Improved extracellular secretion of β -cyclodextrin glycosyltransferase from *Escherichia coli* by glycine supplementation without apparent cell lysis

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Abstract. The use of an effective inducer feeding strategy without causing cell lysis presents significant advantage to enhance the secretion of an enzyme to the culture medium of *Escherichia coli*. The *cgt* gene encoding β -cyclodextrin glycosyltransferase (β -CGTase) was cloned into pQE30xa as an N-terminal His-tagged protein and transformed into *E. coli*. The induction strategy was applied towards enhancing the extracellular secretion of the recombinant β -CGTase by increasing permeability of the outer membrane of *E. coli*. The supplementation of 1.2 mM glycine following 2 h of fermentation at 37°C enhanced the activity of β -CGTase to 38.295 U/mL, which was approximately 1.3-fold higher than the control (without induction). Further flow cytometry analysis was adopted as a rapid and highly reproducible approach to determine the effect of glycine supplementation on the viability of *E. coli* cells. The supplementation of glycine did not contribute to apparent cell lysis, with no adverse effects on cell viability, hence indicating the effectiveness of glycine in enhancing the extracellular secretion of β -CGTase. The recombinant β -CGTase was then purified through a combination of diafiltration and Ni-NTA affinity chromatography with 18.4-fold increase in purity. An effective glycine feeding strategy could enhance the extracellular secretion of β -CGTase without adverse effects on cell viability. This strategy could be applied potentially to enhance the secretion of a recombinant protein to the culture medium from *E. coli* cells without having cell lysis.

Keywords: cell viability, cyclodextrin glycosyltransferase, extracellular secretion, glycine, inducer, membrane permeability

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) is a member of the α -amylase family, also known as glycosyl-hydrolases which catalyse the formation of cyclodextrins (CDs) from starch

(Han *et al.*, 2014). Among the well-characterised CDs which are α -, β - and γ -CDs, β -CD has been extensively used due to its hydrophobic internal cavity and hydrophilic outer surface which provides water soluble properties (Wang *et al.*,

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2017). The predominant production of β -CD is of interest that can reduce the time and cost of purification (Al-Sharawi *et al.*, 2013; Cheirsilp *et al.*, 2010). β -CD has been frequently employed to form inclusion complexes with a variety of guest molecules, for instance to encapsulate antimicrobial compounds for the development of antimicrobial active packaging materials (Abarca *et al.*, 2016), development of CD nanoparticles to be used in drug delivery systems (Lakkakula and Maçedo Krause, 2014), development of CD-based nanosponges to be used as innovative carriers in cosmetics, pharmaceutical, agrochemistry and environmental protection (Atul and Bhushan, 2017) and many others.

However, large amounts of β -CGTase are necessary for an effective production of β -CD through enzymatic synthesis. The low specific activity of the β -CGTase synthesised by the natural host cells is the main drawback in the production of CD at a larger scale. Therefore, the need for enhancement of the CGTase properties has become increasingly necessary to improve its quantity and quality for efficient use in industrial applications. According to Qi and Zimmermann (2005), about 50% of commercialised CGTases were produced through genetic engineering approach. Although a lot of progress has been made to overproduce the enzyme mainly through the development of secretory expression system in *E. coli* as the common expression platform (Zhou *et al.*, 2018), further research on the enhancement of extracellular secretion of CGTase is needed to improve CD production.

The use of *E. coli* as a cell factory is preferred as the genetic properties are well established, simple, inexpensive and fast high-density cultivation (Sørensen and Mortensen, 2005). However, the formation of inclusion bodies, poor growth of host and protein inactivity (Rosano and Ceccarelli, 2014) are the critical issues that need to be tackled in order to produce the recombinant enzymes from *E. coli* effectively. The formation of inclusion bodies could block the translocation channels of pre-proteins and eventually delaying protein secretion (Li *et al.*, 2014). An inadequate release of β -CGTase across two cell membranes of *E. coli* is the main challenge in order to achieve higher enzyme secretion (Li *et al.*, 2010). Therefore, it was suggested that a balance between the rate of pre-protein synthesis and

translocation needs to be maintained for an efficient secretion of extracellular enzyme from *E. coli*.

The optimization of the cultivation process has been frequently employed to increase the level of extracellular production of CGTase (Cheng *et al.*, 2011). The use of inducers presents significant advantages in modifying the integrity of the cell wall of *E. coli*, hence promoting the secretion of recombinant enzyme to the culture medium. The extracellular secretion of the recombinant enzyme from *E. coli* is preferred over periplasmic and cytoplasmic expression, mainly due to high expression level, the benefit of simple and inexpensive downstream processing (Tesfai *et al.*, 2012), substantial yields of correctly formed proteins and to minimise and avoid protease attack (Choi and Lee, 2004). In the present study, we emphasised on the optimisation of the inducer feeding strategy and attempted to enhance the secretion of recombinant β -CGTase to the culture medium by adding glycine, Triton X-100 or xylose. This study was also intended to address the effect of inducer supplementation on the cell viability where healthy cell growth is required to enhance the secretion of β -CGTase.

MATERIALS AND METHODS

Bacterial strain and plasmid. *E. coli* JM109 harbouring pTZCGT-BS from previous study (Ramli *et al.*, 2013) was used for sub-cloning into pQE30xa. *E. coli* JM109 [*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (rk-,mk+), *relA1*, *supE44*, D (*lac-proAB*), F⁺ (*tra* D36, *pro* AB, *lacIqZ* Δ M15)] from Promega (Madison, WI) was used as a host strain that is ideal for propagating pQE plasmids and used as an expression host. Plasmid pQE30xa from QIAGEN (Germany) was used as an expression vector.

Construction of β -CGTase expression system. The amplified PCR fragment encoding the β -CGTase gene was ligated into pQE30xa vector, designated as pQECGT-BS. The amplification of β -CGTase gene was performed under standard PCR conditions with 1 μ g of genomic DNA, 200 μ mol of each forward and reverse primer, 0.01 μ L of *Taq* DNA polymerase in 1 $\times$ reaction buffer and 0.2 mM of dNTPs in a final volume of 20 μ L. The specific primers designed for

amplification of CGTase used for construction of pQECGT-BS expression system were ProB 5'-TTA GGA TCC TTG CTC TAG ATT GAT CAA CCA-3' and P2 5'-GTC AAG CTT TTA CCA ATT AAT CAT AAC CGT-3' for forward and reverse primer, respectively. The forward and reverse primers were designed to contain the restriction site *Bam*HI and *Hind*III (underlined), respectively. The amplified DNA fragment was purified after agarose gel electrophoresis using Hi Yield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan) and digested with *Bam*HI and *Hind*III before ligation into pQE30xa. To study the expression levels, the ligation products were subsequently introduced into *E. coli* JM109. Luria-Bertani (LB)-ampicillin (100 μ g/mL) was used to plate out the transformation mixtures. After overnight growth at 37°C, colonies were chosen for insert confirmation.

β -CGTase expression in *E. coli*. Recombinant *E. coli* JM109 harbouring pQECGT-BS was inoculated into LB broth containing 100 μ g/mL ampicillin and incubated overnight at 37°C in an incubator shaker agitated at 200 rpm until an optical density of 1.5 at 600 nm was obtained. Terrific Broth (TB) supplemented with 100 μ g/mL ampicillin was used as a production medium for the preparation of crude recombinant β -CGTase. The culture was incubated at 37°C, agitated at 200 rpm for 24 h and sampled every 4 h. Cell-free supernatant was collected by centrifuging at 10000 rpm at 4°C for 10 min to determine the profile of enzyme expression. For estimation of cell growth, the pellets were appropriately diluted and the optical density was measured at OD₆₀₀ nm.

Effects of the induction strategies on β -CGTase production. TB media supplemented with either glycine, Triton X-100 or xylose were used to investigate the effects of inducers on the extracellular secretion of the recombinant β -CGTase. Furthermore, the effects of selected inducer supplementation time (0, 1, 2, 3 and 4 h), supplement concentrations (0.1, 0.4, 0.8, 1.2 and 1.6 mM) and post-induction temperatures (25, 30, 47 and 40°C) were determined. The experiments were carried out in triplicates.

Flow cytometry sample preparation. The culture broth was diluted at least 1:10 (v/v) in order to obtain a measurement range of less than 1000 cells/second (Falcioni *et al.*, 2008). The diluted broth was filtered using 40 μ m Nylon cell

strainer (Falcon, NJ, USA) to minimise the possibility of causing a clog in the flow cytometer, followed by vortex for at least five min.

Fluorescent probes and staining procedure. A nucleic acid double staining assay (Barbesti *et al.*, 2000) was applied to distinguish between the populations of viable and dead cells. A 42 μ mol/L of thiazole orange diluted in dimethyl sulfoxide and 4.3 mmol/L propidium iodide diluted in distilled water (BD™ Cell Viability Kit) were used. The unstained samples were prepared as a control to eliminate the assay background noise. The stained samples were instead prepared by adding 5 μ L of each dye into 500 μ L of cell suspension to achieve a final concentration of 43 μ mol/L for propidium iodide and 420 nmol/L for thiazole orange, respectively. Further to that, both stained and unstained samples were added with 5 μ L of EDTA (1 mM, pH 8). The staining process was carried out following the manufacturer's protocol of the BD™ Cell Viability Kit (Cat. No. 349483, Becton Dickinson, San Jose, CA). The samples were incubated in an ice box on an orbital shaker for one hour. A control for the gating process was prepared by heat treatment (Sharuddin *et al.*, 2018) whereby 50 mL of the samples were heated at 80 to 90°C in a water bath for 45 min.

Flow cytometric analyses. The flow cytometric analyses were done using a BD Accuri® C6 cytometer (Becton Dickinson UK Ltd., Oxford, UK) equipped with red (640 nm) and blue (488 nm) lasers, forward-scattered (FSC) and side-scattered light (SSC) and four fluorescence detectors. The propidium iodide fluorescence was collected at the FL3 channel, while the thiazole orange fluorescence was collected at the FL1 channel, with 20,000 cells counted for each sample. The differentiation between viable and dead cells was done through a gating on a dot plot of red versus green fluorescence after excluding the auto-fluorescence. The visualisation of the plots and the gating process were done using the built in CFlow Plus software. The repeated measurements Analysis of Variance (ANOVA) were conducted to test for significant differences of viable and dead cells between no induction, 0 h and 2 h induction with glycine.

Purification of β -CGTase. The crude β -CGTase was washed with equilibrium buffer (10 mM imidazole) with molecular weight cut-off (MWCO) of 30 kDa using AKTA Flux tangential flow filtration system (GE Healthcare, USA). Affinity purification was performed on a Ni-NTA column on an AKTA Avant chromatography system (GE Healthcare, USA) according to the manufacturer's instructions. The sample was incubated for 30 min in the column, followed by washing with buffer containing 20 mM imidazole (pH 8.0). Elution buffer containing 250 mM imidazole (pH 8.0) was used to elute the bound enzyme. The fractions with maximum activity were pooled together and concentrated using Amicon ultrafiltration membrane kit (30 kDa MWCO membrane). The samples were assayed for β -CGTase activity and protein concentration.

Determination of protein concentration and size. Protein concentration was measured using Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as a standard. The protein size was analysed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at constant voltage of 100 V for 1.5 h at room temperature until the band was migrated sufficiently. Gel (8.3 cm X 7.3 cm) was run according to the method of Laemmli (1970). The concentrations of the stacking and resolving gels were 5% and 12%, respectively. Appropriately diluted protein samples (30 μ g) with 4 $\times$ marker containing 50 mM Tris–HCl (pH 6.8), 40% (w/v) of glycerol, 10% (w/v) of SDS, 5% (v/v) of mercaptoethanol and 0.05% (w/v) of bromophenol blue were boiled for 5 min before applied into each lane. The lane was formed by a 10-well comb (Mini-PROTEAN®) and the molecular weight marker (PageRuler™ Unstained Protein Ladder) from Thermo Scientific, USA was used as a standard. The gel was stained with Coomassie Brilliant Blue R-250.

β -CGTase activity. The β -CGTase activity was measured using phenolphthalein assay (Kaneko *et al.*, 1987). Reaction mixture containing 1 mL of 40 mg of soluble starch in 100 mM phosphate buffer (pH 6.0) and 0.1 mL of β -CGTase solution was incubated at 60°C for 10 min in a water bath. The reaction was stopped by the addition of 3.5 mL of 30 mM NaOH. Subsequently, 0.5 mL of 0.02%

(w/v) phenolphthalein in 5 mM Na₂CO₃ solution was added to the reaction mixture and mixed well. After 15 min, the reduction in colour intensity was measured at 550 nm. Blank lacking the CGTase was analysed simultaneously with each batch of samples. As a standard, the soluble starch and enzyme were replaced with 1 mL of β -CD solution and 0.1 mL of water, respectively. A calibration curve was made using 0–0.5 mg/mL of β -CD in 100 mM phosphate buffer, pH 6.0. One unit of enzyme activity was defined as the amount of enzyme that formed 1 μ mol of β -CD per min under the conditions defined above.

Production of cyclodextrin and kinetic parameters. The kinetic parameters, K_m and V_{max} of the recombinant β -CGTase were analysed by linear regression with the linear transformation (Hanes-Woolf plot) of the Michaelis-Menten equation using an Excel curve-fitting program (Microsoft Excel 2010, San Francisco, CA, USA). The K_m and V_{max} values for the recombinant β -CGTase were determined by incubating 0.5 U/mL of β -CGTase with 2–10 mg/mL of soluble starch at 60°C for 10 min in 1 mL of 0.1 M phosphate buffer (according to standard β -CGTase assay) (Kaneko *et al.*, 1987). The concentrations of β -CD were determined by high performance liquid chromatography (HPLC) using a Lichrospher 100 NH₂ column (Merck, USA) eluted with 65:35 acetonitrile:water mixture at 1 mL/min. β -CD was detected by a refractive index detector (Shimadzu, Japan). The experiments were carried out in triplicates and standard deviations were calculated.

RESULTS AND DISCUSSION

Construction of recombinant plasmid and production of β -CGTase. The *egt* gene was constructed in pQE30xa and in frame with 6x-His tag to assist in protein purification using affinity chromatography. The expression vector was successfully constructed and verified by sequencing before it was transformed into *E. coli* JM109. The resulting strains exhibited 30.371 U/mL of β -CGTase activity from the supernatant without any inducer (Figure 1), a 2.6-fold increment of β -CGTase activity as compared to

wild type producer (Ramli *et al.*, 2010). The same *cgt* gene was previously constructed in pTZ57R/T and expressed in *E. coli* JM 109 with the activity of 37.480 U/mL (Ramli *et al.*, 2013). A low enzyme activity detected in the culture medium of *E. coli* without inducer might be due to the accumulation of protein in the periplasmic space (Li *et al.*, 2010). However, the disruption of outer membrane to recover the target protein is not preferred as it may cause the intracellular proteolysis by periplasmic proteases (Choi and Lee, 2004). Moreover, it could also result in cell death and injury, for instance by introducing high pressure to *E. coli* cells to recover proteins (Hauben *et al.*, 1996). The disruption of outer membrane of *E. coli* for the recombinant protein production needs to be handled with care so as not to compromise cellular integrity.

Effects of inducers on extracellular secretion of β -CGTase. Three types of inducers were selected to enhance the extracellular secretion of β -CGTase from *E. coli* which were glycine, Triton X-100 and xylose, in comparison to control culture without any inducer. The glycine, Triton X-100 and xylose were known to have different effects on the integrity of the cell wall of *E. coli* (Li *et al.*, 2014, 2010; Lo *et al.*, 2007). The supplementation of inducers during cultivation offers advantages as compared to the physical methods including osmotic shock, sonication and freezing and thawing where they can only be applied after harvesting cells. Therefore, by adopting the inducer feeding strategy, the additional steps of recombinant proteins recovery after the fermentation process are not necessary.

The induction by glycine increased the extracellular secretion of β -CGTase activity at 12 h of fermentation by 1.1-fold, while the Triton X-100 and xylose induction showed negative effects (Figure 1A). ANOVA test was conducted in this study and demonstrated significant differences of β -CGTase activities between the use of different types of inducers at a confidence level of 0.05 ($p < 0.05$). Glycine's capability to act as a permeability enhancer, resulted in the increased activity of recombinant β -CGTase. The glycine could replace the alanine residues in the peptide component of the peptidoglycan of *E. coli* cell wall, resulting in a more loosely cross-linked peptidoglycan and therefore, enhancing the

permeability of outer membrane of *E. coli* (Yang *et al.*, 2017). The glycine was previously shown to increase the secretion of extracellular α -CGTase from *E. coli* by 11-fold to achieve maximum activity of 28.5 U/mL (Li *et al.*, 2010). However, the supplementation of Triton X-100 and sucrose might have compromised the cellular integrity and the growth of *E. coli*, which resulted in the reduced activity of extracellular β -CGTase. The glycine was thus selected to be used in the optimisation of inducer feeding strategy by single factor design to further increase the extracellular secretion of β -CGTase.

Effect of glycine feeding strategy on extracellular secretion of β -CGTase. The effect of glycine supplement time was conducted to enhance the extracellular secretion of recombinant β -CGTase from *E. coli* (Figure 1B). The addition of 0.1 M glycine at 2 h fermentation has resulted in the 1.1-fold increment of enzyme activity at 12 h of incubation as compared to the induction at the beginning of the culture. The induction at 2 h during early log phase might have increase the leakage of periplasmic proteins to the culture medium, which resulted in the increased activity of β -CGTase. According to Li *et al.* (2010), cell growth impairment caused by glycine could be controlled by delaying the addition of glycine to the culture medium. However, reduction of β -CGTase activities was recorded by increasing the timing of induction to 3 and 4 h of fermentation. The induction at high cell density during stationary phase is not recommended due to the bacterial cell membrane rigidity (Bao *et al.*, 2016).

The amount of glycine necessary to enhance the permeability of outer membrane of *E. coli* was 1.2 mM and showed enhancement of β -CGTase activity by 1.3-fold as compared to the culture without inducer (Figure 1C). However, the supplementation of 1.6 mM glycine inhibited the β -CGTase activity and an excessive amount of glycine is unfavorable to the cell and might inhibit the bacterial growth (Zou *et al.*, 2014). In addition, an approach to increase the production of recombinant proteins by lowering the induction temperature is frequently reported (Lu *et al.*, 2018; Zheng *et al.*, 2016). Although the enzyme expression could be significantly enhanced at lower induction temperature to increase the formation of correctly folded protein, this study

found opposing results where the incubation at 25°C caused a reduction of enzyme activity by 2.4-fold as compared to the incubation at 37°C (Figure 1D). Low incubation temperature at 25°C

might contribute to the impaired growth of *E. coli* (Chang *et al.*, 2017), hence reducing the activity of β -CGTase.

Figure 1. Effects of different types of inducers (A); glycine supplement time (B); glycine concentrations (C); post-induction temperatures (D) on the extracellular secretion of β -CGTase from *E. coli* harbouring pQECGT-BS. Each value represents the mean of three independent measurements and the deviation from the mean is below 5%.

Effect of glycine supplementation on cell viability. To confirm the secretion of β -CGTase is not due to the cell lysis, the live and dead cells were quantified by using nucleic acid double staining assay based on flow cytometry. The secretion of enzyme into the culture medium via cell lysis is undesirable due to the release of proteins and other contaminants from the intracellular space (Shin and Chen, 2008), which might interfere in the purification process. Although different methods were reported to

indicate for cell lysis, for instance by determination of cytoplasmic proteins; β -galactosidase (Bao *et al.*, 2016) and glucose-6-phosphate dehydrogenase (Li *et al.*, 2010) that are expected to be present in very low concentration in the culture medium, they differ in respect to sensitivity and accuracy. In this study, a flow cytometry method was adopted to quantify the concentrations of live and dead cells where it considered as a rapid, sensitive and highly reproducible approach (Fontana *et al.*, 2017). This

approach could also replace the cells quantification method under microscopy that is time-consuming and lacks reproducibility.

Figure 2. (A) Proportion of viable (■) and dead (□) cells of *E. coli* harbouring pQECGT-BS throughout 16 h of fermentation; (B) Representative flow cytometry dot plots showing distribution of dead and viable cells of recombinant *E. coli* with no induction as a control and inductions with 1.2 mM of glycine at 0 h and 2 h. An ANOVA test demonstrated a significant difference of viable and dead cells between no induction, 0 h and 2 h induction with glycine at $p < 0.05$.

As shown in Figure 2, the addition of 1.2 mM glycine at 2-h of fermentation did not show any apparent effect on the viability of the *E. coli* cells where the percentage of viable cells recorded was more than 97%, comparable with the viable cells in the culture without any inducer. Only a slight reduction of the percentage of viable cell was recorded with the addition of 1.2 mM glycine at the beginning of the culture. A higher percentage of live cells recorded following glycine induction indicates the release of recombinant β -CGTase was due to the interference of glycine with the membrane integrity and not due to cell lysis.

Purification and kinetic parameters for determination of the recombinant β -CGTase.

The recombinant β -CGTase was collected and purified from the 12-h culture induced with 1.2 mM glycine at 2 h induction time at 37°C. The recombinant β -CGTase carried an N-terminal

fusion peptide containing a 6x-His sequence that provides metal-binding affinity. Therefore, it was purified through a combination of diafiltration and Ni-NTA affinity chromatography, which resulted in increased purity to 18.4-fold. Ultrafiltration was then applied to concentrate the protein where the purity was increased to 21.6-fold (Table 1). To evaluate the purity of protein, the diafiltrated and purified proteins were resolved by SDS-PAGE. The results showed the expected size band of the purified protein of 78.6 kDa (Figure 3).

Figure 3. SDS-PAGE analysis of the (A) crude protein; (B) purified protein; and (C) diafiltrated protein. The protein samples were loaded with the total concentration of 30 μ g for each well. M: PageRuler unstained protein leader (Thermo Fisher Scientific, USA).

Furthermore, a different range of soluble starch concentrations was tested to produce β -CD by β -CGTase. A maximum β -CD of 16.671 mg/mL was achieved by using 4 mg/mL of starch (Figure 4). However, the production of β -CD was reduced and attained almost a similar concentration with increased starch concentration. At these points, the enzyme could probably has reached saturation with the substrate (Robinson, 2015). The cyclisation reaction of β -CGTase was then described by Michaelis-Menten kinetic parameters (Figure 5) to evaluate the performance of recombinant β -CGTase for β -CD production (Chen *et al.*, 2018; Rakmai and Cheirsilp, 2016). The V_{max} and K_m values were evaluated to be 1.3 mg/mL/min and 0.025 mg/mL, respectively. A small value of K_m could indicate a high affinity for the substrate (Ong *et al.*, 2008). Hence, it was suggested that the recombinant β -CGTase was specific towards

starch and could achieve its maximum catalytic efficiency at low starch concentrations.

Table 1 Purification yield of recombinant β -CGTase from *E. coli* following induction with 1.2 mM glycine at 2 h of fermentation at 37°C.

Purification steps	β -CGTase activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	38.585 ± 1.513	16.175 ± 0.702	2.385 ± 0.197	1	100
Diafiltration	31.354 ± 0.877	10.189 ± 0.456	3.077 ± 0.052	1.290	81.26
Affinity chromatography	19.953 ± 0.143	0.455 ± 0.026	43.882 ± 2.821	18.395	51.71
Ultrafiltration	8.361 ± 0.088	0.163 ± 0.029	51.408 ± 3.021	21.550	21.67

Figure 4. The production of β -cyclodextrin by purified β -CGTase using different starch concentrations. Standard deviations are shown as bars and the deviation from the mean is below 5%.

Figure 5. The Hanes-Woolf plot to identify K_m and V_{max} which were used in the Michaelis-Menten equation. S: substrate, v : velocity.

CONCLUSION

As a conclusion, the present study demonstrated that the supplementation of glycine with correct feeding strategy could enhance the secretion of β -CGTase into the culture medium of *E. coli* without any adverse effect on the viability of *E. coli* cells. This feeding strategy could be applied to enhance the extracellular secretion of a recombinant protein from *E. coli* system at the cultivation process.

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