ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 8 (2): 184-193, 2009
ISSN 1682-296X

Improvement of Excretory Overexpression for *Bacillus* sp. G1 Cyclodextrin Glucanotransferase (CGTase) in Recombinant *Escherichia coli* through Medium Optimization

[1]P.K. Lo, [1]C.Y. Tan, [2]O. Hassan, [3]A. Ahmad, [4]N.M. Mahadi and [1]R.M. Illias
[1]Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[2]School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[3]Department of Chemical Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[4]Malaysia Genome Institute, UKM-MTDC Smart Technology Center, 43600 Bangi, Selangor, Malaysia

Abstract: In the study presented, Design of Experiments (DOE) was combined with statistical analysis such as fractional factorial design and small central composite design Response Surface Methodology (RSM) to significantly increase the extracelullar recombinant CGTase yields in fermentation flasks. The new medium obtained by the statistical analysis for the significant medium components comprised of 12 g L^{-1} NZ Amine A, 24 g L^{-1} yeast extract, 9.44 g L^{-1} KH_2PO_4, 4.4 g L^{-1} K_2HPO_4, 4.58 mL L^{-1} glycerol, 7 mg L^{-1} sucrose and 3 mg L^{-1} $CuCl_2$. Yields were improved about 68% from 9.54 to 16.07 kU mL^{-1} in flasks when using the optimized cultivation medium. The results suggest that the overexpression level of recombinant CGTase excreted into the culture medium using the recombinant *Escherichia coli* could be improved through medium optimization.

Key words: Extracellular recombinant CGTase, medium optimization, *E. coli*, RSM

INTRODUCTION

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) is a unique extracellular enzyme that can degrade starch to form non-reducing cyclic malto-oligosaccharides called cyclodextrin (CDs) (Tonkova, 1998). CDs have a peculiar torus-shaped structure with hydrophobic interior cavity and hydrophilic exterior surface and therefore can form inclusion complexes with a variety of hydrophilic guest compounds (Szetjli, 1998). As a result, CDs can change physical and chemical properties of the encapsulated guest compounds.

Given the potential uses of CGTase and their high demand, requirement exists for the cloning and recombinant clones that will produce CGTase with high volumetric yield and the development of low cost industrial media formulation. Park *et al.* (1997) applied the fed-batch strategy for the fermentation of recombinant *Escherichia coli* to produce *Bacillus macerans* CGTase together with some modifications of the inducer used; a 31-fold increase in the CGTase activity was achieved. Besides, in order to enhance the production of recombinant CGTase, Kim *et al.* (2005) co-expressed the folding accessory proteins for the production of active CGTase of *B. macerans* in recombinant *E. coli*, using fed-batch fermentation and this resulted in 1200 U mL^{-1} CGTase activity. Expression of foreign protein in *E. coli* normally resulted with intracellular protein production. A few studies on the usage of recombinant *E. coli* for excretion of foreign protein into the culture medium were conducted by previous researchers. This include the overexpression of the phytase from *E. coli* as an extracellular production in bioreactor by using *kil* gene (Miksch *et al.*, 2002), secretory expression of thermostable T1 lipase through bacteriocin release protein by Rahman *et al.* (2005) and secretion of heterologous CGTase of *Bacillus* sp. E1 from *E. coli* by Yong *et al.* (1996) with signal peptide. In this study, co-expression of the Bacteriocin Release Protein (BRP) with the expression of CGTase in recombinant *E. coli* enable the excretion of

Corresponding Author: Rosli Md Illias, Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia Tel: 607-5535564 Fax: 607-5581463

recombinant CGTase into the culture medium. However, none of the research regarding the medium optimization for the production of extracellular recombinant β-CGTase in *E. coli* was reported.

Designing a fermentation medium is of critical importance because medium composition will absolutely affect the product concentration, yield and volumetric productivity (Kennedy and Krouse, 1999). Medium optimization by classical methods, which only involves the change of one variable at a time, is definitely time-consuming. Besides, the interaction effects between the variables will be neglected. In this respect, the Response Surface Methodology (RSM), which is used to study the effects of several factors influencing the responses by varying them simultaneously and conducting a limited number of experiments, was the best choice for the medium optimization. This study applied the response surface methodology, the fractional factorial design and small central composite design, for the medium optimization with the objective to maximize the production of the extracellular recombinant CGTase by excretory *E. coli* overexpression system.

MATERIALS AND METHODS

Strains and plasmid: CGTase gene (*cgt*) from *Bacillus* sp. G1 was isolated and cloned in *E. coli* JM109 (F', traD36, proAB, lacIqZΔM15) for the DNA manipulation and propagation. The *E. coli* K12 N3406 (Thr leu thi lacY tonA supE) that carried the pJL3-BRP gene (MoBiTec) was used as the real host for the overexpression of cgt, in which the cgt was placed under the control of the xylA promoter in pWH 1520 (MoBiTec) and was inducible by xylose. Meanwhile, the BRP gene was regulated by lac promoter and was induced by IPTG.

Expression of recombinant CGTase: *Escherichia coli* carrying the appropriate plasmids was grown in an initial medium consisting 12 g L^{-1} NZ Amine, 24 g L^{-1} yeast extract, 8 mL L^{-1} glycerol, 2.2 g L^{-1} KH_2PO_4 and 9.4 g L^{-1} K_2HPO_4, 100 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol, overnight at 37°C on a rotary shaker at 200 rpm as the seed culture. The experiment was carried out with 50 mL of culture shaken in 250 mL flasks at 37°C at 200 rpm. A 1 mL aliquot per 50 mL production medium served as its inoculum. When the cell optical density reached 0.3 at 600 nm, xylose at the final concentration of 1.0 mM and 50 μM IPTG was added and then the temperature was shifted to 20°C. Extracellular recombinant CGTase activity was measured after induction had started for 29 h (Lo *et al*., 2007).

CGTase enzyme assay: CGTase assay was conducted according to the Kaneko method (Kaneko *et al*., 1987). 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 after some appropriate dilution and the mixture was incubated in water bath at 60°C for 10 min. Reaction was stopped by adding 3.5 mL of 30 mM NaOH. Finally, 0.5 mL of 0.02% (w/v) phenolftalein in 5 mM Na_2CO_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 with a spectrophotometer at 550 nm. A blank lacking the enzyme was tested simultaneously with each batch of samples. One unit of enzyme activity is defined as the amount of enzyme that forms 1 μmol of β-CD from soluble starch in 1 min.

Medium optimization by design of experiments: DOE analysis was performed using Design Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 6.0.4). A 2^{15-10} fractional factorial design was applied to determine the statistically significant medium components with 3 center points repeated and incorporated 32 factorial points. Medium optimization was further expanded to a small central composite design with the results obtained from the screening process. The small central composite design consisted of 14 axial points, 5 center points and 22 factorial points. For all analysis, ANOVA test was considered to evaluate the significance for the experimental data using the p-value whereas p-value less than 0.05 indicates that the model terms are significant. Center-points for the factorial designs were applied to test the curvatures as well as the replication error for testing main and two-way interaction effects. Plots for the designs were constructed using the models generated and these plots were based on the best fitted model found by fitting the full quadratic models with the experimental data.

Batch fermentation: The cultivation of the recombinant *E. coli* was performed in the Biostat MD B. Braun stirred tank reactor consisted of barosilicate glass culture vessel with 2 L of total volume. Batch mode bioreactor was carried out for the recombinant CGTase expression. The optimized cultivation conditions (Lo *et al*., 2007) and new optimized medium were applied for the batch mode cultivation. Initial working volume was 75% of the total volume. The stirrer speed was maintained at 200 rpm and the oxygen was controlled around 20%. The overnight cultured inoculum was transferred to the working volume of medium and the cells were first maintained at 37°C. When the cell optical density reached 0.3 at 600 nm,

xylose at the final concentration of 1.0 mM and 50 μM IPTG was added and then the temperature was shifted to 20°C. Extracellular recombinant CGTase activity was measured after induction had started for 36 h.

RESULTS AND DISCUSSION

Selection of medium components: As reported previously, the extracellular recombinant CGTase was successfully overexpressed in an excretory *E. coli* overexpression system; besides, flasks cultivation conditions were optimized in the early studies and the maximum yield for extracellular recombinant CGTase was 9.54 kU mL^{-1} (Lo *et al.*, 2007). In a recent work, modified TB gave the highest expression level when the cultivation temperature was maintained at 37°C (Fig. 1). Previous researchers reported that addition of medium nutrients enhanced the expression level of the recombinant *E. coli*. Hence, the effect of different medium components was investigated and six categories of components were studied in the early one-at-a-time screening stage. The six categories involved are carbon sources (sugars and polyols), nitrogen sources, mineral salts, vitamins, trace elements and some protein folding agents. The original medium was enriched or replaced accordingly with the certain concentration as given by the references. The details and the yield of extracellular recombinant CGTase are shown in Table 1 and Fig. 2.

Carbon sources such as lactose, sucrose, fructose, maltose, glucose, mannitol and sorbitol were added to the original cultivation medium during the experiment. Except for fructose, maltose, glucose, mannitol and sorbitol, the rest of the carbon sources gave satisfactory enzyme activity results (10.15-13.65 kU mL^{-1} enzyme) compared to the control (9.79 kU mL^{-1} enzyme) given by the original cultivation medium (Table 1 and Fig. 2). The lowest yield activity was obtained with glucose (1.21 kU mL^{-1} enzyme), which contradicted with the defined medium well established by Riesenberg *et al.* (1990). As for nitrogen sources, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, casamino acid and tryptophan were screened manually. According to Table 1, enzyme activities of 4.10, 4.36, 8.39 and 9.35 kU mL^{-1} enzyme were obtained with NH_4Cl, NH_4NO_3, casamino acid and tryptophan, respectively, which is lower than the control enzyme activity (9.79 kU mL^{-1}). The best performance was seen for $(NH_4)_2SO_4$ (10.06 kU mL^{-1} enzyme).

In order to study the effects of the mineral salts and vitamins on the expression level of the extracellular recombinant CGTase, the original medium was enriched with salt components such as NaCl (LB), $CaCl_2$, $MgSO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 7H_2O$, NaH_2PO_4 orthophosphate

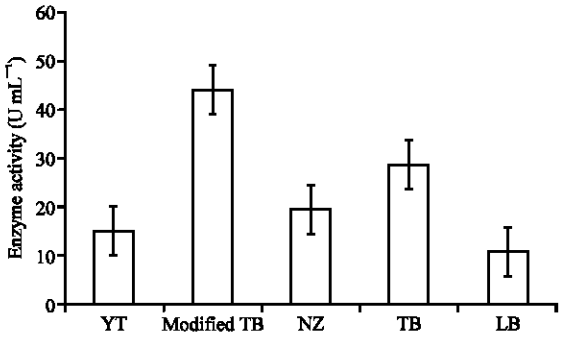

Fig. 1: Comparison for the expression level of the extracellular recombinant CGTase in media selection phase after the post induction for 24 h, before the cultivation conditions optimization. Modified TB obviously gave better performance than the others. (YT: YT medium, TB: Terrific broth, NZ: NZ medium, LB: Luria-Bertani medium)

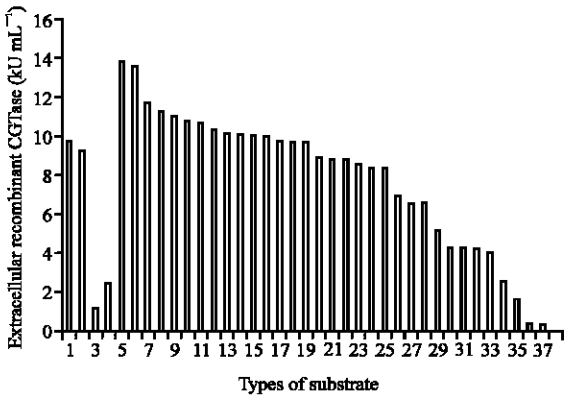

Fig. 2: Effects of substrate components toward the expression level of the extracellular recombinant CGTase (Types of substrate were detailed in Table 1)

NaOH and sodium acetate. However, only $CaCl_2$ gave positive effect towards the expression level of extracellular recombinant CGTase (9.98 kU mL^{-1} enzyme), due to the stabilizing effect of $CaCl_2$ on the CGTase (Han and Tao, 1999). Besides, vitamin B1, proline, trisodium citrate and citric acid together with glucose were also added and only vitamin B1 enhanced the expression level to 10.11 kU mL^{-1} enzymes. Finally, trace elements such as $CoCl_2 \cdot 6H_2O$, EDTA, $FeCl_3$, $FeCl_2$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CuCl_2$, $CuSO_4$, CuN_2O_3, H_3BO_3 and some protein folding agents such as urea and Triton X-100 were added to the original medium. Better performance was seen for $CoCl_2 \cdot 6H_2O$, EDTA, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CuCl_2$ and $CuSO_4$, which

Table 1: Categories and the concentrations of the medium components involved

Components	Concentration studied (Manually screening)	Yield manually screening (U mL^{-1})	Label as No.	Concentration studied (Screening)	Concentration studied (Optimization)
Sugar (Carbon)					
Lactose*	3 g L^{-1}	10145.01	13, X_7	0-6 g L^{-1}	
Sucrose (1)	10 g L^{-1}	10305.51	12		
Sucrose (2)*	3 g L^{-1}	13649.39	6, X_8	0-6 g L^{-1}	0-3 g L^{-1}
Fructose	3 g L^{-1}	2588.06	34		
Maltose	3 g L^{-1}	5218.53	29		
Glucose (Glycerol was substituted)	10 g L^{-1}	1205.67	3		
Polyols (Carbon)					
Mannitol	0.5 M	4349.27	31		
Sorbitol	0.5 M	6606.22	27		
Glycerol*	8 mL L^{-1}	9786.17	1, X_5	0-16 mL L^{-1}	0-8 mL L^{-1}
Nitrogen					
NH_4Cl	1 g L^{-1}	4102.74	33		
$(NH4)_2SO_4$*	4 g L^{-1}	10055.33	15, X_{13}	0-8 g L^{-1}	
NH_4NO_3	4 g L^{-1}	4355.42	30		
Casamino acid	2 g L^{-1}	8385.25	25		
Tryptophan (NZ Amine was substituted)	12 g L^{-1}	9345.33	2		
NZ Amine*	12 g L^{-1}	9786.17	1, X_1	0-24 g L^{-1}	12-24 g L^{-1}
Yeast extract*	24 g L^{-1}	9786.17	1, X_2	0-48 g L^{-1}	24-48 g L^{-1}
Vitamins					
Vitamin B1*	0.1 g L^{-1}	10107.85	14, X_6	0-0.2 g L^{-1}	
Proline	0.05 g L^{-1}	8610.97	23		
Trisodium citrate	1 g L^{-1}	6580.75	28		
Citirc acid (together with glucose)	1.7 g L^{-1}	2534.10	4		
Salts					
NaCl	10 g L^{-1}	8918.09	20		
$CaCl_2$	1.2 g L^{-1}	9981.70	16		
$MgSO_4 \cdot 7H_2O$	1.2 g L^{-1}	6977.22	26		
$Na_2HPO_4 \cdot 7H_2O$	6 g L^{-1}	4307.63	32		
NaH_2PO_4 orthophosphate	1 mM	9752.19	18		
NaOH	3 g L^{-1}	1731.88	35		
Sodium Acetate	2.5 g L^{-1}	410.89	37		
KH_2PO_4*	2.2 g L^{-1}	9786.17	1, X_4	0-4.4 g L^{-1}	2.2-4.4 g L^{-1}
K_2HPO_4*	9.4 g L^{-1}	9786.17	1, X_3	0-18.8 g L^{-1}	9.4-18.8 g L^{-1}
Trace elements					
$CoCl_2 \cdot 6H_2O$*	2.5 mg L^{-1}	10720.56	11, X_9	0-5 mg L^{-1}	
EDTA*	5 mg L^{-1}	10796.27	10, X_{10}	0-10 mg L^{-1}	
$FeCl_3$	0.025 g L^{-1}	8430.14	24		
$FeCl_2$	0.025 g L^{-1}	9776.07	17		
$MnCl_2 \cdot 4H_2O$	16 mg L^{-1}	8816.95	22		
$ZnSO_4 \cdot 7H_2O$*	2 mg L^{-1}	11262.58	8, X_{11}	0-4 mg L^{-1}	
$FeSO_4 \cdot 7H_2O$*	0.2 g L^{-1}	11027.54	9, X_{12}	0-0.4 g L^{-1}	
$CuCl_2$*	1.5 mg L^{-1}	11739.64	7, X_{15}	0-3 mg L^{-1}	1.5-3 mg L^{-1}
$CuSO_4$*	1.5 mg L^{-1}	13821.57	5, X_{14}	0-3 mg L^{-1}	
CuN_2O_3	1.5 mg L^{-1}	9722.88	19		
H_3BO_3	3 mg L^{-1}	8885.48	21		
Denaturing agents					
Urea	60.06 g L^{-1}	60.37	38		
Triton X-100	0.5% v/v	474.53	36		

*The selected medium components for the fractional factorial medium screening stage

showed enzyme activities of 10.72, 10.80, 11.26, 11.03, 11.74 and 13.82 kU mL^{-1}, respectively (Table 1). All the protein folding agents reduced the expression level of the recombinant enzymes. Finally, the 10 components that gave the highest performance of enzyme activity were included in the fractional factorial design, together with the components from the original medium, as shown in Table 1.

Fractional factorial design for screening process: In order to formulate a better medium for the growth of recombinant *E. coli* harbouring plasmid pWH 1520-cgt and hence increase the expression and production of the extracellular recombinant CGTase, effects of medium components were further investigated through the fractional factorial design to study the interaction effects between the components remained. Besides, the first order model generated by the fractional factorial design was able to identify the significant components for the medium optimization small central composite design.

A total of 15 medium components were chosen after the one-at-a-time screening method in which the initial

medium components included are detailed in Table 1, together with the concentration studied. The statistical model predicting the production of extracellular recombinant CGTase with the medium components is given in the first order model, Eq. 1:

$$\sqrt{(Y+164.41)} = 9.6013 + 2.1175X_1 + 0.6717X_2 + 1.5241X_3 + 6.2939X_4 + 1.2804X_5 + 23.6550X_6 - 0.8403X_7 - 1.6801X_8 - 1.5633X_9 - 0.5422X_{10} + 1.6503X_{11} + 81.9179X_{12} + 3.2418X_{13} - 1.2006X_{14} - 11.3194X_{15} - 7.9399X1X_{12} - 0.2029X1X_{13} + 0.5847X1X_{15} \quad (1)$$

where, Y is the extracellular recombinant CGTase activity (kU mL^{-1}) and the model was used to predict the enzyme activity.

The fitness of the regression Eq. 1 was confirmed by the determination coefficient R^2 where R^2 implies that the samples variation of 95.83% for the extracellular recombinant CGTase was attributed to the medium components investigated. The corresponding Analysis of Variance (ANOVA) is presented in Table 2, where only the significant components are considered where their p-values (Probability>F) are less than 0.05. Components X_1 (NZ Amine A), X_2 (yeast extract), X_3 (K_2HPO_4), X_4 (KH_2PO_4), X_5 (glycerol), X_8 (sucrose) and X_{15} ($CuCl_2$) show p-value less than 0.05 and were considered to be significant while the rest having p-value more than 0.05 and hence were considered insignificant for the enhancement of expression level for the extracellular recombinant CGTase. Furthermore, the significance of the curvature with p-value less than 0.0001 anticipated an optimum concentration for each significant component within the range studied. In order to achieve a more accurate concentration for the components, an optimization design is required.

Small central composite design for optimization process: The seven selected significant medium components, NZ Amine A (X_1), yeast extract (X_2), K_2HPO_4 (X_3), KH_2PO_4 (X_4), glycerol (X_5), sucrose (X_8) and $CuCl_2$ (X_{15}), were, further optimized using a small central composite design, with the new range of concentration determined (Table 1). The software Design Expert (Stat-Ease Inc., Minneapolis, USA, Version 6.0.4) was used for the data analysis and quadratic model building (Eq. 2) and the theoretical values of CGTase activity could be obtained with the model fitting techniques:

$$(Y+131.31)^{1.15} = 3.26E+005 - 29818.09X_1 - 14790.39X_2 - 38246.24X_3 + 1.83E+005X_4 + 79466.31X_5 - 1.61E+005X_8 + 1.29E+005X_{15} - 34.77X_1^2 - 32.12X_2^2 - 601.16X_3^2 - 8037.59X_4^2 - 932.89X_5^2 - 2029.20X_8^2 + 9856.90X_{15}^2 + 578.24X_1X_2 + 1365.43X_1X_3 + 301.99X_1X_4 - 1228.69X_1X_5 + 3467.09X_1X_8 - 4420.38X_1X_{15} + 924.67X_2X_3 - 2006.02X_2X_4 - 1052.87X_2X_5 + 1574.95X_2X_8 + 1345.12X_2X_{15} - 4816.61X_3X_4 + 1274.35X_3X_5 + 4482.57X_3X_8 - 1081.82X_3X_{15} + 4927.22X_4X_5 - 2684.02X_4X_8 - 1247.94X_4X_{15} + 3370.67X_5X_8 - 22097.46X_5X_{15} - 9927.33X_8X_{15} \quad (2)$$

where, Y represents the predicted CGTase activity (kU mL^{-1}). The statistical model (Eq. 2) adequacy was analyzed by p-value and the correlation coefficient R is tabulated in Table 3. The p-value of the lack of fit is 0.8625 which is not significant indicating the experimental data achieved a reliable response. Adequate precision that measures the signal to noise ratio obtained is 15.803, which is greater than 4 and is desirable to indicate the responses achieved are reliable.

Maximization of the regression model (Eq. 2) was carried out using iterative procedure (Rao *et al*., 2000; Rosenbrock, 1960) to obtain the optimum concentrations of each independent variable. The 3D response surface plots of the variables presented in Fig. 3-6 provide a method to visualize the relationship between the response and experiment levels of each variable. The p-value of the regression model (Eq. 2) obtained in Table 3 (0.0023) indicates that the global maximum in the recombinant protein yield was confined within the experimental range. From Table 3 and based on the p-value, K_2HPO_4 (X_3) has

Table 2: ANOVA for the fractional factorial screening design

Source	Sum of squares	df	Mean	F-value	Prob >F
Model	48474.50	18	2693.03	19.15	<0.0001
Curvature	3879.69	1	3879.69	27.60	<0.0001
Lack of Fit	2079.80	13	159.98	10.99	0.0864
X_1 (NZ Amine A)	1631.82	1	1631.82	11.61	0.0039
X_2 (yeast extract)	8315.91	1	8315.91	59.15	<0.0001
X_3 (K_2HPO_4)	6567.85	1	6567.85	46.72	<0.0001
X_4 (KH_2PO_4)	6135.25	1	6135.25	43.64	<0.0001
X_5 (glycerol)	3357.28	1	3357.28	23.88	0.0002
X_8 (sucrose)	812.94	1	812.94	5.78	0.0296
X_{15} ($CuCl_2$)	1332.86	1	1332.86	9.48	0.0076

df: Degree of Freedom

Table 3: ANOVA of the small composite optimization design and interaction effect of the medium components

Source	Prob>F	Source	Prob>F
Model	0.0023	X_2X_3	0.0178
Lack of Fit	0.8625	X_2X_4	0.1362
X_1	0.4763	X_2X_5	0.0050
X_2	0.0091	X_2X_8	0.4221
X_3	0.0010	X_2X_{15}	0.2665
X_4	0.0192	X_3X_4	0.1692
X_5	0.0642	X_3X_5	0.1785
X_8	0.2336	X_3X_8	0.0347
X_{15}	0.0027	X_3X_{15}	0.9118
X_1X_2	0.0137	X_4X_5	0.2917
X_1X_3	0.0038	X_4X_8	0.7648
X_1X_4	0.7943	X_4X_{15}	0.9743
X_1X_5	0.1181	X_5X_8	0.3970
X_1X_8	0.0900	X_5X_{15}	0.0681
X_1X_{15}	0.1303	X_8X_{15}	0.5670

R^2 = 99.18%, R = 99.59%. Adequacy precision = 15.803

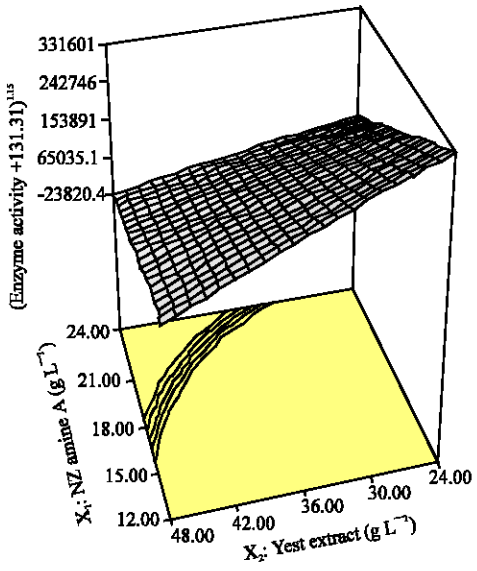

Fig. 3: Response surface plot of extracellular recombinant CGTase activity: Effect of NZ Amine A (X_1) and yeast extract (X_2) while the other variables were fixed at their predicted concentration (Table 4)

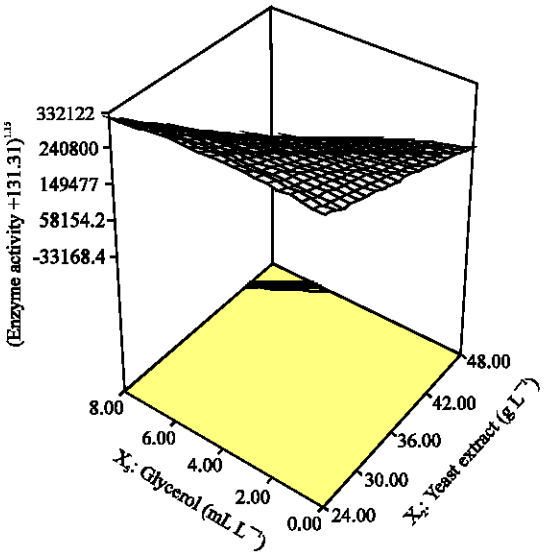

Fig. 5: Response surface plot of extracellular recombinant CGTase activity: Effect of yeast extract (X_2) and glycerol (X_5) while the other variables were fixed at their predicted concentration

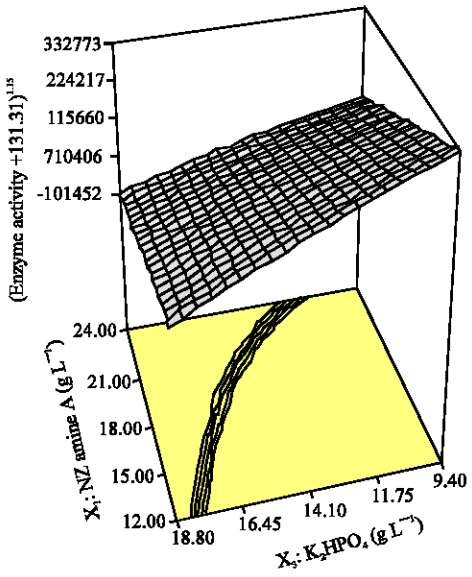

Fig. 4: Response surface plot of extracellular recombinant CGTase activity: Effect of NZ Amine A (X_1) and K_2HPO_4 (X_3) while the other variables were fixed at their predicted concentration

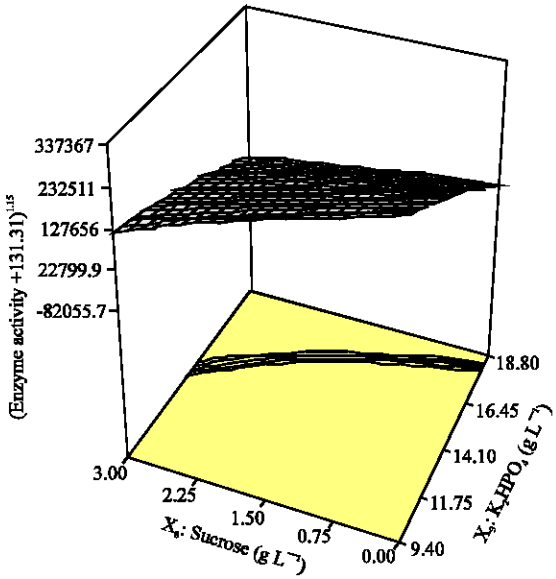

Fig. 6: Response surface plot of extracellular recombinant CGTase Activity: Effect of K_2HPO_4 (X_3) and Sucrose (X_8) while the other variables were fixed at their predicted concentration

the greatest impact towards the expression level (p-value is 0.0010) while NZ Amine A (X_1) has the least impact towards the expression level with p-value obtained is 0.4763, the largest p-value obtained among the medium components studied. The p-value in Table 3 shows that yeast extract (X_2) influences the expression level more than NZ Amine A (X_1); while the nitrogen sources, the yeast extract and NZ Amine A give more impact on the expression level more than the carbon sources such as glycerol and sucrose. The interaction effect of X_1X_3 (NZ Amine A and K_2HPO_4) gave the greatest influence towards the production of extracellular recombinant

CGTase with the p-value of 0.0178 when compared to all the interaction effects observed. The observation of the p-value is reasonable in this study as the recombinant *E. coli* requires a lot of nitrogen sources to synthesize the recombinant CGTase and potassium (K^+) is required by all organisms especially for those involved in protein synthesis (Madigan *et al*., 2003).

From Eq. 2, the negative fitted constant of X_1 (NZ Amine A) suggests that the concentration should be fixed at a low level (12 g L^{-1}) as well as the variable X_2 (yeast extract) and variable X_3 (K_2HPO_4), the concentrations for each variable were fixed at 24 g L^{-1} (Fig. 3) and 9.44 g L^{-1} (Fig. 4), respectively. Based on Fig. 5, when the concentration of yeast extract was fixed at 24 g L^{-1}, in order to achieve the highest enzyme activity, a concentration of 4.6 mL L^{-1} glycerol was selected. When variable X_3 (K_2HPO_4) was set at 9.4 g L^{-1}, Fig. 6 predicts that the highest enzyme activity would be achieved by fixing the sucrose (X_8) at 70 mg L^{-1}. The positive fitted constant of variable X_4 (KH_2PO_4) showed in Eq. 2 confines that the concentration for KH_2PO_4 should be chosen at the high level (4.4 g L^{-1}), as well as for the $CuCl_2$ (X_{15}), the high level concentration of 3 mg L^{-1} was selected finally. There are 2 new added medium components to the original medium after the medium optimization was carried out.

As a summary, the predicted optimum concentration for the medium components is NZ Amine A (X_1) 12 g L^{-1}; yeast extract (X_2) 24 g L^{-1}; K_2HPO_4 (X_3) 9.44 g L^{-1}; KH_2PO_4 (X_4) 4.4 g L^{-1}; glycerol (X_5) 4.6 mL L^{-1}; sucrose (X_8) 70 mg L^{-1}; $CuCl_2$ (X_{15}) 3 mg L^{-1}, while the predicted maximum yield for the extracellular recombinant CGTase was 14.78 kU mL^{-1}. For further verification, the new medium formulation was implemented in the cultivation and an optimal yield of extracellular recombinant CGTase activity at 16.07 kU mL^{-1} was attained. Before the new medium optimization was applied for the production of extracellular recombinant CGTase, an activity of 9.54 kU mL^{-1} was achieved with the optimized cultivation condition (Lo *et al*., 2007) and 1.68-fold increment in the activity yield was observed with the new formulated medium. Table 4 show the new medium optimization and the extracellular recombinant CGTase activity obtained. NZ Amine A and yeast extract remained the same concentration as the original medium, while the concentration for K_2HPO_4 and glycerol was quite similar with the original medium (Table 4). However, the concentration for KH_2PO_4 increases from 2.2 to 4.4 g L^{-1}. In this case, the potassium phosphate is considered to supply nutrient K^+ and to remain as a buffer as well.

There have been a number of studies conducted on formulation of medium components for both cultivation of recombinant clone and wild type, or selection of the best medium for some overexpression studies. Khushoo *et al.* (2004) compared some media such as LB, 2xYT and TB, which are well known for the expression of recombinant protein and finally found that TB resulted in 4-fold higher extracellular L-asparaginease II in recombinant *E. coli*. In this case, modified TB was chosen as the initial medium for the cultivation. Modified TB consisting of phosphate buffer for better production of extracellular recombinant CGTase compared to TB. K_2HPO_4 and KH_2PO_4, the main components in the phosphate buffer, which help to maintain the pH of the culture along the cultivation period, were added in the modified TB used. After a long cultivation period, the pH of the fermentation medium normally changes to acidic due to the production of acetic acid caused by the glucose metabolism (March *et al*., 2002; Park *et al*., 1997). Due to that condition, K_2HPO_4 (X_3) and KH_2PO_4 (X_4) remained significant in this case. Based on Fig. 6, which reveals that only minor changes of pH occurred during the cultivation period indicates that the advantages of using phosphate buffer solution consisting of K_2HPO_4 and KH_2PO_4 towards the production of extracellular recombinant CGTase. This suggests that phosphate buffer is capable of neutralizing the acids generated by *E. coli* in the course of metabolizing lactose as a partial carbon source (Ma *et al*., 2006). A similar concept was also applied by Xu *et al.* (2000) when the

Table 4: Summary of the optimized medium

			Extracellular recombinant CGTase activity (kU mL^{-1})		
				After optimize	
Medium components	Concentration before optimize	Concentration after optimize	Before optimize	$Y_{predicted}$	$Y_{experiment}$
NZ Amine A	12 g L^{-1}	12 g L^{-1}	9.54	14.78	16.07
Yeast extract	24 g L^{-1}	24 g L^{-1}			
K_2HPO_4	9.4 g L^{-1}	9.44 g L^{-1}			
KH_2PO_4	2.2 g L^{-1}	4.4 g L^{-1}			
Glycerol	4 mL L^{-1}	4.6 mL L^{-1}			
Sucrose	-	70 mg L^{-1}			
$CuCl_2$	-	3 mg L^{-1}			

phosphate salts, K_2HPO_4 and KH_2PO_4, were added into the medium for the production of human Epidermal Growth Factor (hEGF) in the recombinant *E. coli* K12. Maximal hEGF expression was detected at the initial pH of about 6.8 (Xu *et al*., 2000).

Trace elements that are essential to microbial nutrition were investigated in this study. However, $CaCl_2$ and $MgSO_4$ were excluded due to the interference of these elements with the autolytic process caused by the Bacteriocin Release Protein (BRP) (Dekker *et al*., 1999) and thus will inhibit the production of extracellular recombinant CGTase where BRP mainly helps to excrete the expressed CGTase into the culture medium. Among the trace elements studied, only $CuCl_2$ remained as the important component that will influence the production of recombinant CGTase. In order to maintain the integrity of periplasmic and outer membrane components especially for the proper folding of Bacteriocin Release Protein (BRP), RpoE of *E. coli*, which is a sigma factor of the extracytoplasmic function protein family, is activated by Cu(II) ion (Egler *et al*., 2005). In this study, which involved the co-expression of BRP to obtain the extracellular recombinant CGTase, $CuCl_2$ included in the new medium is important.

The carbon source content in the medium will affect the cell density of *E. coli* and the new medium containing 2 carbon sources, which are glycerol and sucrose. This is different with the typical medium for recombinant *E. coli* that normally consisting glucose as the main carbon source. It has been shown by a few research groups (Wong *et al*., 2008; March *et al*., 2002; Liu *et al*., 2000; Lee, 1996) that, during the biochemical synthesis of proteins, production of nonessential metabolites can waste carbon and energy that might otherwise be directed towards the protein product, for example the formation of acetate and acetic acid during aerobic growth of *E. coli* on glucose. Two carbon sources (glucose and glycerol) were compared for cell growth and IGF-1 production of recombinant *E. coli* (Yoon *et al*., 1997), glycerol was found to be a better carbon source than glucose for IGF-1 expression in high-cell density culture. Besides, in comparison with different carbon sources in the medium, Liu *et al*. (2000) found that the specific levels of PGA (penicillin G Acylase) activity obtained in the glucose medium were the lowest, which were likely due to the catabolic repression. Lower rate of transport for glycerol into the cell, compared with the glucose, caused a reduction in the flux of carbon through glycolysis, which greatly reduces the acetate formation (Lee, 1996). Addition of polyols such as glycerol, mannitol and sorbitol improved the production of active recombinant CGTase expressed in *E. coli* (Kim *et al*., 1999). Nevertheless, sucrose (X_8) instead of glucose was selected as one of the significant carbon sources for the new medium as glucose was screened out in the very first manually screening stage. There have been several attempts to introduce cheap carbon source especially sucrose substrates such as beet and cane molasses in order to obtain the protein produced by recombinant *E. coli* more economically (Lee and Chang, 1993). The use of sucrose or any other non-metabolizing sugars were found to prevent the aggregation of β-lactamase and thus improved the production of this recombinant enzyme in *E. coli* (Gowden and Georgiou, 1988). In this study, two carbon sources: glycerol (X_5) and sucrose (X_8) have significant influence towards the production of extracellular recombinant CGTase based on the screening results.

Bioreactor scale production of extracellular recombinant CGTase: In the study, the production scheme could be scaled up and same expression level could be obtained as the shake flask scale and the batch fermentation was performed in 2 L bioreactor using the optimized medium. As shown in Fig. 7, the pH of the culture was maintained between 7.21-7.08 using the optimum medium, while the pH of the culture dropped from 7.14-6.51 using the original medium. The observation confirms that the new potassium phosphate buffer solution maintained the culture pH along the fermentation hour better than the original medium. Besides, with the optimized medium concentration, the cell density was increased successfully in the shake flask scale (Fig. 7) to an optical density reading 9.56 while by using the original medium (Lo *et al*., 2007), the highest cell density observed was less than 8. However, the cell density decreased when the cultivation was performed in the bioreactor scale (Fig. 7), as well as the expression level (Fig. 8). Based on Fig. 8, 18.11 kU mL^{-1} enzyme activity was observed during the shake flask scale at 36 h post induction time. However, only 12.94 kU mL^{-1} enzyme activity was observed

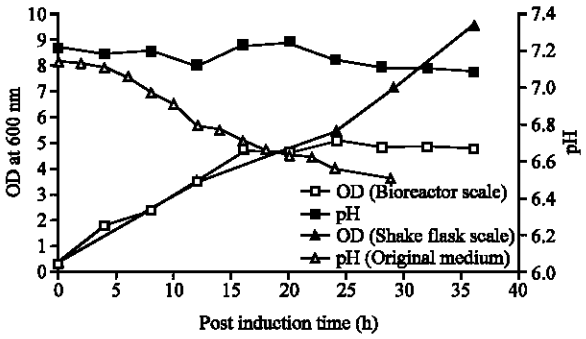

Fig. 7: Comparisons for the pH profile (between the new and original medium) and the cell density profile (between the shake flask scale and the bioreactor scale) along the fermentation time

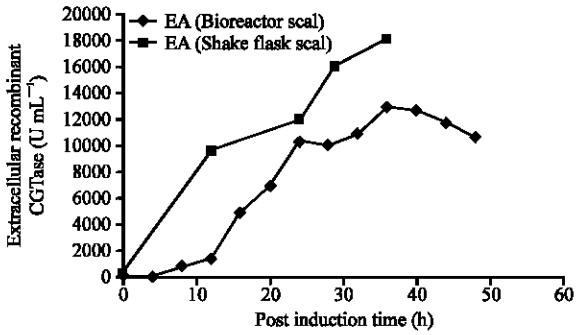

Fig. 8: Comparisons for the production yield (between the shake flask scale and the bioreactor scale) along the fermentation time (EA: Enzyme activity)

at the bioreactor scale and as much as 28.5% of product yield reduction was obtained. This production yield appears to be largely reduced and the results are similar with the initial scaling-up study reported by Chen *et al.* (2007). Notably, the product formation rate was affected by the scale-up scheme for the expression level of the extracellular recombinant CGTase and the decrement might be due to the changes on scale-up process such as oxygen transfer rate, heat transfer surface-to-volume ratio, quality of mixing, shear and superficial air velocity (Humphrey, 1998).

CONCLUSION

Medium optimization was successfully carried out by using the Fractional Factorial Design (FFD) and small Central Composite Design (SCCD) which is a Response Surface Methodology (RSM). The new medium consisted of 12 g L^{-1} NZ Amine A, 24 g L^{-1} yeast extract, 9.44 g L^{-1} KH_2PO_4, 4.4 g L^{-1} K_2HPO_4, 4.58 mL L^{-1} glycerol, 70 mg L^{-1} sucrose and 3 mg L^{-1} $CuCl_2$. A predicted 14.78 kU mL^{-1} extracellular recombinant CGTase was obtained. After an experimental verification, an activity of 16.07 kU mL^{-1} was attained. The production of extracellular recombinant CGTase increased nearly 1.68-fold when the new medium was utilized.

REFERENCES

Chen, P.T., C.J. Chiang and Y.P. Chao, 2007. Medium optimization for the production of recombinant Nattokinase by *Bacillus subtilis* using response surface methodology. Biotechnol. Prog., 23: 1327-1332.

Dekker, N., J. Tommassen and H.M. Verheij, 1999. Bacteriocin release protein triggers dimerization of outer membrane phospholipase *in vivo*. J. Bacteriol., 181: 3281-3283.

Egler, M., C. Grosse, G. Grass and D.H. Nies, 2005. Role of the extracytoplasmic function protein family sigma factor RpoE in metal resistance of *Escherichia coli*. J. Bacteriol., 187: 2297-2307.

Gowden, G.A. and G. Georgiou, 1988. The effect of sugars on β-lactamase aggregation in *Escherichia coli*. Biotechnol. Prog., 4: 97-101.

Han, N.S. and B.Y. Tao, 1999. A simple method to express soluble, highly active cyclodextrin glycosyltransferase in recombinant *E. coli*. Biotechnol. Technol., 13: 631-635.

Humphrey, A., 1998. Shake flask to fermentor: What have we learned? Biotechnol. Prog., 14: 3-7.

Kaneko, T., T. Kato, N. Nakamura and K. Horikhoshi, 1987. Spectrophotometric determination of cyclozation activity of β-cyclodextrin-forming cyclodextrin glucanotransferase. J. Japan Soc. Starch Sci., 34: 45-48.

Kennedy, M. and D. Krouse, 1999. Strategies for improving fermentation medium performance: A review. J. Ind. Microbiol. Biotechnol., 23: 456-475.

Khushoo, A., Y. Pal, B.N. Singh and K.J. Mukherjee, 2004. Extracellular expression and single step purification of recombinant *Escherichia coli* l-asparaginase II. Prot. Exp. Purif., 38: 29-36.

Kim, M.H., J.K. Lee, H.L. Kim, C.B. Sohn and T.K. Oh, 1999. Overexpression of cyclodextrin glycosyltransferase gene from *Brevibacillus brevis* in *Escherichia coli* by control of temperature and mannitol concentration. Biotechnol. Tech., 13: 765-770.

Kim, S.G., D.H. Kweon, D.H. Lee, Y.C. Park and J.H. Seo, 2005. Coexpression of folding accessory proteins for production of active cyclodextrin glycosyltransferase of *Bacillus macerans* in recombinant *Escherichia coli*. Prot. Exp. Purif., 41: 426-432.

Lee, S.Y. and H.N. Chang, 1993. High cell density cultivation of *Escherichia coli* W using sucrose as a carbon source. Biotechnol. Lett., 15: 971-974.

Lee, S.Y., 1996. High cell density culture of *Escherichia coli*. Trends Biotechnol., 14: 98-105.

Liu, Y.C., L.C. Liao and W.T. Wu, 2000. Cultivation of recombinant *Escherichia coli* to achieve high cell density with a high level of penicillin G acylase activity. Proc. Natl. Sci. Counc. Repub. China B., 24: 156-160.

Lo, P.K., O. Hassan, A. Ahmad, N.M. Mahadi and R.M. Illias, 2007. Excretory over-expression of *Bacillus* sp. G1 cyclodextrin glucanotransferase (CGTase) in *Escherichia coli*: Optimization of the cultivation conditions by response surface methodology. Enzym. Microbiol. Technol., 40: 1256-1263.

Ma, X., W. Zheng, T. Wang, D. Wei and Y. Ma, 2006. Optimization and high-level expression of a functional GST-tagged rHLT-B in *Escherichia coli* and GM1 binding ability of purified rHLT-B. J. Microbiol., 44: 293-300.

Madigan, M.T., J.M. Martinko and J. Parker, 2003. Brock Biology of Microorganisms. 10th Edn., Prentice Hall, USA., pp: 104-105.

March, J.C., M.A. Eiteman and E. Altman, 2002. Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in *Escherichia coli*. Applied Environ. Microbiol., 68: 5620-5624.

Miksch, G., S. Kleist and E. Flaschel, 2002. Overexpression of the phytase from *Escherichia coli* and its extracellular production in bioreactors. Applied Microbiol. Biotechnol., 59: 685-694.

Park, Y.C., C.S. Kim, C.I. Kim, K.H. Choi and J.H. Seo, 1997. Fed-batch fermentations of recombinant *Escherichia coli* to produce *Bacillus macerans* CGTase. J. Microbiol. Biotechnol., 7: 323-328.

Rahman, R.N.Z.R.A., T.C. Leow, M. Basri and A.B. Salleh, 2005. Secretory expression of thermostable T1 lipase through bacteriocin release protein. Prot. Exp. Purif., 40: 411-416.

Rao, K.J., C.H. Kim and S.K. Rhee, 2000. Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology. Proc. Biochem., 35: 639-647.

Riesenberg, D., K. Menzel, V. Schulz, K. Schumann, G. Veith, G. Zuber and W.A. Knorre, 1990. High cell density fermentation of recombinant *Escherichia coli* expressing human interferon alpha 1. Applied Microbiol. Biotechnol., 34: 77-82.

Rosenbrock, H.H., 1960. An automatic method for finding the greatest or least value of a function. Comp. J., 3: 175-184.

Szetjli, J., 1998. Introduction and general overview of cyclodextrin chemistry. Chem. Rev., 98: 1743-1753.

Tonkova, A., 1998. Bacterial cyclodextrin glucanotransferase. Enzym. Microbiol. Technol., 22: 678-686.

Wong, M.S., S. Wu, T.B. Causey, G.N. Bennett and K.Y. San, 2008. Reduction of acetate accumulation in *Escherichia coli* cultures for increased recombinant protein production. Metab. Eng., 10: 97-108.

Xu, Z., G. Liu, P. Cen and W.K.R. Wong, 2000. Factors influencing excretive production of human epidermal growth factor (hEGF) with recombinant *Escherichia coli* K12 system. Bioproc. Eng., 23: 669-674.

Yong, J., J.N. Choi, S.S. Park, C.S. Park, K.H. Park and Y.D. Choi, 1996. Secretion of heterologous cyclodextrin glycosyltransferase of I>Bacillus sp. E1 from *Escherichia coli*. Biotechnol. Lett., 18: 1223-1228.

Yoon, S.H., H.H. Wong, S.Y. Lee, B.H. Chung and Y.I. Lee, 1997. Efficient production of IGF-1 by fed-batch culture of recombinant *Escherichia coli*. Proceedings of the APBioCheC'97, Oct. 1997, Beijing, China, pp: 1-1.