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ORIGINAL PAPER

An effective extracellular protein secretion by an ABC transporter system in *Escherichia coli*: statistical modeling and optimization of cyclodextrin glucanotransferase secretory production

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Abstract Direct transport of recombinant protein from cytosol to extracellular medium offers great advantages, such as high specific activity and a simple purification step. This work presents an investigation on the potential of an ABC (ATP-binding cassette) transporter system, the hemolysin transport system, for efficient protein secretion in *Escherichia coli* (*E. coli*). A higher secretory production of recombinant cyclodextrin glucanotransferase (CGTase) was achieved by a new plasmid design and subsequently by optimization of culture conditions via central composite design. An improvement of at least fourfold extracellular recombinant CGTase was obtained using the new plasmid design. The optimization process consisted of 20 experiments involving six star points and six replicates at the central point. The predicted optimum culture conditions for maximum recombinant CGTase secretion were found to be 25.76 μM IPTG, 1.0% (w/v) arabinose and 34.7°C post-induction temperature, with a predicted extracellular CGTase activity of 68.76 U/ml. Validation of the model gave an extracellular CGTase activity of 69.15 ± 0.71 U/ml, resulting in a 3.45-fold increase compared to the initial conditions. This corresponded to an extracellular CGTase yield of about 0.58 mg/l. We showed that a synergistic balance of transported protein and secretory pathway is important for efficient protein transport. In addition, we also demonstrated the first successful removal of the *C*-terminal secretion signal from the transported fusion protein by thrombin proteolytic cleavage.

Keywords Cyclodextrin glucanotransferase · Response surface methodology · Hemolysin transport system · Protein secretion · *Escherichia coli*

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Introduction

Protein production in a bacterial cytoplasm, such as *E. coli*, is a widely used and probably the simplest strategy for recombinant protein overexpression. In contrast to cytoplasmic expression, extracellular secretion of recombinant protein offers simple detection and purification, avoids inclusion body formation, and provides a better folding environment free from proteolytic degradation [3]. The *Bacillus subtilis* expression system is generally used for hyper-secretion of target proteins at significant concentration. However, the system suffers from quality control posed by proteases that could often reduce protein production yield [41]. An alternative host system is *E. coli*. Recent development in engineering *E. coli* cell for secretory production of functionally active enzymes or protein may offer great potential for large-scale application [2, 27].

There are four general strategies to engineer *E. coli* to produce extracellular protein: (1) use a dedicated secretion system; (2) use a carrier protein with no known translocation mechanisms; (3) use cell envelope mutants; (4) co-expression a lysis-promoting protein [27]. One of the most extensively studied protein secretion systems is the α-hemolysin (HlyA) system in *E. coli*. The system transports a substrate molecule directly from the cytoplasm to the extracellular medium, bypassing the formation of a periplasmic intermediate [11]. Efficient protein secretion requires three membrane protein components: HlyB (an ATP-binding cassette (ABC) protein), HlyD (a membrane fusion protein), and TolC (an outer membrane protein), and a *C*-terminal secretion signal located at the *C*-terminal 50–60 amino acids of HlyA. Heterologous protein fused to the HlyA signal sequence (HlyAs) can be recognized and secreted by the hemolysin translocator. In contrast to the Sec pathway, the *C*-terminal secretion signal is not removed after the target protein is transported to the extracellular medium. Recent successes in engineering the hemolysin transport system [23, 35] for improved secretion level have opened up the possibility of large-scale secretory production of recombinant protein in *E. coli*.

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is a unique enzyme that synthesizes non-reducing cyclic oligosaccharides called cyclodextrins (CDs) from starch and other related substrates, such as dextrin, glycogen, amylose, and amylopectin [37]. CDs are highly valuable modified starches that are extensively used as molecular chelating agents in the cosmetic, pharmaceutical, food, agricultural, and chemical industries [24]. In 1986, the recombinant production of a *Bacillus* CGTase in *E. coli* was carried out by Kato and Horikoshi [14]. Since then, many CGTase genes have been isolated and expressed in *E. coli* [16, 26], including a β-CGTase from *Bacillus* sp. G1 isolated by our group [28]. However, recombinant CGTases expressed in *E. coli* are often found in inclusion bodies, and in vitro refolding is required to restore the biological activity of the enzyme [15]. To increase the soluble production of recombinant CGTase, strategies such as manipulation of cultivation conditions [29] and co-expression of folding accessory proteins [17] have been developed. Alternatively, functionally active CGTase could be obtained by extracellular secretion in *E. coli*. Recently, Ding et al. [4] described the use of medium additives for extracellular secretion of active α-CGTase from *E. coli*. No study regarding the secretion of recombinant CGTase in *E. coli* by utilizing the hemolysin transport system has been reported.

One of the main strategies to improve productivity and secretion efficiency of recombinant protein is by the enhancement of gene expression rates [34]. In line with this, increasing the amount of secretion machineries is a possible approach to promote over-secretion of a target protein. However, caution must be taken to avoid growth retardation or undesired cell death because overexpression of integral membrane-translocator protein(s) is detrimental to the host cell [18]. Dlugolecka et al. [5] described the use of a dual-plasmid system for the enhanced secretion of esterase in *E. coli* via an ABC transporter. However, the authors did not evaluate the effect of secretion machinery expression on target protein secretion or its optimization.

In the present study, we investigated the possibility of high yield secretory production of an important industrial enzyme, β-CGTase from a locally isolated *Bacillus* sp. G1 [28], by the hemolysin transport system in *E. coli*. We assessed the effect of plasmid design and culture conditions, including concentrations of inducers and post-induction temperature, on the secretory production of recombinant CGTase. We developed a statistical model that reveals a synergistic relationship between factors for efficient protein secretion. In addition, our plasmid system enables the removal of the *C*-terminal HlyA signal sequence from the fusion protein by the proteolytic cleavage of thrombin.

Materials and methods

Bacterial strains and plasmids construction

The *E. coli* strains and oligonucleotides used in this study are shown in Table 1. JM109 was used for cloning; BL21 (DE3), Rosetta 2 (DE3), Rosetta blue (DE3), and Rosetta-gami2 (DE3) (Novagen) were used for recombinant protein production; J96 contained the alpha-hemolysin gene coding region (American Type Culture Collection). Standard recombinant DNA manipulation techniques and PCR were performed as described [33].

The coding region of the mature *cgt* gene (GenBank accession no. AY770576; from *Bacillus* sp. G1) lacking a stop codon was PCR-amplified from our laboratory clone [28] using primers CGT-F and CGT-R. The resulting 2,022-bp DNA fragment was digested with *Kpn*I and ligated into the corresponding site of pET-29(a) (Novagen), yielding pCGT23. All genes to be expressed were cloned into a single plasmid for a single-plasmid system. The coding region of the *hly* operon, from the final 61 codons of *hlyA* ($HlyAs_{61}$) to the 3′ end of *hlyD*, was PCR-amplified from total genomic DNA isolated from *E. coli* J96 using primers FHlyAs and pET_AsBD_R. The resulting 3,838-bp DNA fragment was digested with *Bam*HI and *Sac*I before being ligated into the corresponding sites of pCGT23, yielding pCGT-AsBD.

Two different plasmids were constructed for a dual-plasmid system. $HlyAs_{61}$ was cloned into pCGT23 using

Table 1 Bacterial strains and oligonucleotides used in this study

Name	Relevant characteristics
Bacteria strain	Genotype
JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 (r_k^-, m_k^+) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F′ *tra*D36 *pro*AB + *laq*I^{q}ZΔM15]
BL21(DE3)	[F$^-$ *omp*T *hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)]
Origami 2 (DE3)	[Δ*ara–leu*7697 Δ*lac*X74 Δ*pho*A *PvuII pho*R *ara*D139 *ahp*C *gal*E *gal*K *rps*L F ′[*lac*$^+$ *lacI*^{q} *pro*] (DE3) *gor*522::Tn*10 trxB* (Str^R, Tet^R)]
Rosetta 2 (DE3)	[F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE2 (Cam^R)],
Rosetta blue (DE3)	*end*A1 *hsd*R17(r_{K12}^- m_{K12}^+) *sup*E44 *thi-1 rec*A1 *gyr*96 *rel*A1 *lac* (DE3) F'[*pro*A$^+$B$^+$ *lacI*^{q}*ΔZ* M15::Tn]*10* pRARE (Cam^R, Tet^R)
Rosetta-gami2 (DE3)	Δ*ara–leu*7697 Δ*lac*X74 Δ*pho*A *PvuII pho*R *ara*D139 *ahp*C *gal*E *gal*K *rps*L (DE3) F [*lac*$^+$ *lacI*^{q} *pro*] *gor*522::Tn*10 trxB* pRARE2 (Cam^R, Str^R, Tet^R)
J96	Serotype O4:K6
Oligonucleotide	Sequence (5′–3′)[a]
FHlyAs	GGCCGGATCCGATGCATTAGCCTATGGAAGT
RHlyAs	GCGCGAAGCTTTTATGCTGATGCTGTCAAAG
pET_AsBD_R	GGCCGAGCTCTTAACGCTCATGTAAACTTTCTGT
CGT-F	GCGGTACCGACGTAACAAACAAAGTC
CGT-R	GGCCGGTACCCCAATTAATCATAACCGT
pBAD_BD_F	GGGCCGAGCTCAATGGATTCTTGTCATAAAATTGATTATG
pBAD_BD_R	GGCCGGTACCTTAACGCTCATGTAAACTTTCTGTTACAGA

[a] Relevant restriction sites used for cloning of PCR products are underlined

primers FHlyAs and RHlyAs, yielding pCGT-As. The *hlyB* and *hlyD* genes were cloned from total genomic DNA of *E. coli* J96 using primers pBAD_BD_F and pBAD_BD_R into pBAD/*Myc*-His (Invitrogen), yielding pBAD-BD. All PCR-amplified sequences were confirmed by nucleotide sequencing.

Expression of Hly-secretion machinery proteins and CGTase

A single colony of *E. coli* harboring plasmids pCGT-As and pBAD-BD (dual-plasmid system) or pCGT-AsBD (single-plasmid system) was inoculated in Luria–Bertani (LB) medium and grown at 37°C. The overnight inoculum (5%) was inoculated into expression medium (LB, terrific broth (TB) or M9 minimal salt (M9) medium [33]), supplemented with ampicillin (100 μg/ml) and/or kanamycin (30 μg/ml). Cultures were grown in 250-ml Erlenmeyer flasks filled with 50 ml of expression medium at 37°C in a rotary shaker (200 rpm). Gene expression was initiated by the addition of arabinose and/or isopropyl-*β*-D-thiogalactopyranoside (IPTG) at a cell density of about A_{600} 1.0, and then cells were incubated at a specific post-induction temperature.

CGTase enzyme assay

CGTase activity was determined using the phenolphthalein assay [10]. The reaction mixture containing 1.0 ml of 4% (w/v) soluble starch in 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of enzyme solution was incubated at 60°C for 10 min. The reaction was stopped by the addition of 3.5 ml of 0.03 M NaOH. We then added 500 μl of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 to the reaction mixture, which was incubated at room temperature for 15 min. The reduction in color intensity was measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that formed 1 μmol *β*–CD/min.

Statistical model for optimum Hly-mediated protein secretion

Response surface methodology (RSM) was applied to optimize the culture conditions for the over-secretion of recombinant CGTase in *E. coli*. The optimization was designed with three variables consisting of two blocks with a total of 20 runs (14 combinations with six replications of the central point). The first block (Block 1) was based on a two-level full factorial design with a total of 12 experiment trials that involved four replicates at the central point. The second block (Block 2) was designed from augmentation of the full factorial design (Block 1) into a central composite design (CCD) with the addition of eight experiment trials that involved six star points and two replicates at the central point. The star point was set with an *α* value of 1.31607 from the coded center point. Design-Expert 6.0.4 (State-Ease, USA) was used to analyze the obtained results. The culture conditions investigated were concentration of

IPTG (A), concentration of arabinose (B), and post-induction temperature (C). Details of the lower limit and the upper limit are shown in Table 2. The variables are coded according to the following equation [39]:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad i = 1, 2, 3, \ldots, j \tag{1}$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value. Extracellular CGTase activity was considered the dependent variable or response (Y_i). A second-order polynomial equation was employed to fit the experimental data presented in Table 3. The proposed model for the response Y_i is given in the following equation:

$$Y_i = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + + \beta_{13} AC + \beta_{23} BC \tag{2}$$

where i = predicted response; A, B and C = independent variables; β_0 = offset term; β_1, β_2 and β_3 = linear effects; β_{11}, β_{22} and β_{33} = squared effects and β_{12}, β_{13} and β_{23} = interaction term effects. The experimental data were analyzed using analysis of variance (ANOVA). A p value (probability $> F$) less than 0.05 indicated that the model terms were significant. The adequacy of the model was further analyzed.

Single-step purification and removal of the *C*-terminal signal sequence from fusion CGTase by thrombin cleavage

Crude supernatant containing recombinant CGTase was purified by starch adsorption [32] using wheat starch (Merck). CGTase was eluted in distilled water at 50°C with shaking. The sample was further concentrated with an Amicon concentrator (30-kDa MWCO, Millipore). Protein content was measured using Bradford reagent (Sigma) with bovine serum albumin as standard. SDS–PAGE was performed using a 5% (w/v) stacking gel and a 10% (w/v) separating gel [33]. Thrombin cleavage was carried out in a 100-µl reaction volume consisting of ~150 ng of protein sample, 1 U of thrombin (Novagen), and 1× thrombin reaction buffer, at 20°C. Starch-SDS–PAGE zymography [25] was performed to analyze the effect of thrombin cleavage towards the enzymatic activity of recombinant CGTase.

Results and discussion

Plasmid design

Two expression systems were constructed and evaluated for efficient CGTase extracellular secretion: the single-plasmid system and the dual-plasmid system (with two different inducible promoters). The single-plasmid system consisted of the mature *cgt* gene fused upstream of the partial *hly* operon, which ran from $HlyAs_{61}$ through the 3′ end of *hlyD*. Gene expression was controlled by a *T7lac* promoter. In contrast, the dual-plasmid system consisted of two different plasmids; the *T7lac* promoter system was used for the recombinant CGTase (mature *cgt* gene fused to the $HlyAs_{61}$; CGT-$HlyAs_{61}$) expression in recombinant plasmid pCGT-As, and the P_{BAD} promoter system was used for the *hlyB-hlyD* expression in recombinant plasmid pBAD-BD. A thrombin cleavage sequence (LVPRGS), located between *N*-terminal CGTase and *C*-terminal $HlyAs_{61}$, was present in both expression systems to permit cleavage of the fusion protein.

Selection of the expression medium and *E. coli* strain for CGTase secretion

Preliminary expression screening of recombinant *E. coli* cells on starch plates indicated that a functionally active CGTase enzyme was secreted by both systems to the extracellular medium, specifically by using the hemolysin transport pathway (data not shown). Optimization was thus performed to evaluate both systems' capacity to oversecrete active recombinant CGTase. Initially, selection of the expression medium and the host strain was done using the classical one-factor-at-a-time (OFAT) method. The secretion level of CGT-$HlyAs_{61}$ in LB, TB, and M9 medium were compared for the single-plasmid system (pCGT-AsBD; unregulated *hlyB-hlyD* expression) and the dual-plasmid system (pCGT-As/pBAD-BD) using *E. coli* BL21(DE3) as the host cell. The dual-plasmid system

Table 2 Process variables and the levels studied in the optimization design

Factors	Notation	Unit	Low star point $-\alpha$	Low level -1	Center point 0	High level $+1$	High star point $+\alpha$
Concentration of IPTG	A	µM	3.68	10	30	50	56.32
Concentration of arabinose	B	% (w/v)	0.07	0.20	0.60	1.00	1.13
Post-induction temperature	C	°C	28.89	30.0	33.5	37.0	38.11

Table 3 Experiment design for central composite design and the response for extracellular CGTase activity

Run[a]	Block	Coded value			Extracellular CGTase activity (U/ml)	
		A (IPTG concentration, μM)	B (arabinose concentration, %)	C (post-induction temperature, °C)	Actual	Predicted
1	1	−1	−1	1	42.17	45.16
2	1	1	1	−1	29.71	32.38
3	1	1	−1	−1	24.17	26.42
4	1	−1	−1	−1	18.71	21.51
5	1	0	0	0	60.83	63.42
6	1	0	0	0	58.59	63.42
7	1	−1	1	−1	32.32	35.64
8	1	0	0	0	61.93	63.42
9	1	0	0	0	62.13	63.42
10	1	1	1	1	49.51	52.37
11	1	−1	1	1	55.15	58.57
12	1	1	−1	1	44.79	47.13
13	2	0	0	+α	53.68	50.47
15	2	0	0	0	62.21	63.42
16	2	+α	0	0	54.34	52.30
17	2	−α	0	0	57.03	53.17
18	2	0	0	0	68.80	63.42
19	2	0	+α	0	70.69	67.02
20	2	0	−α	0	56.51	54.19

[a] Run 14 has been removed from the design due to inadequacy of the outlier-T

outperformed the single-plasmid system in all tested media, for at least a fourfold higher CGT-HlyAs$_{61}$ secretion level (Fig. 1). The highest CGT-HlyAs$_{61}$ secretion was obtained using *E. coli* BL21(DE3)/pCGT-As/pBAD-BD in TB medium, at 15.54 ± 1.46 U/ml. M9 medium resulted in the lowest CGT-HlyAs$_{61}$ secretion, at 1.33 ± 0.40 U/ml for the dual-plasmid system. No CGTase activity was detected for the single-plasmid system in M9 medium. TB medium contains a higher amount of tryptone, yeast extract, and carbon source (glycerol) than the other two mediums tested. Therefore, a rich nutrient medium significantly enhances protein expression. A similar observation was reported by Lo et al. [22], in which modified TB medium exhibited the highest recombinant CGTase secretion among all tested media (TB, YT, NZ, and LB media). Sunitha et al. [36] also showed that higher composition of tryptone and yeast extract increased phytase production in *E. coli*. Therefore, *E. coli* harboring recombinant plasmids pCGT-As and pBAD-BD in TB medium was selected for the subsequent expression study.

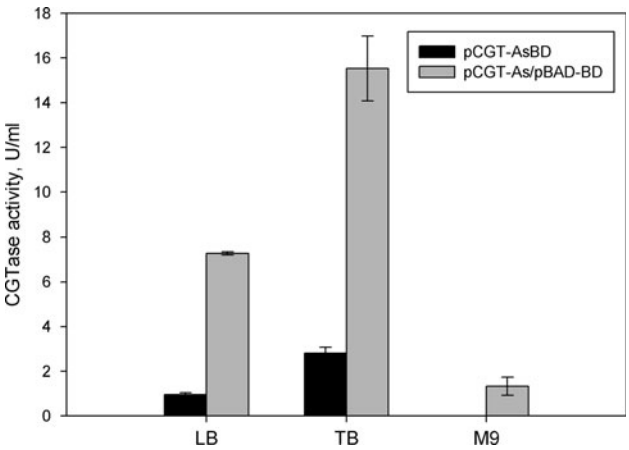

Fig. 1 Comparison of CGT-HlyAs$_{61}$ secretion using LB, TB, and M9 minimal media in *E. coli* BL21(DE3). *E. coli* cells were induced with arabinose (0.02%, w/v) and/or IPTG (50 μM) at cell density of ~A_{600} 1.0 and incubated at 30°C for 4 h. The level of secreted CGT-HlyAs$_{61}$ was determined by CGTase assay. Each *bar* represents the mean ± standard error

Attempts to achieve over-secretion of CGT-HlyAs$_{61}$ using the single-plasmid system did not yield a satisfactory outcome. This may have been due to a low amount of functional ABC transporter (HlyB-HlyD) to facilitate secretion [1]. Thus, increasing the expression of HlyB-HlyD seems to be a straightforward solution to achieve efficient target protein secretion. However, over-expression of integral membrane proteins is difficult [6]. Blight et al. [1] reported that neither high-copy-number plasmid nor expression using strong promoters (i.e., λP_R, ptrp and T7 promoter) results in over-expression of HlyB. Indeed,

co-expression of *hlyB-hlyD* regulated by the *T7lac* promoter was found to result in about ninefold lower recombinant CGTase secretion than that of the dual-plasmid, single-promoter system (coexpression of CGT-HlyAs$_{61}$ and HlyB-HlyD were driven by *T7lac* promoter in two plasmids; our unpublished data). We circumvented this problem by using a novel dual-plasmid system: an independent gene co-expression system in which the ABC transporter genes (*hlyB* and *hlyD*) were co-expressed under the tightly regulated P_{BAD} promoter system. Increased secretion of CGT-HlyAs$_{61}$ using the dual-plasmid system (Fig. 1) may have been due to an increased amount of functional HlyB-HlyD translocator complex (see "discussion" below).

Recombinant *E. coli* BL21(DE3) cells exhibited the highest CGT-HlyAs$_{61}$ secretion among other tested strains. Recombinant Origami 2(DE3), Rosetta-gami 2(DE3), and Rosetta 2(DE3) yielded a CGT-HlyAs$_{61}$ secretion of 44, 41, and 32% of BL21(DE3), respectively (data not shown). Origami 2 (DE3) contains *trxB* and *gor* mutations for enhanced protein disulphide bond formation, Rosetta 2 (DE3) and Rosetta-Blue (DE3) provide rare codon supplementation, while Rosetta-gami 2 (DE3) has both of the above features. Our finding suggests that disulphide bond formation and/or rare codon supplementation may not be important for the expression of membrane transporter and CGT-Hly As$_{61}$. Indeed, *E. coli* BL21(DE3) is one of the most preferred *E. coli* strain for functional membrane protein expression studies [6] and recombinant CGTase production [13, 29].

Effect of CGTase and Hly-secretion machinery expression level and post-induction temperature on CGTase secretion

The OFAT method was used to investigate the effect of inducer concentrations (IPTG and arabinose) and post-induction temperature on CGT-HlyAs$_{61}$ secretion and to determine the effective range of each significant variable for the subsequent RSM experiment. The concentrations of IPTG and arabinose varied in the range of 10 μM–1.0 mM and 0.0002–1% (w/v), respectively, while post-induction temperature was set between 20 and 37°C. The results were intriguing (Fig. 2). CGT-HlyAs$_{61}$ secretion was proportional to arabinose concentration; 1% arabinose gave the highest secretion level, at 23.66 U/ml, compared to 6.28 U/ml when induced with 0.0002% arabinose. Increased arabinose induction may induce increased formation of functional HlyB-HlyD translocator complexes, which in turn enable greater CGT-HlyAs$_{61}$ secretion. In contrast to previous findings with λP_R, ptrp, and T7 promoter [1], we found that the P_{BAD} promoter was capable of over-expressing HlyB-HlyD membrane proteins. It is tempting to speculate how P_{BAD} promoter enables higher level of functional HlyB-HlyD. Efficient repression, modulation, and moderately high expression by the P_{BAD} promoter [8] may be important factors for the efficient expression of the integral membrane proteins. Indeed, these characteristics enable cells to grow despite the metabolic burden from the expression of heterologous proteins [20].

Temperature is one of the most important environmental factors in microbial fermentations affecting gene expression and metabolic reaction rates of cells [9]. We found that a higher post-induction temperature resulted in higher CGT-HlyAs$_{61}$ secretion (Fig. 2). Maximum CGT-HlyAs$_{61}$ secretion (37 U/ml) was obtained at 37°C, in contrast to 0.86 U/ml at 20°C. This result contradicts previous studies that emphasized the importance of low culture temperature for soluble and active production of recombinant CGTase in *E. coli*. Jin et al. [13] demonstrated that lowering the culture temperature from 37 to 30°C can increase the soluble fraction of *B. macerans* CGTase by at least threefold. Similarly, Park et al. [29] achieved twofold higher CGTase activity at a culture temperature of 25°C compared to 37°C. In addition, maximum CGTase secretion by co-expressing bacteriocin release protein (BRP) has been obtained at a post-induction temperature of 20°C [21]. The main reason for these observations is that a lower expression temperature reduces the expression rate (transcription and translation) and facilitates the correct folding of the aggregation-prone recombinant CGTase. Despite this evidence, our findings suggest a different scenario for secretory protein production: secretion-competent proteins/peptides

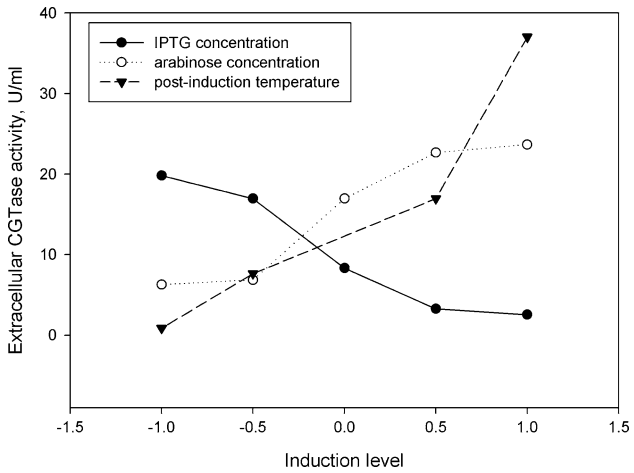

Fig. 2 Effect of inducers (IPTG and arabinose) and post-induction temperature on CGT-HlyAs$_{61}$ secretion in *E. coli* BL21(DE3). Cells were induced at a cell density of ~A_{600} 1.0. The *induction level bar* represents the high (+1) and low (−1) range of each variable. The values investigated for each variable were: 1.0, 0.5, 0.1, 0.05, and 0.01 mM for IPTG; 1, 0.2, 0.02, 0.002, and 0.0002% (w/v) for arabinose; and 37, 30, 25, and 20°C for the post-induction temperature. The constant value for each variable was 0.05 mM IPTG, 0.02% (w/v) arabinose and a 30°C post-induction temperature. Extracellular CGTase activity was measured 4 h post-induction

(unfolded or partially folded) are recognized and transported by the HlyBD-TolC complex before they are folded into their native conformations or aggregate into inclusion bodies in the cytoplasm. Secreted proteins/peptides are then folded in the relatively oxidized extracellular milieu. A high culture temperature (30°C and higher) may induce higher protein expression (i.e., CGT-HlyAs$_{61}$ and HlyB-HlyD) or may be most effective for protein translocation, which in turn promotes a higher secretion level of the target protein.

Interestingly, the CGT-HlyAs$_{61}$ secretion level was inversely proportional to the concentration of IPTG. The highest extracellular CGTase activity (19.81 U/ml) was observed at 10 μM IPTG, compared to 2.55 U/ml at 1.0 mM IPTG. Because recombinant CGTase was prone to aggregation when expressed in *E. coli*, we expect that a lower gene induction (CGT-HlyAs$_{61}$ by IPTG) increases the secretion-competent pool of CGT-HlyAs$_{61}$ by reducing the concentration of the recombinant protein in the cytoplasm. Similarly, high gene induction would promote inclusion body formation and overwhelm the secretion translocation pathway, thus impairing protein translocation. This was more evident when a highly efficient polymerase (i.e., T7 RNA polymerase) was used. Indeed, reduced HlyA aggregation due to a slower translation rate results in hypersecretion of active HlyA in *E. coli* [7].

Our OFAT screening indicated that inducer (arabinose and IPTG) concentrations and post-induction temperature were significant factors for CGT-HlyAs$_{61}$ secretion by the hemolysin transport system in *E. coli*. Therefore, the optimum levels of these factors were further determined by RSM to maximize CGT-HlyAs$_{61}$ secretion.

CCD for CGTase secretion optimization

CCD was applied to determine the optimum value of the three variables (concentrations of IPTG and arabinose and post-induction temperature) for CGT-HlyAs$_{61}$ secretion. Experiments with different combinations of the culture conditions involved (labeled A, B, and C) were performed (Table 2). Extracellular CGTase activity was determined as the response or actual value, as shown in Table 3.

The responses were analyzed with ANOVA (Table 4). The p values for the model (less than 0.0001) and for 'lack of fit' (0.9363) indicated that the obtained experimental data fit well with the model. In other words, the model fit all of the design points well and was desirable for the subsequent analysis. Model terms B, C, AB, A^2, and C^2 were significant factors that influenced the secretion of CGT-HlyAs$_{61}$ according to the p values, which were less than 0.05.

The robustness of the model was expressed by the R^2 (multiple correlation coefficient) value of 0.9881, indicating that 98.81% of the variability in the response could be explained by the model. Meanwhile, the coefficient of determination (adjusted R^2) was calculated to be 0.9748, indicating that only 2.52% of the total variation was not included in the model. The predicted R^2 (0.9625) being in reasonable agreement with the adjusted R^2 indicates a good agreement between the observed and the predicted values of CGT-HlyAs$_{61}$ secretion. In addition, the model had an adequate precision value of 32.027, indicating that the model could be used to navigate the design space. Furthermore, a low CV (4.13%) showed that the experiments performed were highly reliable. Therefore, the regression model was applied to calculate the predicted values; the usefulness of the model is shown in Table 3, where the predicted values closely match the experimental values. In other words, the model obtained was applicable to predicting the optimum culture conditions that would maximize CGT-HlyAs$_{61}$ secretion.

The response of extracellular CGT-HlyAs$_{61}$ secretion, (Y), U/ml, by *E. coli* can be expressed in terms of the following regression equation:

$$\begin{aligned} Y = & -1525.5186 + 1.4139A + 31.1175B + 89.6293C \\ & - 0.2552AB - 0.0105AC - 0.1295BC \\ & - 0.0154A^2 - 10.0168B^2 - 1.2854C^2 \end{aligned} \quad (3)$$

All terms, regardless of their significance, are included in the above equation, where A is the concentration of IPTG, B is the concentration of arabinose, and C is the post-induction temperature.

The ANOVA showed that the concentration of arabinose (B) and the post-induction temperature (C) were the most significant factors affecting CGT-HlyAs$_{61}$ secretion ($p < 0.0001$). IPTG concentration (A) was not significant ($p = 0.6145$) (Table 4). Our findings are similar to those of Lo et al. [21], who found that BRP expression and post-induction temperature, and not CGTase expression, were the significant factors that affected recombinant CGTase secretion. Our results strengthen the view that the availability of secretion channels and host cell metabolism are the main factors influencing secretory production of recombinant protein in *E. coli*.

The application of a statistical model for bioprocess optimization is increasing in the current biotechnology field. One of the main advantages of RSM is to reveal relationships between process variables that are difficult or impossible when using a traditional OFAT approach [40]. Interactions among the culture conditions can be easily predicted from Table 4. The interaction between the concentrations of IPTG and arabinose (AB) gave the most significant effect, with a p value of 0.0247. Figure 3 shows that CGT-HlyAs$_{61}$ secretion increased when either one of the inducers, or both, were increased. However, a further increase in target protein expression decreased

Table 4 ANOVA for response surface quadratic model for CGT-HlyAs$_{61}$ secretion

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*p* value (probability > *F*)	Significant term based on *p* value[a]
Model	2,919.42	9	324.38	74.12	<0.0001	Significant
A (IPTG concentration)	1.20	1	1.20	0.27	0.6146	
B (arabinose concentration)	268.80	1	268.80	61.42	<0.0001	Significant
C (temperature)	979.33	1	979.33	223.77	<0.0001	Significant
AB	33.33	1	33.33	7.62	0.0247	Significant
AC	4.31	1	4.31	0.98	0.3502	
BC	0.26	1	0.26	0.06	0.8126	
A^2	233.31	1	233.31	53.31	<0.0001	Significant
B^2	15.73	1	15.73	3.59	0.0946	
C^2	959.19	1	959.19	219.17	<0.0001	Significant
Residual	35.01	8	4.38			
Lack of fit	5.39	4	1.35	0.18	0.9363	Not significant
Pure error	29.63	4	7.41			
Correlation total	4,011.79	18				
Standard deviation	2.09	*R*-squared	0.9881			
Mean	50.70	Adj *R*-squared	0.9748			
C.V. %	4.13	Pred *R*-squared	0.9625			
PRESS	110.90	Adeq precision	32.027			

[a] *p* value (probability > *F*) less than 0.05 indicated that the model terms are significant

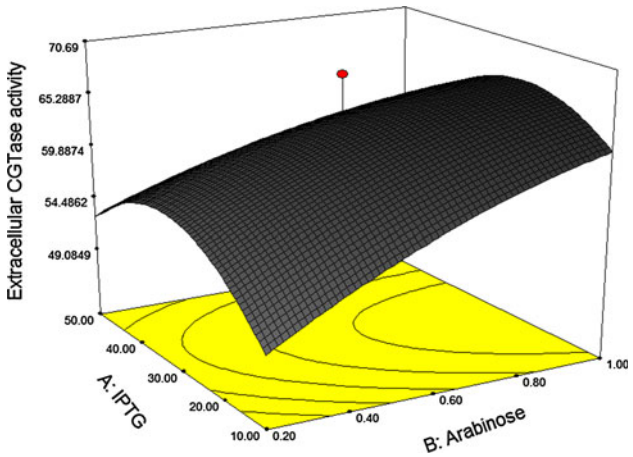

Fig. 3 Response surface plot of CGT-HlyAs$_{61}$ secretion showing the interactive effects of inducer concentration [IPTG (**a**) and arabinose (**b**)] at a post-induction temperature of 33.5°C

CGT-HlyAs$_{61}$ secretion when HlyB-HlyD expression level was held a constant level. An optimum expression level of CGT-HlyAs$_{61}$ and HlyB-HlyD facilitated efficient protein secretion. Our findings underscore the synergistic relationship between target protein and secretion machinery expression, in which an optimal balance of both factors should be determined for efficient protein secretion.

Optimization and model verification

The optimum culture conditions for maximal extracellular CGT-HlyAs$_{61}$ secretion were predicted by the 'Numerical Optimization' function. The condition comprised 25.76 μM IPTG, 1.0% arabinose and 34.71°C post-induction temperature. To confirm the optimization results, the time course of CGTase production under the optimized conditions was investigated. Figure 4 shows that extracellular CGT-HlyAs$_{61}$ secretion reached a maximum level of 75.52 U/ml 6 h post-induction and remained constant until 24 h post-induction. Meanwhile, intracellular CGTase reached a maximum CGTase activity of 15.48 U/ml at 2 h post-induction before it declined to 2.6 U/ml after 4 h incubation and remained constant until 24 h post-induction. A relatively low level of cytoplasmic CGT-HlyAs$_{61}$ indicates active translocation of the recombinant enzyme to the extracellular medium. The level of CGTase secretion at 4 h post-induction was 69.15 ± 0.71 U/ml, which was very close to the predicted value (68.764 U/ml). A 3.45-fold increment of extracellular CGTase activity was achieved under the optimized conditions (Table 5). This corresponded to an extracellular CGTase yield of about 0.58 mg/l, which is comparable with the secretory production yield of other recombinant proteins by the hemolysin transport system in *E. coli* [27]. In addition, the

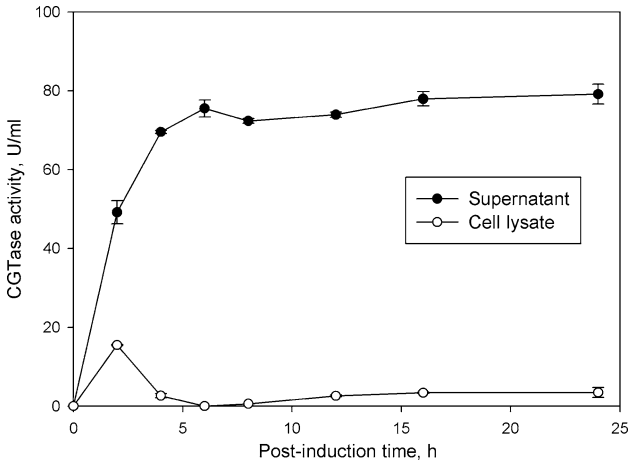

Fig. 4 Secretory production of CGT-HlyAs$_{61}$ by *E. coli* BL21(DE3)/pCGT-As/pBAD-BD under the optimized conditions. The level of secreted CGT-HlyAs$_{61}$ was determined by CGTase assay. Each *point* represents the mean ± standard error

Table 5 Summary of the optimized and initial culture conditions for CGT-HlyAs$_{61}$ secretion

Cultivation conditions	Optimized	Initial
Concentration of IPTG	25.76 μM	50 μM
Concentration of arabinose	1.0%	0.02%
Post-induction temperature	34.7°C	30°C
Extracellular CGTase activity	69.15 ± 0.71 U/ml	15.54 ± 1.46 U/ml
Secretion rate[a]	17.29 U/ml/h	3.89 U/ml/h
Secretion efficiency, %[b]	96.34	85.59
Secretion fold	4.45	1

[a] Secretion rate = CGTase activity in culture supernatant/post-induction time; refers to 4 h post-induction time

[b] Secretion efficiency = CGTase activity in culture supernatant/(CGTase activity in culture supernatant + CGTase activity in cell lysate) × 100

secretion rate increased significantly from 3.89 to 17.29 U/ml/h under the optimized conditions.

Extracellular production of CGTase is usually achieved by expression in the native *Bacillus* host or by targeting the protein to the periplasmic space followed by release to the extracellular medium through the weakening of *E. coli* cell envelope. For example, Rahman et al. [31] achieved a maximum *β*-CGTase production of 49.25 and 17.8 U/ml by the parental strain *Bacillus* sp. TS1-1 and recombinant *E. coli*, respectively. Recently, Ding et al. [4] reported a maximum extracellular α-CGTase activity of 12.89 U/ml in recombinant *E. coli* by supplementation of SDS, Na^{+}, glycine and Ca^{2+}. Li et al. [19] obtained a maximum enzyme activity of 22.5 U/ml of *Paenibacillus macerans* α-CGTase by recombinant *E. coli* after 90 h post-induction. Our work is the first report showing the highest

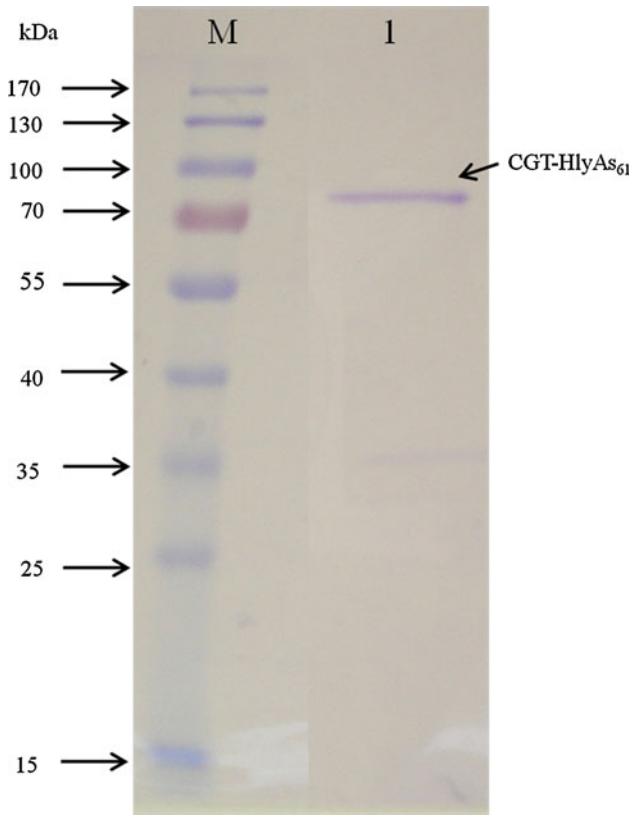

Fig. 5 Purification of secreted CGT-HlyAs$_{61}$. Ten microliters of sample was loaded. *M* prestained protein marker (Fermentas), *lane 1* purified CGT-HlyAs$_{61}$

extracellular production of a *β*-CGTase by the hemolysin transport system in *E. coli*. While a significant amount of CGTase was obtained by these approaches, contamination by endogenous proteins (either due to native secreted protein of *Bacillus* host or the release of endogenous periplasmic protein of *E. coli*) poses a problem to downstream processing. In contrast, direct transport of target protein across the inner and outer membrane by a dedicated protein secretion system (such as the hemolysin transport system) may greatly eliminate this problem [27]. Besides, crude supernatant with a high target-protein-specific activity devoid of proteolytic activity may be used directly in biotransformation application. In addition, an *E. coli* cell with intact cell envelop is more robust, and thus is more likely to withstand bioreactor fermentation process. These striking characteristics could serve as advantages for successful large-scale protein secretory production platform.

Purification and release of *C*-terminal HlyAs$_{61}$ by thrombin cleavage

Absorption of CGT-HlyAs$_{61}$ on wheat starch (Merck) was more pronounced than on other starches (tapioca, potato, sago, and soluble starches) (data not shown). CGTase

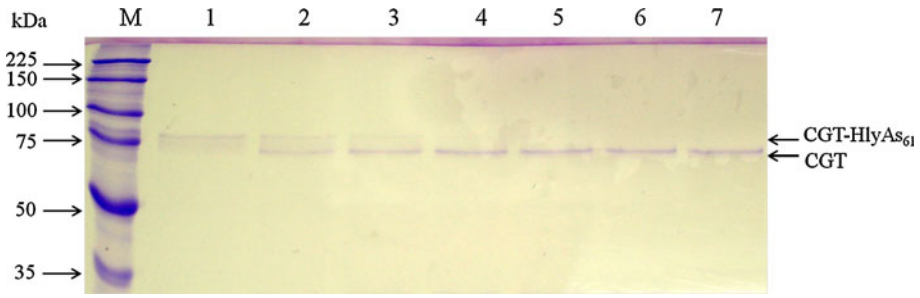

Fig. 6 Thrombin cleavage analysis of CGT-HlyAs$_{61}$. Approximately 5 µl of sample was loaded into each well. *M* broad range protein molecular weight markers (Promega), *lane 1* 0 h, *lane 2* 0.5 h, *lane 3* 1 h, *lane 4* 1.5 h, *lane 5* 2 h, *lane 6* 3 h, *lane 7* 4 h

absorption level on tapioca, sago, and potato starch were 97, 51, and 45% of wheat starch, respectively. Soluble starches, obtained from Merck and Goodrich Chemical Enterprise, yielded the lowest absorption, at 29 and 32%, respectively, compared to wheat starch. Further concentration of the protein sample resulted in near homogeneity of CGT-HlyAs$_{61}$ enzyme sample (Fig. 5), with a specific activity of 113.926 kU/mg.

Thrombin has been used successfully in the removal of fusion partners or tags [12], such as glutathione-S-transferase, maltose-binding protein and hexahistidine tags, from chimeric proteins. As stated previously in the section "Plasmid design", a thrombin cleavage site was present between the *N*-terminal CGTase and *C*-terminal HlyAs$_{61}$ to facilitate the removal of the signal sequence. Thrombin cleavage followed by SDS–PAGE revealed the presence of two protein bands in which the larger-sized protein was the uncleaved, fusion CGT-HlyAs$_{61}$, while the smaller-sized protein was the cleaved CGTase (Fig. 6), suggesting that the *C*-terminal HlyAs$_{61}$ was removed from the fusion CGT-HlyAs$_{61}$. Complete removal of HlyAs$_{61}$ was observed after 1.5 h incubation, based on SDS–PAGE, and prolonged incubation up to 24 h did not cause non-specific proteolysis of the recombinant CGTase (data not shown). Starch zymography analysis was performed to evaluate the effect of thrombin cleavage towards the enzymatic activity of fusion CGTase. Samples were taken at specific time intervals during thrombin digestion. It was demonstrated that the intensity of starch hydrolysis on starch-SDS–PAGE was comparable for the un-cleaved (CGT-HlyAs$_{61}$, control) and the cleaved CGTase (up to 4 h post-incubation) (data not shown), suggesting that the proteolytic activity of thrombin did not significantly affect CGTase enzyme activity. These findings show the usefulness of our expression system, because proteolytic cleavage of the fusion protein may have resulted in a truncated target protein [30] or inefficient and non-specific digestion [38], leading to a non-functional protein. While numerous studies have demonstrated the removal of fusion proteins by the use of specific proteases (such as thrombin and factor Xa), this is the first report, to our knowledge, of the successful removal of a *C*-terminal HlyAs from heterologous CGTase fusion protein secreted by the hemolysin transport pathway.

Conclusions

The results obtained in this study demonstrated that the Hly transport system is an attractive alternative to achieve high-level extracellular recombinant CGTase production in *E. coli*. The optimization of cultural condition, especially manipulating and balancing expression of translocation machinery and target protein, can improve the secretion level of the recombinant enzyme. The system, when coupled with an easy and simple purification step, should be suitable for large-scale protein production.

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