation of  $\alpha$ -CGTase.

### 3.6.3. Effects of additives on the fatty acid composition in the cell membrane

The length and saturation degree of fatty acyl of membrane lipid is one of the most important factors affecting the fluidity and integrity of the cell membrane [29]. To understand the changes of compositions of membrane fatty acids in *E. coli* under the optimal and control cultivation conditions, GC–MS analysis of membrane fatty acids was performed. In the results of GC–MS chromatography, 10 peaks of fatty acids were observed. Because different fatty acids are homologues, the order of peaks in the chromatograph was in accordance with the length of the carbon chain and regularity. Each fatty acid peak was then analyzed qualitatively by comparing with NIST spectral library and using of area-method to determine its relative content. The results were summarized in Table 3.

The percentage of fatty acid C16:1, C18:1, and C19:1 in the *E. coli* cells was increased from 1.3%, 13.41%, and 2.28% in those grown under control condition to 2.34%, 20.85% and 23.27%, respectively; while the percentage of fatty acid C14:1 and C17:0 was reduced from 0.29% and 27.45 to 0.11% and 26.11%, respectively. These alterations resulted in the complete change of the ratio between saturated fatty acids and unsaturated fatty acids in the membrane lipids. The unsaturation ratio of fatty acids in the cell membrane was increased from 37% in the *E. coli* grown under control condition to 47% in that under optimal condition. Therefore, the mobility of cell membrane in the *E. coli* grown under optimal condition was increased, the rigidity of cell membrane was reduced, and the permeability of cell membrane was enhanced, resulting in the enhanced extracellular production of recombinant  $\alpha$ -CGTase.

## 4. Conclusion

In this study, the effects of different additives on the extracellular production of  $\alpha$ -CGTase were investigated. Addition of SDS, glycine, calcium ions and sodium ions can increase the extracellular yield of  $\alpha$ -CGTase. The optimal condition to achieve maximal secretion of  $\alpha$ -CGTase was determined to be the supplementation of the culture with 0.03% SDS, 400 mM Na<sup>+</sup>, 0.3% glycine and 10 mM Ca<sup>2+</sup>. Under this optimal condition, the permeability, fluidity and membrane content of PG of *E. coli* cell membrane were increased and thus, those  $\alpha$ -CGTases accumulated in the cytoplasm and the periplasmic compartment may be more easily secreted into the medium, resulting in a high level of extracellular enzymatic activity.

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