Excretory over-expression of *Bacillus* sp. G1 cyclodextrin glucanotransferase (CGTase) in *Escherichia coli*: Optimization of the cultivation conditions by response surface methodology

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Abstract

Co-expression of cyclodextrin glucanotransferase (CGTase) from *Bacillus* sp. G1 (*cgt*) with bacteriocin release protein (BRP) in *Escherichia coli* system resulted in the expression and excretion of the enzyme into the culture medium. The cultivation conditions were then optimized with the objective to enhance the production of extracellular recombinant CGTase using response surface methodology (RSM) that based on rotatable central composite design (CCD). The process consisted of a total of 50 experiments involving 10 star points and 8 replicates at the central points. The optimum predicted cultivation conditions for the maximum expression of extracellular recombinant CGTase were found to be comprised of: 20 °C post-induction temperature, induction-starting time when cell optical density is 0.3 at 600 nm, 1.0 mM xylose, 50 μM IPTG and 29 h post-induction time, with a predicted extracellular recombinant CGTase activity of 9144.28 U/ml. The experimental extracellular recombinant CGTase activity obtained was 9542.30 U/ml, which was very close to the predicted value. The expression of extracellular recombinant CGTase improved about 151-fold after the optimization was conducted.
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1. Introduction

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) is an extracellular enzyme that converts starch, and other polysaccharides such as dextrins, amylose and amylopectin to form cyclodextrin, CD [1]. Major producers of CGTases belong to *Bacillus* sp. especially aerobic alkalophilic types. The most common available CDs to be synthesized are composed of 6, 7 and 8 glucose units named α-, β- and γ-CDs, respectively [2]. The distribution of α-, β- and γ-CDs is highly dependent on the CGTases used [3].

Genes encoding CGTase have been cloned and identified by several researchers. In 1987, molecular cloning of the CGTase gene form an alkalophilic *Bacillus* and its expression in

Escherichia coli and *Bacillus subtilis* was carried out by Kimura et al. [4]. Furthermore, CGTase gene of *Bacillus macerans* was subcloned down-stream of yeast *ADH1* promoter and expressed in *Saccharomyces cerevisiae*, 0.28 U/ml CGTase was observed in the extracellular medium after 48-h cultivation [5]. Recently, Kim et al. [6] successfully overexpressed the active CGTase of *B. macerans* in recombinant *E. coli*. By co-expressing the folding accessory proteins, molecular chaperones, and human peptidyl–prolyl *cis–trans* isomerase (PPIase) and with a pH-stat fed-batch strategy, 1200 U/ml CGTase activity was obtained. Many CGTase genes were isolated and mostly expressed in *E. coli*. However, formation of inclusion bodies and low yield are major issues that need to be solved. Refolding of the CGTase inclusion bodies were carried out by Kim et al. [7] and Lee et al. [8]. However, other conditions such as changing the culture temperature and co-expression of the folding accessory proteins were favoured by most researchers. The excretory production of recombinant proteins by *E. coli* is desirable over the

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intracellular production as low expression level, inclusion bodies or production lethality always occurs. However, none of the studies regarding the expression of active extracellular CGTase in recombinant *E. coli* by co-expressing the BRP and statistical optimization of the cultivation conditions for the extracellular CGTase expression have been reported.

Excretion of active recombinant CGTase could simplify the enzyme purification and able to achieve the high-yield expression by solving the solubility problem. When referring to the established routes of extracellular excretion for the recombinant protein, co-expression of bacteriocin release protein (BRP) without modifying the existing secretion pathway of *E. coli* is a possible method to promote a excretory *E. coli* over-expression system and express an extracellular recombinant CGTase. However, during such recombinant CGTase expression, caution must be taken in order to avoid the full-lysis or undesired cell death, which is caused by the over-induction of the recombinant BRP. Induction must be independent of the product gene promoter and as a result, co-expression of both BRP and CGTase genes is carried out under a different promoter system.

Production of extracellular recombinant CGTase is mainly dependent on the cultivation conditions such as post-induction temperature, induction-starting time that was determined by the cell optical density at 600 nm, concentration of xylose, concentration of IPTG and post-induction time. In this study, response surface methodology based on rotatable central composite design was applied to identify and optimize the critical, crucial and significant cultivation conditions that will maximize the expression of extracellular recombinant CGTase.

2. Materials and methods

2.1. Construction of the over-expression plasmid

Amplification of the cyclodextrin glucanotransferase gene (*cgt*) with the forward and reverse primers by the polymerase chain reaction (PCR) method was applied. The forward primer used was synthesized based on the sequence starting from the mature gene (5′-tcg-gga-tcc-gac-gta-aca-aac-aaa-gta-aat-tac-3′) and the reverse primer was designed based on the sequence of the termination codon (5′-tcg-gca-tgc-tta-cca-att-aat-cat-aac-cgt-atc-3′). The forward primer was designed to contain the restriction site *Bam*HI and the reverse primer was prepared with the addition of the restriction site *Sph*I.

The amplified CGTase gene fragment was digested with the corresponding restriction enzymes and was ligated with the *Bam*HI-*Sph*I-digested pWH1520 shuttle vector (MoBiTec) to produce pWH1520-*cgt* recombinant plasmid. The resulting recombinant plasmid was transformed into *E. coli* according to the heat shock method [9]. *E. coli* JM109 (F′, *tra*D36, *pro*AB, *lacIqZ*ΔM15) and

E. coli K12 N3406 (*Thr leu thi lacY tonA supE*) that carried the pJL3-BRP gene (MoBiTec) were used for the DNA manipulation and over-expression, respectively. Co-expression of bacteriocin release protein (BRP) was carried out and BRP enables the excretion of the recombinant CGTase into the liquid medium.

2.2. Induction and expression of recombinant CGTase

Modified terrific broth (TB) used for the over-expression containing 12 g/l NZ amine, 24 g/l yeast extract, 8 ml/l glycerol, 2.2 g/l KH$_2$PO$_4$ and 9.4 g/l K$_2$HPO$_4$. One hundred microgram per milliliter of ampicilin and 34 μg/ml chloramphenicol were added into the medium to allow the growth of the plasmid-carrying strain. For solid agar medium preparation, LB medium [9] with 1.5% (w/v) agar powder concentration added was used for the recombinant clone.

In order to observe the recombinant CGTase secretion on the solid media, individual clones were cultured on the LB agar plates [9] that were supplemented with 0.5% soluble starch, 100 μg/ml ampicilin, 34 μg/ml chloramphenicol and 0.5 mM xylose. The plates were incubated at 37 °C overnight for cell growth. Then the recombinant BRP was induced by 20 μl IPTG (40 μM). The plates were incubated at 37 °C overnight again and then stained with iodine solution to visualize the regions of starch digestion.

In order to analyze the expression level of the CGTase, the recombinant clone was cultured at 37 °C in modified TB medium as the seed culture. The seed culture was grown overnight at 37 °C on a rotary shaker at 200 rpm. Fifty milliliters of culture was shaken in 250 ml flasks at 37 °C at 200 rpm before induction. Xylose and IPTG were added as the inducers at different concentration of the cell to induce the expression for the recombinant CGTase and BRP, respectively. Recombinant CGTase was expressed at different temperature for the corresponding post-induction time.

2.3. CGTase enzyme assay

Enzyme assay was carried out according to the Kaneko method [10]. The reaction mixture containing 1 ml of 40 mg of soluble starch in 0.1 M potassium phosphate buffer (pH 6.0) and 0.1 ml of the crude enzyme from the culture, and the mixture was incubated in water bath at 60 °C for 10 min. The reaction was stopped with 3.5 ml of 30 mM NaOH. Finally, 0.5 ml of 0.02% (w/v) phenolftalein in 5 mM Na$_2$CO$_3$ was added and mixed well. After leaving the mixture to stand for 15 min at room temperature, the reduction in colour intensity was measured at 550 nm. A blank lacking the enzyme is tested simultaneously with each batch of samples. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-CD from soluble starch in 1 min.

2.4. Experimental design and optimization

Response surface methodology [11] was applied to optimize the cultivation conditions for the excretory over-expression of recombinant CGTase in *E. coli*. The optimization was designed based on a rotatable central composite design (CCD) with a total of 50 experimental trials that involving 10 star points and 8 replicates at the central points. DESIGN EXPERT (State-Ease, USA) was applied to analyze the obtained results. The cultivation conditions investigated are post-induction temperature (X_1), induction-starting time (X_2), concentration

Table 1
Cultivation conditions and the levels studied in the optimization design

Factors	Notation	Unit	Low star point	Low level	Center point	High level	High star point
			$-\alpha$	-1	0	$+1$	$+\alpha$
Post-induction temperature	X_1	°C	20.00	25.80	30.00	34.20	40.00
Induction-starting time[a]	X_2	OD	0.30	0.79	1.15	1.51	2.00
Concentration of xylose	X_3	mM	0.00	0.29	0.50	0.71	1.00
Concentration of IPTG	X_4	mM	0.00	0.03	0.05	0.07	0.10
Post-induction time	X_5	h	0.00	13.90	24.00	34.10	48.00

[a] Induction-starting time is dependent on the optical density of the cell at 600 nm.

of xylose (X_3), concentration of IPTG (X_4) and post-induction time (X_5), with the CGTase enzyme activity collected as the responses (Y). Details of lower limit and the upper limit are shown in Table 1.

For statistical calculations, the relation between the coded values and actual values were as described in Eq. (1):

$$X_i = \frac{A_i - A_0}{\Delta A} \tag{1}$$

where X_i is a coded value of the variable; A_i is the actual value, A_0 is the actual value of A_i at the center point, and the star point was set with α of 2.378 from the coded center point. Experiments were conducted as per design matrix (Table 2) that was arranged by DESIGN EXPERT and a mathematical model was developed. The significance among the groups of the experimental data was analyzed using the ANOVA test and a P-value (Probability $> F$) less than 0.05 indicated that the model terms are significant. Adequacy of the model developed was further analyzed.

Table 2
Central composite design matrix, the predicted and experimental value obtained for the expression of CGTase from recombinant *E. coli*

Standard[a]	Coded values					Enzyme activity (U/ml)			
	X_1	X_2	X_3	X_4	X_5	Actual value[b]	Predicted value[c]Z ($\acute{Y}$)	Actual value[c] (Y)	Residual ($Y - \acute{Y}$)
1	−1	−1	−1	−1	−1	673.69	6.66	6.69	0.03
2	1	−1	−1	−1	−1	46.43	5.27	5.17	−0.10
3	−1	1	−1	−1	−1	604.88	6.42	6.60	0.18
4	1	1	−1	−1	−1	77.98	5.22	5.34	0.12
5	−1	−1	1	−1	−1	487.18	6.55	6.43	−0.12
6	1	−1	1	−1	−1	108.14	5.21	5.47	0.27
7	−1	1	1	−1	−1	434.86	6.35	6.34	0.01
8	1	1	1	−1	−1	54.61	5.20	5.22	0.02
9	−1	−1	−1	1	−1	447.48	6.33	6.36	0.03
10	1	−1	−1	1	−1	49.61	4.99	5.19	0.20
11	−1	1	−1	1	−1	239.32	6.10	5.91	−0.18
12	1	1	−1	1	−1	42.81	4.95	5.15	0.20
13	−1	−1	1	1	−1	397.73	6.29	6.27	−0.02
14	1	−1	1	1	−1	18.53	5.00	5.00	0.00
15	−1	1	1	1	−1	384.41	6.10	6.24	0.14
16	1	1	1	1	−1	20.92	5.01	5.02	0.01
17	−1	−1	−1	−1	1	770.92	6.88	6.80	−0.08
18	1	−1	−1	−1	1	101.85	5.40	5.45	0.05
19	−1	1	−1	−1	1	566.13	6.66	6.55	−0.11
20	1	1	−1	−1	1	85.46	5.37	5.37	0.00
21	−1	−1	1	−1	1	719.75	6.78	6.74	−0.04
22	1	−1	1	−1	1	56.90	5.35	5.23	−0.12
23	−1	1	1	−1	1	481.63	6.61	6.42	−0.19
24	1	1	1	−1	1	110.53	5.38	5.48	0.10
25	−1	−1	−1	1	1	506.05	6.52	6.46	−0.06
26	1	−1	−1	1	1	19.55	5.09	5.01	−0.08
27	−1	1	−1	1	1	397.56	6.31	6.27	−0.04
28	1	1	−1	1	1	34.80	5.07	5.10	0.03
29	−1	−1	1	1	1	417.96	6.49	6.31	−0.18
30	1	−1	1	1	1	39.01	5.12	5.13	0.01
31	−1	1	1	1	1	417.82	6.33	6.31	−0.02
32	1	1	1	1	1	35.49	5.15	5.11	−0.04
33	−α	0	0	0	0	5818.72	8.41	8.69	0.28
34	α	0	0	0	0	29.13	5.35	5.07	−0.28
35	0	−α	0	0	0	422.38	6.23	6.31	0.08
36	0	α	0	0	0	228.79	5.97	5.88	−0.09
37	0	0	−α	0	0	399.99	6.35	6.27	−0.08
38	0	0	α	0	0	459.11	6.30	6.38	0.08
41	0	0	0	0	−α	5.06	5.23	4.91	−0.32
42	0	0	0	0	α	264.34	5.65	5.98	0.33
43	0	0	0	0	0	234.97	5.99	5.90	−0.09
44	0	0	0	0	0	208.91	5.99	5.83	−0.16
45	0	0	0	0	0	381.16	5.99	6.24	0.25
46	0	0	0	0	0	295.58	5.99	6.05	0.06
47	0	0	0	0	0	284.24	5.99	6.03	0.04
48	0	0	0	0	0	282.92	5.99	6.02	0.03
49	0	0	0	0	0	248.32	5.99	5.94	−0.05
50	0	0	0	0	0	251.15	5.99	5.94	−0.05

[a] Standards 39 and 40 have been removed from the design due to the inadequacy of the outlier-T.
[b] Experimental enzyme activity.
[c] Enzyme activities that have been transformed according to the requirement of the statistical analysis.
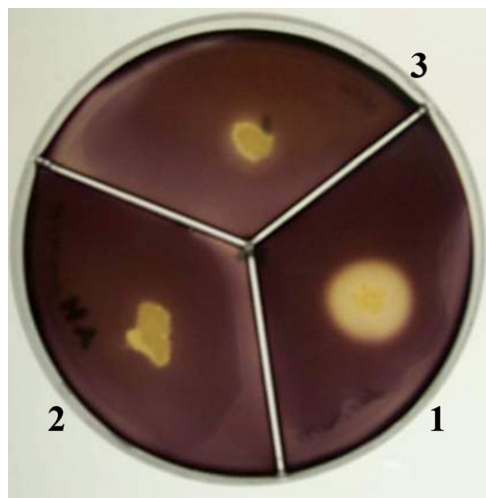
Fig. 1. Extracellular recombinant CGTase overexpressed by *E. coli* expression system. 1: recombinant clone *E. coli* that harboured both vector and *cgt* gene insertion; 2: *E. coli* that harboured both vector but without *cgt* gene insertion; 3: *E. coli* that carried only pJL3-BRP.

3. Results and discussion

3.1. Excretory over-expression of recombinant CGTase in E. coli

A local isolated cyclodextrin glucanotransferase (*cgt*) gene from *Bacillus* sp. G1 was amplified, cloned and expressed in the *E. coli* expression system. In order to obtain the extracellular recombinant CGTase, co-expression of bacteriocin release protein (BRP) was carried out with the amplified *cgt* gene. The BRP gene is regulated by the *lac* promoter in the recombinant plasmid pJL3 and the *cgt* gene was placed downstream of the *xyl* promoter in the recombinant plasmid pWH 1520, and so called pWH 1520-cgt. The recombinant plasmid pWH 1520-cgt was determined as the vector backbone for the over-expression of recombinant CGTase. The signal peptide of the *cgt* gene was removed during the PCR amplification by flanking the region coding for *cgt* mature gene and incorporating restriction enzymes *Bam*HI and *Sph*I. In order to determine whether the recombinant CGTase could be excreted onto solid media, iodine-staining method was applied to visualize the region of starch digestion. The colonies containing both genes clearly excreted the recombinant CGTase as evidenced by the halo of digested starch around the colonies (Fig. 1). Based on Fig. 1, *E. coli* that carried only pJL3-BRP, and *E. coli* that harboured both vector but without *cgt* gene insertion did not produce any extracellular recombinant CGTase. The formation of the halos resulting from starch digestion (Fig. 1) indicated that the excretory *E. coli* expression system was successfully obtained by simply co-expressing both BRP gene and *cgt* gene.

In most cases, targeting protein to the periplasmic space or to the culture medium facilitates downstream processing, folding, and in vivo stability, enabling the production of soluble and biologically active proteins at a reduced process cost [12]. Besides, in order to be distinguished from total cell lysis, which must generally be avoided due to the difficulty of reproducible

and control, targeting the recombinant protein into the culture medium is the major subject in over-expression study [13]. Co-expression of the bacteriocin release protein (BRP) is a possible method to promote a excretory *E. coli* over-expression system. The release of bacteriocin molecules is accompanied by the release of a subset of cytoplasmic proteins and many periplasmic proteins [14], including the recombinant CGTase that was overexpressed as an extracellular protein (Fig. 1). However, care must be exercised during such recombinant protein production so as not to over-induce the recombinant BRP, which will cause full-lysis and undesired cell death. Due to the potential quasi-lysis effect, co-expression must be carried out under a tightly repressible promoter and induction must be independent of the product gene promoter [12]. As a result, two different promoter regulation systems (*xyl* and *lac* promoters) were chosen to express both *cgt* and BRP genes, respectively. The possibility for the undesired low yield extracellular recombinant CGTase shall be reduced and the limited concentration of the IPTG would not be a limitation for the expression of the extracellular recombinant CGTase.

3.2. Effects of the cultivation conditions on the expression level of extracellular recombinant CGTase

In order to enhance the expression level of the extracellular recombinant CGTase, the rotatable central composite design (CCD) was applied to predict the optimum culture conditions for the recombinant *E. coli*. The experiments with different combination of cultivation conditions involved (X_1–X_5) as stated previously in Table 1 were performed. Extracellular recombinant CGTase was collected as the response or actual value as shown in Table 2. Enzyme activity collected ranged from a minimum value of 5.16 U/ml to a maximum value of 5818.72 U/ml based on the experiments. According to Myers and Montgomery [11], a ratio that is greater than 10 indicates transformation is required. Therefore, a natural log transformation for the model was performed with quadratic as the original design model.

The responses were analyzed by using the analysis of variance (ANOVA) and the details are shown in Table 3. The *P*-value for the model source, each model terms and interactions were detailed in the ANOVA (Table 3). The *P*-value less than 0.0001 indicate that the regression model as shown in Eq. (2) is significant. There is only 0.01% chance that the model failure occurs due to noise. Model terms X_1, X_4, X_5, X_1^2, X_3^2, X_4^2 and X_5^2 are significant factors that influence the expression of recombinant CGTase due to the *P*-value, which is less than 0.05. *P*-value for term 'lack of fit' in Table 3, which is greater than 0.05 indicates that the 'lack of fit' of model is insignificant. In other words, the model is fit with the responses data collected and is desirable for the following experiment.

An actual model as shown in Eq. (2) was developed to predict the optimum cultivation conditions that are to maximize the expression of extracellular recombinant CGTase. All terms regardless of their significance are included in the following equation, where *Y* is the response, that is, the enzyme activity (U/ml) and X_1–X_5 are the actual values of the test variables: the post-induction temperature, induction-starting time, concentra-

Table 3
ANOVA for response surface quadratic model for the expression of extracellular recombinant CGTase from recombinant E. coli

	Sum of squares	Degree of freedom	Mean square	F-value	P-value* (Probability > F)
Source model	22.20	20	1.11	32.71	<0.0001
X_1 (post-induction temperature)	17.90	1	17.90	527.56	<0.0001
X_2 (induction-starting time)	0.12	1	0.12	3.63	0.0676
X_3 (concentration of xylose)	4.83E−003	1	4.83E−003	0.14	0.7090
X_4 (concentration of IPTG)	0.62	1	0.62	18.36	0.0002
X_5 (post-induction time)	0.35	1	0.35	10.26	0.0035
X_1^2	1.26	1	1.26	37.12	<0.0001
X_2^2	0.02	1	0.02	0.52	0.4753
X_3^2	0.18	1	0.18	5.22	0.0304
X_4^2	0.94	1	0.94	27.72	<0.0001
X_5^2	0.49	1	0.49	14.35	0.0008
$X_1 X_2$	0.08	1	0.08	2.26	0.1443
$X_1 X_3$	6.66E−003	1	6.66E−003	0.20	0.6613
$X_1 X_4$	5.54E−003	1	5.54E−003	0.16	0.6893
$X_1 X_5$	0.02	1	0.02	0.44	0.5126
$X_2 X_3$	4.54E−003	1	4.54E−003	0.13	0.7175
$X_2 X_4$	1.51E−004	1	1.51E−004	4.49E−003	0.9473
$X_2 X_5$	1.87E−003	1	1.87E−003	0.06	0.8163
$X_3 X_4$	0.01	1	0.01	0.30	0.5884
$X_3 X_5$	7.27E−004	1	7.27E−004	0.02	0.8847
$X_4 X_5$	1.89E−003	1	1.89E−003	0.06	0.8154
Residual	0.92	27			
Lack of fit	0.81	0.81	0.04	2.63	0.0967
Pure error	0.11	0.11	7	0.02	
Correlation total	47	47			

* P-value (Probability > F) less than 0.05 indicated that the model terms are significant.

tion of xylose, concentration of IPTG and post-induction time:

$$Ln(Y + 130.00)$$

$$= 18.5580 - 0.7261X_1 - 1.6069X_2 - 2.3086X_3$$

$$+ 58.3450X_4 + 0.0684X_5 + 8.8728E - 003X_1^2$$

$$+ 0.1459X_2^2 + 1.3308X_3^2 - 709.3003X_4^2 - 9.5761E$$

$$- 004X_5^2 + 0.0326X_1X_2 + 0.0163X_1X_3 + 0.1489X_1X_4$$

$$- 5.0934E - 004X_1X_5 + 0.1585X_2X_3 + 0.2891X_2X_4$$

$$+ 2.1183E - 003X_2X_5 + 4.0354X_3X_4 + 2.2464E$$

$$- 003X_3X_5 - 0.0362X_4X_5 \qquad (2)$$

The fitness of the model was expressed by the R^2 value which is 0.9604, indicating that 96.04% of the variability in the response can be explained by the model. Meanwhile, the coefficient of determination (adjusted R^2) was calculated to be 0.9310, indicating that only 6.90% of the total variation were not included in the model. This value indicates a good agreement between the observed and the predicted values of recombinant CGTase production. Therefore, the regression model was applied to calculate the predicted values and the usefulness of the model is shown in Table 2, where the predicted values are closely matched with the experimental values after the transformation. In other words, the model obtained is applicable to predict the optimum cultivation conditions that will maximize the expression of extracellular recombinant CGTase.

The ANOVA (Table 3) shows that the post-induction temperature (X_1) gave the most significant effect to the expression

of recombinant CGTase ($P < 0.0001$). This significant effect also can be easily observed from Fig. 2 where the lower post-induction temperature enhances the expression of extracellular recombinant CGTase, and being the reverse, the higher temperature reduces the desire expression of extracellular recombinant CGTase. Wang et al. [15] optimized the culture conditions for the production of a new recombinant sea anemone neurotoxin by rotatable central composite design, with soluble fusion protein as the response data. From their findings, post-induction temperature significantly influences the production of a new
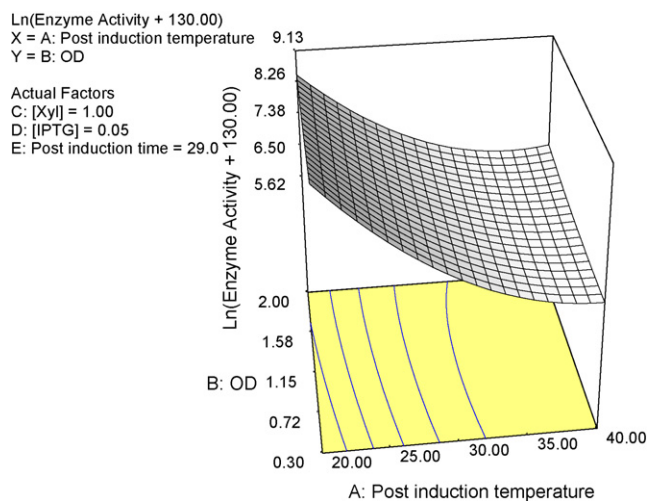

Fig. 2. Response surface plot of extracellular recombinant CGTase production: post-induction temperature vs. induction-starting time with constant level for concentration of xylose, concentration of IPTG and post-induction time.

recombinant sea anemone neurotoxin. Analysis for the expression of the *Trichoderma harzianum ech42* gene in two isogenic clones of *E. coli* by using the surface response methodology determined that the post-induction temperature was important to increase the expression level for the endochitinase [16]. In recombinant bacteria, protein aggregation is an ordinary consequence of thermal stress [17]. Therefore, by simply lowering the cultivation temperature, production of soluble recombinant protein in bacteria will be enhanced [18]. As a result, post-induction temperature is an important factor that will influence the expression level of extracellular recombinant CGTase. In order to obtain high yield of CGTase, Fig. 2 reveals that 20 °C is the best post-induction temperature for the expression of extracellular recombinant CGTase.

The concentration of IPTG (X_4) is also a significant factor ($P = 0.0002$) that influences the expression of the extracellular recombinant CGTase (Table 3). When *E. coli* cells were grown in LB broth without IPTG as reported by Fu et al. [19], almost no F1 protease was released into the medium. Obviously, IPTG is a significant factor to express the BRP and enable the recombinant protein to be excreted into the medium. Inducer also has a significant effect towards the expression level of recombinant endochitinase [16]. However, high concentration of IPTG did not significantly improve the *hk2a* fusion protein levels [15] and recombinant phytase production [20]. In this study, the concentration of xylose (X_3) gave an insignificant effect towards the expression level of the extracellular recombinant CGTase (Table 3), which is similar to the reports given by Wang et al. [15] and Sunitha et al. [20]. The contrast effect of the two inducers (IPTG and xylose) involved in this co-expression system indicated that the expression of BRP and CGTase are independent due to the various promoter regulation systems (Table 3). In other words, increasing the concentration of xylose (X_3) would not influence the effect of concentration for IPTG (X_4) towards the expression of both genes. Consequently, full-lysis of the cells when the over-induction of BRP occurred can be avoided.

The last significant factor in this study was the post-induction time (X_5) where the *P*-value is 0.0035. Based on Fig. 3, the expressed extracellular recombinant CGTase was lower with shorter post-induction time; in contrast, the longer duration resulted in a higher extracellular recombinant CGTase expression. Although longer cultivation time will enhance the expression level for extracellular recombinant CGTase, but it is not economical from the production perspective. Therefore, based on Fig. 3, the optimum 29 h post-induction time was selected as one of the optimized cultivation conditions.

Interactions among the cultivation conditions that were investigated can be easily predicted by the ANOVA (Table 3). The *P*-value that is greater than 0.05 indicated that the interactions between the cultivation conditions that will influence the production of extracellular recombinant CGTase are insignificant and could be ignored in the following study. Generally, the interactions between the cultivation conditions would not give a significant effect towards the production of the enzyme. Nevertheless, the interactions between the substrates normally gave a significant effect toward the production. As reported by Wong et al. [21], initial screening of the fermentation variable for the
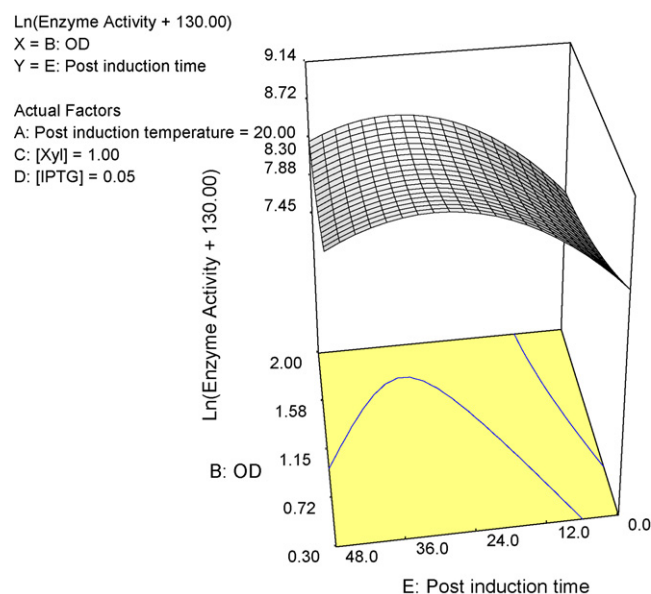

Fig. 3. Response surface plot of extracellular recombinant CGTase production: induction-starting time vs. post-induction time with constant level for post-induction temperature, concentration of xylose and concentration of IPTG.

production of CGTase from local isolated *Bacillus stearothermophillus* HR1 also indicated that the interactions between the cultivation conditions (temperature, pH and agitation speed) are insignificant. However, the majority of the interaction effects between the medium components are significant for the production of β-CGTase [22], which is contradictory to the cultivation conditions studied by Wong et al. [21]. The insignificant interaction effects simplify the scale-up process for the enzyme production and are desirable for most of the large-scale production.

3.3. Optimization of the cultivation conditions for the expression of extracellular recombinant CGTase

After investigating the effect of the post-induction temperature (X_1), induction-starting time that based is on the cell density at 600 nm (X_2), concentration of xylose (X_3), concentration of IPTG (X_4) and the post-induction time (X_5), contour plots and response surface plots that were calculated according to the model (Eq. (2)) are shown in Figs. 2–5. By using the plots, the effects of the cultivation conditions towards the expression level of the extracellular recombinant CGTase were analyzed and the optimum value for each factor investigated was predicted.

The response surface plots have shown that the optimum predicted cultivation conditions for the maximum production of extracellular recombinant CGTase were found to be comprised of: 20 °C post-induction temperature (Fig. 2), induction-starting time when cell optical density is 0.3 at 600 nm (Fig. 4), 1.0 mM xylose (Fig. 4), 50 μM IPTG (Fig. 5) and 29 h post-induction time (Fig. 3).

The maximum value of enzyme activity calculated from the model according to the predicted optimum cultivation condition was 9144.28 U/ml. In order to confirm the predicted optimized cultivation condition, numbers of experiment with
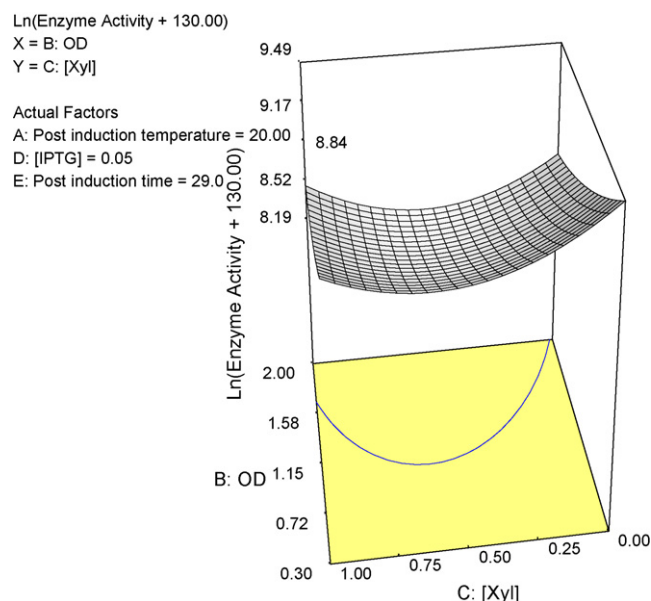
Fig. 4. Response surface plot of extracellular recombinant CGTase production: induction-starting time vs. concentration of xylose with constant level for post-induction temperature, concentration of IPTG and post-induction time.

the cultivation conditions proposed by the model had been carried out. The final extracellular recombinant CGTase activity obtained was 9542.30 U/ml, which was very close to the predicted value. The determined predicted and experimental enzyme activity that was maximized by the optimized cultivation condition is detailed in Table 4.

Based on Table 4, the expression of extracellular recombinant CGTase was successfully optimized and improved about 151-fold after the optimization was carried out. The profile for the expression of extracellular recombinant CGTase was shown in Fig. 6.
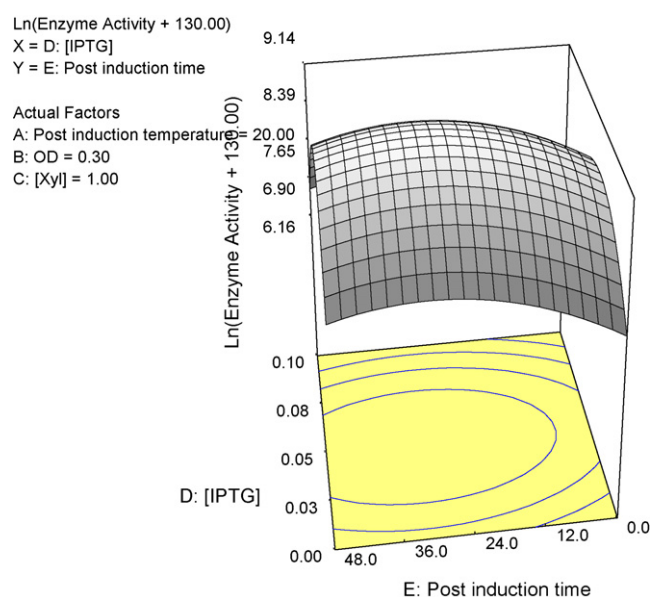

Fig. 5. Response surface plot of extracellular recombinant CGTase production: concentration of IPTG vs. post-induction time with constant level for post-induction temperature, induction-starting time and concentration of xylose.

Table 4
Summary of the optimized cultivation conditions for the production of extracellular recombinant CGTase

Cultivation conditions	Optimum value	Original value
Post-induction temperature	20 °C	37 °C
Induction-starting time	When cell optical density is 0.3 at 600 nm	When cell optical density is 0.5 at 600 nm
Concentration of xylose	1.0 mM	0.5 mM
Concentration of IPTG	50 μM	40 μM
Post-induction time	29 h	84 h

Extracellular recombinant CGTase for optimum value (U/ml): predicted, 9144.28; experimental, 9542.30 and for original value: experimental, 63.
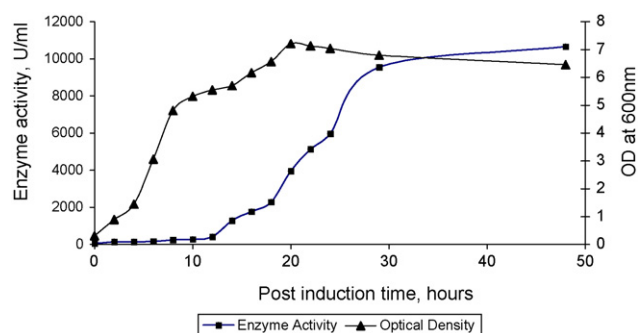

Fig. 6. Enzyme activity profile for the expression of extracellular recombinant CGTase under the optimized cultivation condition.

From Fig. 6, the expression level of extracellular recombinant CGTase occurred at the first 12 h after induction was not significant. The slow expression for the recombinant CGTase might due to the alteration of the cultivation temperature, where the optimum value 20 °C is not the optimum growth temperature (37 °C) for most of the cells especially for *E. coli*. However, low cultivation temperature will enhance the production of the desirable soluble recombinant protein in bacteria. Besides, excretion enables the production of soluble and biologically active recombinant protein. As a result, with the aids of both excretory *E. coli* expression system and low cultivation temperature, the high yield of the extracellular recombinant CGTase was achieved.

4. Conclusion

Excretory over-expression of CGTase from *Bacillus* sp. G1 was successfully carried out by co-expression with BRP (bacteriocin release protein) gene in *E. coli*. The optimization of the cultivation conditions to improve the expression and production of the recombinant CGTase was achieved by using response surface methodology. The optimized cultivation conditions are 20 °C post-induction temperature, induction-starting time when cell optical density is 0.3 at 600 nm, 1.0 mM xylose, 50 μM IPTG and 29 h post-induction time. The maximum value achieved after the optimization is 9542.30 U/ml, which is 151-fold increment of CGTase expression compared before the optimization (63 U/ml).

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