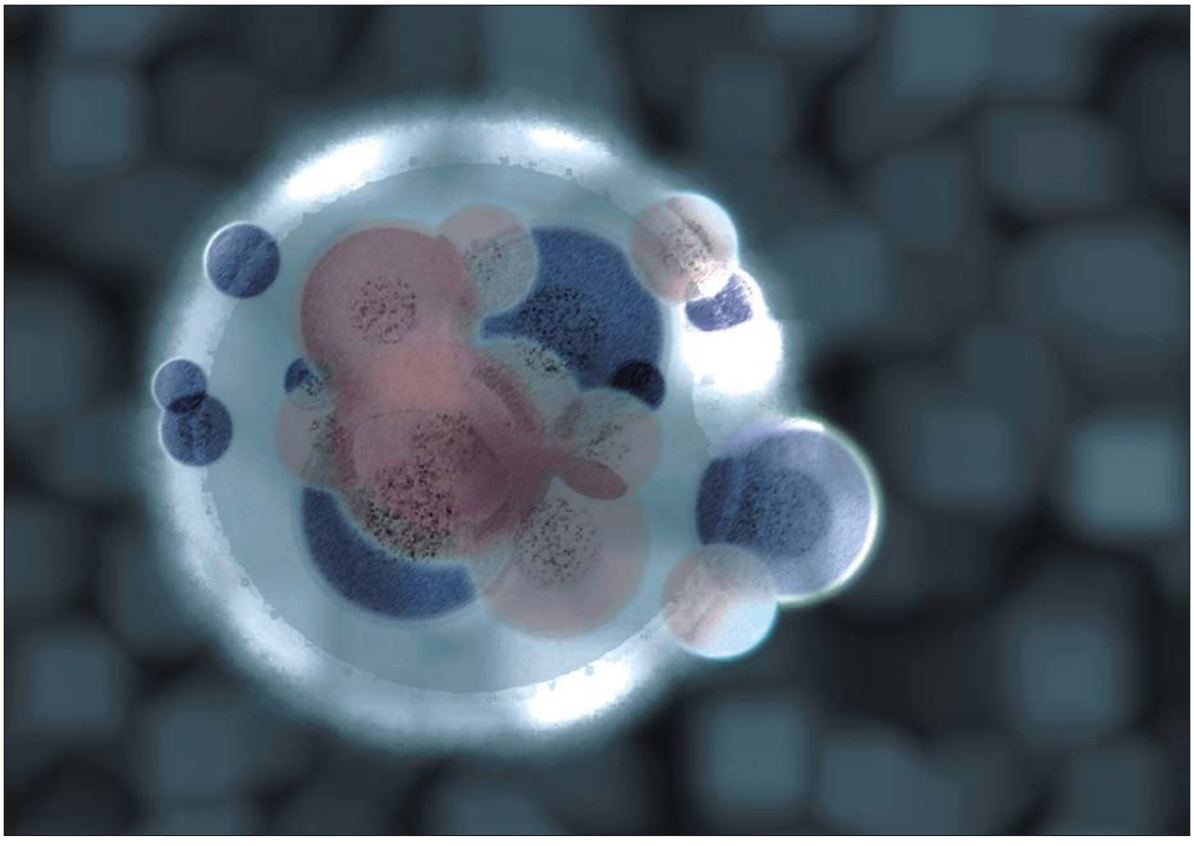

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Application of Statistical Experimental Design for Optimization of Novel α-amylase Production by *Anoxybacillus* Species

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Abstract: *Anoxybacillus* sp. DT3-1 is a newly isolated bacterium with amylolytic activity. The gene that encodes the α-amylase was recently cloned and expressed in *E. coli* system. However, the expression level was far too low to be used for further analysis. The main objective of this study is to enhance the recombinant α-amylase (ADTA) expression level extracellularly. In medium comparison, LB/amp medium was found to be the best medium to support the cell growth and extracellular ADTA production. Subsequently, three factors that affect the ADTA expression which are cells absorbance during induction, concentrations of IPTG and yeast extract were screened using 2^3 full factorial design. Cells absorbance during induction and IPTG concentration were found to be the significant variables that affected the ADTA production. In the consequently Central Composite Rotatable Design (CCRD), the optimized condition for maximum extracellular ADTA production was determined as $OD_{600\ nm}$ 1.52, 0.01 mM IPTG and 0.30% (w/v) yeast extract. The extracellular ADTA production was successfully increased from 30 U in the original medium to 82.29 U.

Key words: *Bacillus*, *Geobacillus*, factorial design, central composite design, response surface methodology

INTRODUCTION

Amylase is one of the most important industrial enzymes. Amylase cover approximate 30% of the enzyme market share (Van Der Maarel *et al*., 2002). This enzyme has been employed in various industries such as food, beverages, detergents, textiles and paper industries for more than 150 years (Gupta *et al*., 2003).

Amylases can be divided into two groups according to the anomeric type of sugars produced by their enzymatic reaction, i.e., the endoamylase and exoamylase. An example of endoamylase is α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1). It cleaves at the α-1,4 glycosidic bonds present in starch in a random fashion (Prakash and Jaiswal, 2010). The final enzymatic products of endoamylases include glucose, maltose, linear oligosaccharides and α-limit dextrin. Exoamylases such as the β-amylase, amyloglucosidase, glucoamylase and glucosidase cleave both α-1,4 and/or α-1,6 bonds to produce maltose and β-limit dextrin (Gupta *et al*., 2003).

Amylase is used in food or starch industries manufacturing maltose, high fructose syrup, oligosaccharide mixtures, maltotetraose syrups and high molecular weight of branched dextrins (Synowiecki, 2007). In addition, amylase can be utilized as anti-staling agent, production of cyclodextrins, removal of starch sizer from textiles, direct fermentation of starch to ethanol, treatment of starch industry effluents and clarification of haziness in beer and fruit juices (Gupta *et al*., 2003). To meet the above-mentioned industries demand, a number of potential industrial amylases have been reported (Hmidet *et al*., 2008; Prakash and Jaiswal, 2010; Kikani and Singh, 2011).

Genetic engineering has become a successful approach to increase the reproducibility of amylase expression in workhorse like *Escherichia coli* or *Saccharomyces cerevisiae* (Prakash and Jaiswal, 2010). Numerous studies have been reported on the cloning and characterization of the amylase gene from different type of bacteria (Sivaramakrishnan *et al*., 2006). Hundreds of *Bacillus* amylase genes are currently known. However, the genus *Anoxybacillus*-a group of alkalitolerant, moderate thermophilic bacteria draws less attention from global researchers. The limited studies of *Anoxybacillus* α-amylases include those from *A. flavithermus* strains

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(Bolton *et al*., 1997; Ozdemir *et al*., 2012; Tawil *et al*., 2012), *A. amylolyticus* (Poli *et al*., 2009) and *A. contaminans* (Vikso-Nielsen *et al*., 2006).

Anoxybacillus sp. DT3-1 is a locally isolated bacteria strain that exhibit amylolytic activity (Chai *et al*., 2012a, b). To the best of knowledge, this is the first reported amylase gene from *Anoxybacillus* species (Goh *et al*., 2013). The gene was recently cloned and expressed in *E. coli* system (Chai *et al*., 2012a). The α-amylase from *Anoxybacillus* sp. DT3-1 is designated as ADTA and the abbreviation will be throughout the report. Based on the amino acid sequence, ADTA has low similarity to other known α-amylase sequences in the public database. Among the characterized α-amylases, the most similar α-amylase is from *Anaerobranca gottschalkii* (68% similarity). On the other hand, sequence comparison with well-known α-amylases such as those from *Aspergillus oryzae*, *Bacillus licheniformis* and *Geobacillus stearothermophilus*, reveal that ADTA is significantly different from them with low similarity ranging from 37-49% (Chai *et al*., 2012a).

It was found that the preliminary recombinant ADTA expression level was far too low to be used for further analysis. Hence, optimization of the expression conditions was carried out to enhance the ADTA activity. Individual optimization of the expression conditions is needed because the behavior of cell and protein is different in each case of interest.

The present study aims to enhance the recombinant α-amylase expression level by two types of statistical experimental designs. In the prescreening study, a complex medium and a defined medium were compared based on the suitability in promoting ADTA expression extracellularly. Two-level full factorial design was used as the first statistical approach to evaluate the importance of three variables: cells absorbance during induction, isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration and yeast extract concentration. Secondly, Central Composite Rotatable Design (CCRD) was used to locate the optimum conditions for all significant variables.

MATERIALS AND METHODS

Study period: The present study was conducted from June 2010 to December 2010 at the faculty laboratory.

Microorganism and inoculum preparation: The *E. coli* transformant used in this work containing the α-amylase gene from *Anoxybacillus* sp. DT3-1 (Chai *et al*., 2012a), isolated from the Dusun Tua hot spring in Malaysia (Chai *et al*., 2012b). The *E. coli* transformant was streaked on Luria-bertani (LB) medium containing 10.0 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract and 10.0 g L^{-1} sodium chloride. The medium was solidified with 1.5% (w/v) agar. A 100 mg mL^{-1} sterile ampicillin (amp) was added to the autoclaved medium. All plates were incubated at 37°C for 24 h.

For inoculum preparation, a single colony was inoculated in 100 mL LB/amp broth inside a 500 mL conical flask. The inoculum was incubated at 37°C with 200 rpm for 18 h. The broth culture was centrifuged at room temperature for 15 min at 8000 rpm. The supernatant was then discarded and the cell pellet was resuspended with fresh sterile medium.

Comparison of complex and defined medium to ADTA expression: A 10 mL inoculum was aseptically transferred to a 500 mL conical flask containing 90 mL of sterile medium of one of the following types: LB and Chemical Defined Medium (CDM). The CDM was prepared with 9.5 g L^{-1} KH_2PO_4, 3.0 g L^{-1} $(NH_4)_2HPO_4$, 15.0 g L^{-1} glucose and 25.0 g L^{-1} $MgSO_4.7H_2O$ (Tabandeh *et al*., 2008). 100 mg mL^{-1} amp was added to both media after autoclaving. All flasks were incubated at 25°C with shaking at 200 rpm for approximately 18 h until the optical density at 600 nm reached 1.0. Then, 1.0 mL of 100 mM sterile IPTG was added into each flask to induce the ADTA expression.

For every two hours, 4.0 mL of broth culture from each flask of IPTG-induced culture was sampled up to 68 h. The sample was then centrifuged at 8000 rpm for 15 min at 4°C. The cell-free supernatant was then used for the enzyme assay.

Analytical procedures: The analysis of ADTA activity was done according to Goyal *et al*. (1995), with slight modifications. The iodine reagent for this analysis was prepared by mixing 0.2% (w/v) of iodine with 2.0% (w/v) potassium iodine solution. A 250.0 μL of enzyme solution was added with 250.0 μL of 0.2% (w/v) soluble starch, which was gelatinized in 100 mM sodium phosphate buffer, pH 6.5. The reaction was allowed to perform at 60°C for 30 min. Immediately after the incubation, 250.0 μL of 1.0 M HCl was added to stop the reaction. This is followed by the addition of 250.0 μL iodine solution and 4.0 mL of distilled water. The mixture was measured for absorbance intensity at 690 nm using a 100 UV-Vis spectrophotometer (Jenway, Staffordshire, UK). One unit (U) of amylase activity was defined as the amount of amylase needed to reduce the colour of starch-iodine compound by 1% changes. All assays were performed in triplicate, unless otherwise specified.

Statistical analysis: Design Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 6.0.4) was used to optimize the ADTA expression. Two

Table 1: Actual and predicted values for ADTA production in 2^3 full factorial design

	Actual variable level and coded variable level			Extracellular ADTA activity	
Std. order	Cell absorbance during induction, $OD_{600\ nm}$ (X_1)	IPTG concentration, mM (X_2)	Yeast extract concentration, % (w/v) (X_3)	Actual value (U)	Predicted value (U)
1	0.3 (-1)	0.01 (-1)	0.05 (-1)	58.17±0.23	58.15
2	1.5 (1)	0.01 (-1)	0.05 (-1)	71.83±0.14	71.80
3	0.3 (-1)	0.1 (1)	0.05 (-1)	28.25±0.07	28.24
4	1.5 (1)	0.1 (1)	0.05 (-1)	65.51±0.15	65.50
5	0.3 (-1)	0.01 (-1)	0.3 (1)	50.90±0.22	50.92
6	1.5 (1)	0.01 (-1)	0.3 (1)	79.01±0.35	79.03
7	0.3 (-1)	0.1 (1)	0.3 (1)	35.46±0.56	35.46
8	1.5 (1)	0.1 (1)	0.3 (1)	58.27±0.15	58.27
9	0.9 (0)	0.06 (0)	0.17 (0)	57.77±0.24	57.76
10	0.9 (0)	0.06 (0)	0.17 (0)	57.77±0.23	57.76
11	0.9 (0)	0.06 (0)	0.17 (0)	57.77±0.20	57.76
12	0.9 (0)	0.06 (0)	0.17 (0)	57.77±0.22	57.76
13	0.9 (0)	0.06 (0)	0.17 (0)	57.71±0.21	57.76
14	0.9 (0)	0.06 (0)	0.17 (0)	57.77±0.24	57.76

Values in bracket are coded variable level, Actual values are mean from triplicates±standard error

Table 2: Actual and predicted values for ADTA production in CCRD design

	Actual variable level and coded variable level		Extracellular ADTA activity	
Std. order	Cell absorbance during induction, $OD_{600\ nm}$ (X_1)	IPTG concentration, mM (X_2)	Actual value (U)	Predicted value (U)
1	1.37 (-1)	0.008 (-1)	52.03±0.32	51.89
2	1.73 (1)	0.008 (-1)	36.76±0.25	36.68
3	1.37 (-1)	0.014 (1)	57.25±0.33	57.29
4	1.73 (1)	0.014 (1)	43.57±0.38	43.67
5	1.30 (-1.414)	0.011 (0)	59.22±0.27	59.28
6	1.80 (-1.414)	0.011 (0)	38.93±0.31	38.91
7	1.55 (0)	0.007 (-1.414)	41.15±0.26	41.29
8	1.55 (0)	0.015 (1.414)	50.16±0.33	50.05
9	1.55 (0)	0.011 (0)	82.35±0.12	82.39
10	1.55 (0)	0.011 (0)	82.08±0.15	82.39
11	1.55 (0)	0.011 (0)	82.41±0.17	82.39
12	1.55 (0)	0.011 (0)	82.81±0.15	82.39
13	1.55 (0)	0.011 (0)	82.54±0.13	82.39
14	1.55 (0)	0.011 (0)	82.15±0.16	82.39

Values in bracket are coded variable level, Actual values are mean from triplicates±standard error

experimental designs which are 2^3 full factorial design and CCRD were adopted to study three independent variables: cells absorbance during induction (X_1), IPTG concentration (X_2) and yeast extract concentration (X_3).

Full factorial design: A 2^3 full factorial design was used to show the statistical significance of cells absorbance during induction (X_1), IPTG concentration (X_2) and yeast extract concentration (X_3) towards the production of ADTA. Full factorial design is a statistical method that involves simultaneous adjustment of experimental factors at two levels: high (+1) and low (-1) (Kennedy and Krouse, 1999). The range for the factors was based primarily on the investigation of single factors (screening process) and literature review. A total of 14 sets of experiments with two replicates and six replicated center points were employed in this study to determine the significant factors that affect the ADTA activity (U) (Table 1).

Central composite rotatable design (CCRD): This study was further expanded to a CCRD. CCRD is one of the designs in Response Surface Methodology (RSM). This design involves five different levels: high (+1), low (-1), centre point (0) and two axial points (-1.414 and +1.414). The coding of variables was conducted according to Eq. 1:

$$\text{Coded value} = \frac{\text{Actual value-(high level+low level)/2}}{\text{(high level-low level)/2}} \quad (1)$$

Based on the screening of variables using 2^3 full factorial design, a full CCRD was developed for variables significantly affecting the expression of ADTA. The significant variables identified by 2^3 full factorial design were cells absorbance during induction (X_1) and IPTG concentration (X_2). Therefore, a total of 14 sets of experiments with two replication, six center point replication and four axial points were carried out as shown in Table 2. The quadratic model for predicting the optimal point was expressed according to Eq. 2:

$$Y = \beta_0+\Sigma\beta_i x_i+\Sigma\beta_{ii} x_{ii}^2+\Sigma\beta_{ij} x_i x_j+\varepsilon \quad (2)$$

The symbols used in the equation are represent as Y = predicted response for extracellular ADTA activity (U), β_0 = constant, β_i = linear effect, β_{ii} = quadratic effect, β_{ij} = interaction effect, x = coded level of the independent variables and ε = random error. The statistical significance of the second order model equation was determined by a significant F-value, an insignificant lack-of-fit F-value and a good multiple coefficient of determination, R^2.

RESULTS AND DISCUSSION

Comparison of complex and defined medium to ADTA expression: Several media have been proposed for *E. coli* expression system. A good medium should sustain the propagation of *E. coli* cells besides promoting the recombinant protein expression level. In the present study, the effect of ADTA expression in two commonly used media for *E. coli* system was compared. The medium used were LB and CDM, both with the addition of amp. LB is a complex medium while CDM is a defined medium. The comparison was done by growing the cells in each medium at 25°C for 68 h. The culture in all media was induced simultaneously with 1.0 mL of 100 mM IPTG.

Figure 1 shows the effect of each medium on *E. coli* growth and extracellular ADTA expression. The extracellular ADTA activity (U) is the mean from triplicates±standard error. The difference in both media can be clearly seen as the growth in CDM/amp medium reached approximately half the biomass quantity of the cell growth in richer medium (LB/amp medium).

Assay used to estimate the ADTA activity is based on the ability of amylase to degrade starch. Promising ADTA expression level was observed in medium LB/amp compared to CDM/amp. The ADTA activity for the first 20 h was similar in both media; however, the expression kept on increased in LB/amp (Fig. 1).

LB/amp medium was shown more favorable to be used in expressing the ADTA and also in promoting the growth of *E. coli* cells. This is because LB/amp medium is a richer medium compared to CDM/amp medium. LB medium contained two organic nitrogen sources (yeast extract and tryptone) that contribute to a number of organic molecules that are needed for bacterial growth and these nitrogen sources are required for protein/enzyme synthesis.

Full factorial design: As mentioned previously, LB/amp medium was the best medium to promote the expression of ADTA at extracellular level. With the aim to engineer the recipe of the medium, optimization was then carried out using full factorial design. Three variables which are the cells absorbance during induction (X_1), IPTG concentration (X_2) and yeast extract concentration (X_3) were studied and correlated with extracellular ADTA activity (U). Table 1 tabulates the experimental design along with the actual and predicted values. The responses were analyzed with analysis of variance (ANOVA) using the Design Expert Software as shown in Table 3.

Based on the ANOVA (Table 3), an equation relating the recombinant ADTA expression with the independent

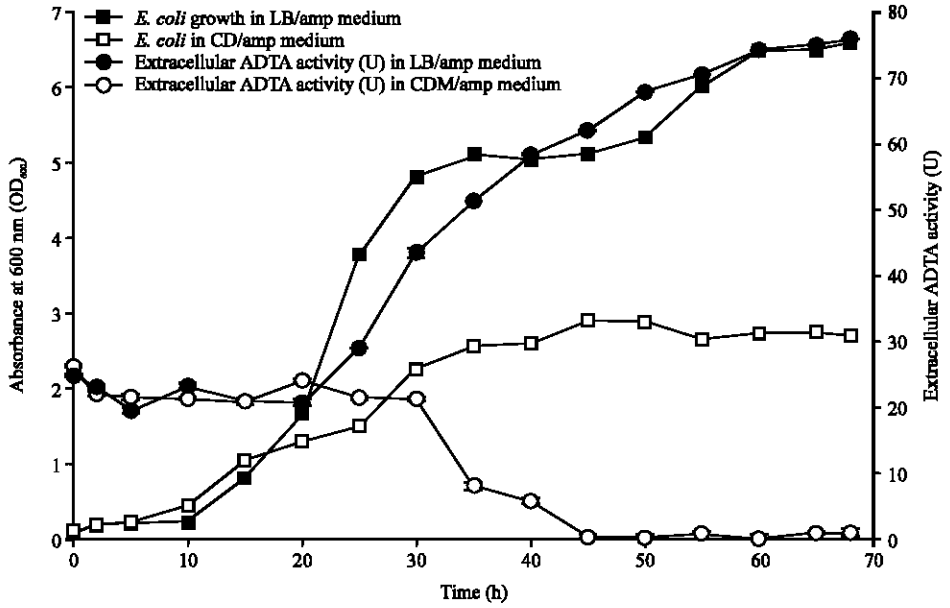

Fig. 1: Time course of *E. coli* growth and extracellular ADTA expression in different medium

Table 3: Statistical analysis (ANOVA) for 2^3 full factorial design where X_1 refers to cell absorbance during induction (OD_{600nm}) and X_2 refers to IPTG concentration (mM)

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	2098.5300	4	524.6300	760548.58	<0.0001*
X_1	1296.3400	1	1296.3400	1879275.31	<0.0001*
X_2	655.7900	1	655.7900	950684.44	<0.0001*
X_1X_2	41.9200	1	41.9200	60777.04	<0.0001*
$X_1X_2X_3$	104.4800	1	104.4800	151457.51	<0.0001*
Curvature	11.5500	1	11.5500	16741.56	<0.0001*
Residual	0.0055	8	0.0007		
Lack-of-fit	0.0023	3	0.0008	1.15	0.4146**
Pure error	0.0033	5	0.0007		
Corrected total	2110.0800	13			
Standard deviation	0.0300	R-squared, R^2	1.0000		
Mean	56.7100	Adjusted R^2	1.0000		
CV	0.0500	Predicted R^2, |R|	1.0000		
PRESS	0.0200	Adequate precision	2954.2200		

*Significant ($p<0.05$), **Insignificant ($p>0.05$)

variables, X_1, X_2 and X_3, is given in the first order polynomial model. The response for Eq. 3 is the extracellular ADTA activity (U):

$$\text{Extracellular ADTA activity (U)} = +55.92+12.73\ X_1-9.05\ X_2+2.29\ X_1X_2-3.61\ X_1X_2X_3 \quad (3)$$

As shown in Table 3, the F-values of 760548.58 suggest that this model is significant. A very low p-value ($p_{model}<0.0001$) also implies that this model adequately fit the experimental data. The F-values indicate the reliability of the results obtained and whether the model is significant. In addition, the p-values corresponding to F-values signify the relative significance of the coefficients where this is vital in understanding the pattern of the mutual interactions between the variables under study (Rezaei *et al*., 2010). Smaller p-values ($p<0.05$) result in larger significance of the corresponding coefficient. The insignificant lack-of-fit, F-value of 1.15 implies that the model obtained is adequate for prediction within the range of variables employed. In contrast, the coefficient of variance (CV) shows the degree of precision of the comparison of the treatments (Gangadharan *et al*., 2008). This model has a relatively low value of CV (0.05) which indicates a high precision and reliability of the conducted experiments.

The robustness of this model was verified by the value of coefficient of determination, R^2 (Table 3). The R^2 value for this model was 1.0000. The data collected in this study is good as a regression model with high R^2 value is considered as a good model (Sharma and Satyanarayana, 2011). Higher value of the predicted R^2, |R| for this model which is 1.0000 also suggests a reasonable agreement between experimental and predicted values. Adequate precision on the other hand, measures the experimental signal-to-noise ratio. It acts as a tool to compare the range of the predicted values to the average prediction error at each design points. A ratio greater than 4.00 indicates the design is good (Kalishwaralal *et al*., 2010). In this study, the model ratio of 2954.22 was obtained. This indicates an adequate signal and again shows that this model is significant and acceptable.

According to Table 3 and Eq. 3, all linear and interaction terms were found to be significant except for yeast extract concentration (X_3). They either acted as an inhibitor or enhancer towards the extracellular ADTA activity (U). In Eq. 3, the coefficient factor for cell absorbance during induction (X_1) is the greatest and positive sign in the equation means that X_1 is the major enhancer factor. This shows that the amount of cells present in the medium play an important role towards ADTA expression by the *E. coli* system.

Based on the model prediction, the maximum ADTA activity (U) that can be obtained extracellularly was 78.97 U in the optimal conditions of cells absorbance during induction, OD_{600nm} 1.50, 0.01 mM concentration of IPTG and 0.30% (w/v) of yeast extract. A curvature with p-value of <0.0001 was also predicted by this model. This implies that there is a significant curvature in the design space. Thus, a continuation of the experiment using a second-order model should be performed to estimate the location of curvature and to obtain the optimum conditions. Since cells absorbance during induction (X_1) and IPTG concentration (X_2) were found to be the significant linear term (Table 3, Eq. 3), both variables were further optimized using a CCRD.

Central composite rotatable design (CCRD): The CCRD approach is one of the most popular designs used in RSM design collection. The optimum level for each significant factor was determined using a CCRD. CCRD contains five levels that are high level (+1), low level (-1), center point (0) and two axial points (-1.414 and +1.414). Both axial points will help in finding the curvature in each interaction and thus reveal the optimum point.

Based on the preceding data, two variables (cells absorbance during induction (X_1) and IPTG concentration (X_2)) were significant factors affecting the expression of ADTA extracellularly. The range of cells absorbance during induction (X_1) and IPTG concentration (X_2) were narrowed down in order to determine the optimum point. It is a general practice to revise the range for each significant factor to locate the curvature if the original range was not suitable (Mahat *et al*., 2004; Stephenie *et al*., 2007). The range for each factors were based primarily on literature review and screening process previously done using 2^3 full factorial design. As yeast extract concentration (X_3) was found to be the insignificant factor affecting the ADTA expression in previous model, the yeast extract concentration was set at 0.30% (w/v). Table 2 shows the range for each factors and responses (actual and predicted value) in CCRD. ANOVA was then used to analyze all the responses and elucidated in Table 4. A quadratic equation was derived based on the second order polynomial as Eq. 4:

$$\text{Extracellular ADTA activity (U)} = +82.39-7.20\ X_1+3.10\ X_2-16.65\ X_1^2-18.36\ X_2^2+0.40\ X_1X_2 \quad (4)$$

All linear, quadratic and interaction terms were significant based on the Eq. 4 and Table 4. The quadratic equation for CCRD model shows a positive linear effect for cells absorbance during induction (X_1) while IPTG concentration (X_2) gives a negative linear effect, respectively. This indicates that X_1 is an enhancer factor while X_2 has inhibitor effect towards the production of ADTA. Similar prediction was observed in 2^3 full factorial design when ADTA was expressed by the *E. coli* system. Cells absorbance during induction (X_1) was found to be the most significant in model presented by Eq. 4 with F-value and p-value of 7792.68 and <0.0001, respectively. In addition, the interaction term of X_1X_2 also shows a similar prediction with previous design whereby this term was defined as the enhancer factor for extracellular ADTA production.

The adequacy of the model was determined using the standard ANOVA as elucidated in Table 4. This model exhibited a high F-value of 17661.65 and a low p-value of <0.0001 which signify a good predictor of experimental results. The calculated value of R^2 was 0.9999, indicating that only 0.01% of the total variation in the experiment was not explained by the model. The R^2 value justified an excellent correlation between the predicted and experimental values. The lack-of-fit F-value of 0.3498 and p-value of 0.7917 implies that the test is not significantly relative to the pure error. Insignificant lack-of-fit is a good sign that the model developed for this experiment is adequate.

Optimal levels of the variables: The optimal levels of variables were determined by generating response surface plot (Fig. 2) based on the Eq. 4. The function of response surface plot is to visualize the interaction of two variables while keeping other variables at centre point level. The response surface plot is also a very useful tool in order to pinpoint the optimum level for the studied variable. From this plot, the extracellularly expression level of ADTA by *E. coli* system can be clearly observed in the experimental range.

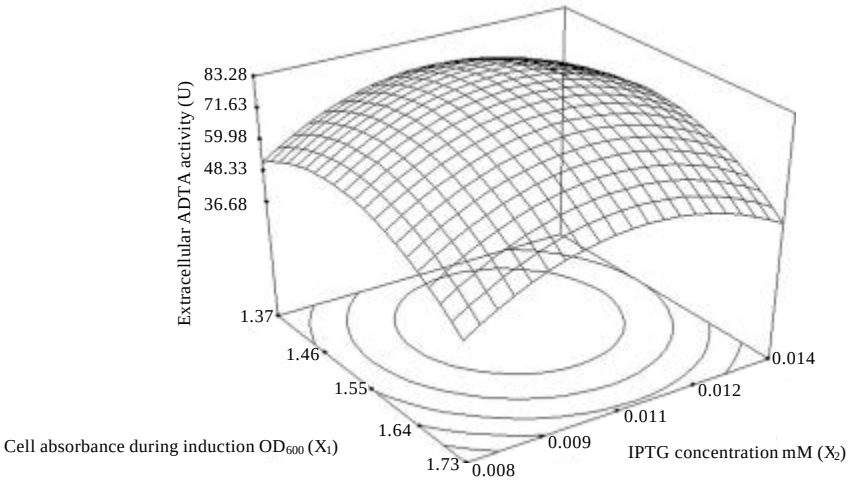

Fig. 2: Response surface plot for extracellular ADTA activity (U)

Table 4: Statistical analysis (ANOVA) for CCRD where X_1 refers to cell absorbance during induction ($OD_{600\ nm}$) and X_2 refers to IPTG concentration (mM)

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	4705.8900	5	941.1800	17661.65	<0.0001*
X_1	415.2700	1	415.2700	7792.68	<0.0001*
X_2	76.7100	1	76.7100	1439.49	<0.0001*
X_1^2	2046.5700	1	2046.5700	38404.86	<0.0001*
X_2^2	2489.0100	1	2489.0100	46707.44	<0.0001*
X_1X_2	0.6300	1	0.6300	11.82	0.0089*
Residual	0.4263	8	0.0533		
Lack-of-fit	0.0740	3	0.0247	0.35	0.7917**
Pure error	0.3524	5	0.0705		
Standard deviation	0.2300	R-squared, R^2	0.9999		
Mean	62.3900	Adjusted R^2	0.9999		
CV	0.3700	Predicted R^2, |R|	0.9998		
PRESS	1.0300	Adequate precision	302.4300		

*Significant ($p<0.05$), **Insignificant ($p>0.05$)

Table 5: Summary of optimum condition for each variable in different designs where actual value refers to the experimental response and predicted value refers to the calculated response based on the model equation

	Variable			Extracellular ADTA activity	
Order	Cell absorbance during induction, $OD_{600\ nm}$ (X_1)	IPTG concentration, mM (X_2)	Yeast extract concentration, % (w/v) (X_3)	Actual value (U)	Predicted value (U)
Unoptimized condition	-	-	-	30.00±0.50	-
Original LB/amp medium	1.00	1.00	0.50	72.03±0.52	-
2^3 full factorial design	1.50	0.01	0.30	79.93±0.19	78.97
CCRD	1.52	0.01	0.30	82.29±0.06	82.90

Actual values are mean from triplicates±standard error

As observed in Fig. 2, the optimum cells absorbance during induction (X_1) at $OD_{600\ nm}$ was 1.50 and IPTG concentration (X_2) of 0.01% (w/v). This model predicted a maximum extracellular ADTA activity of 82.90 U can be produced by *E. coli* using the optimum conditions. The maximum ADTA activity was represented by the top of the concaved shape in the response surface plot.

Verification of the model: The Design Expert software predicted the optimal ADTA expression for 2^3 full factorial design and CCRD using the function of numerical optimization and desirability. A validation experiment was performed to certify the values given by each prediction. The experiment was conducted in a 500 mL conical flask containing 90 mL sterile engineered LB/amp medium under conditions suggested by the software for both models.

Table 5 summarizes the optimum point for each variables predicted by both models while Fig. 3 shows the increment of ADTA expression using different statistical designs. Error bars in Fig. 3 indicates the mean from triplicates±standard error. Prior to this work, the expression achieved was approximately 30 U. During the prescreening process, the expression has improved to 72.03 U. The 2^3 full factorial design further increased the ADTA activity to 79.93 U. Subsequent optimization process using CCRD further enhanced the ADTA expression to 82.29 U.

The present study notion to optimize the ADTA expression outside the *E. coli* host is important for several

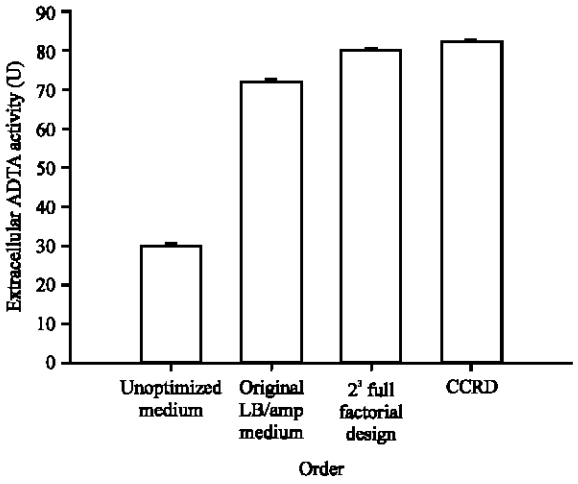

Fig. 3: Comparison of extracellular ADTA expression in different statistical experimental designs

reasons. (1) This is to avoid the formation of inclusion body inside the cells. (2) Expressing higher extracellular recombinant enzyme is relatively cost effective and less time consuming in large scale. (3) Intracellular recombinant enzyme expression is a fundamental burden in downstream processing. Process of breaking the cell wall to obtain the intracellular enzyme is very tedious if done physically or chemically. Purification from extracellular component also becomes much easier with less background proteins present. Sometimes, the background proteins in the medium reduce the purity of

the recovery, as they might bind to the chromatography columns and elute together with the target enzyme of interest. (4) Although extracellular expression of recombinant enzyme is theoretically good, the total enzyme expression may dropped compared to the expression in their origin organisms. Thus, further optimization is needed to improve the expression level for each enzyme of interest.

CONCLUSION

LB/amp medium is found better than the defined medium tested in terms of extracellular expression of ADTA and cell propagation. The 2^3 full factorial design, cells absorbance during induction and IPTG concentration were found to be significant factors that determining ADTA expression. The subsequently CCRD has successfully increased the novel recombinant ADTA activity from 30 U (before optimization) to approximately 82 U (after optimization).

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