Growth Optimization Of *Escherichia coli* Harboring Superoxidase Dismutase Using Response Surface Methodology

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ABSTRACT

A preliminary study on the Response Surface Methodology (RSM) was employed to optimize the growth of recombinant E. coli BL21 (DE3) pLysS (ESOD) harboring superoxide dismutase (SOD) on three variables (nitrogen source, carbon source and initial pH) in shake flask cultures. Full Factorial Design (FFD) was applied as a screening method to the independent variables, and maximum cell concentration (X_{max}) achieved was 3.59g/L at a combination of 7 C/N and initial pH of 7.5. The combined effect of each variables and optimized condition were obtained using Central Composite Design (CCD). The optimized nitrogen and carbon sources derived from the RSM regression were 21.86g/L and 4.89g/L, respectively. The optimal initial pH of the medium was 7.84. The highest cell concentration achieved based on the model developed from RSM was 7.39g/L, which compared very well with the maximum predicted value.

Keywords: *Optimization, superoxide dismutase, response surface methodology*

Introduction

Superoxide dismutase (SOD) is a metaloenzyme that acts as an antioxidant defense mechanism against oxidative stress. SOD catalyzes the dismutation of the toxic superoxide anion (O_2-). O_2- is formed during the reduction of oxygen molecule (O_2). SOD is available in every biological organism as *E. coli* itself has expresses a Cu,Zn SOD and its compositional analysis is similar to the human SOD (Benov et al., 1996). Superoxide dismutase (SOD) (E.C 1.15.1.1) is believed to play a major role in the metabolism of reactive oxygen species. SOD converted superoxide into hydrogen peroxide and however, superoxide itself is not innocuous. The superoxide ion interact with and inactive both catalase and glutathione peroxidase (Huber, 1994). A variety of approaches have now suggested that manipulation of SOD alone can have significant effect on both longevity and the onset of age-related pathology.

The SOD families consist of four metalloforms; two containing copper and zink (CuZn), one manganese (Mn) and one iron (Fe) (Yi Sun, 1990). Cu,ZnSOD is found in the cytosol of most eukaryotic cells (Fridovich, 1975) a different form of Cu,ZnSOD is found in extracellular fluids, where it is called EC-SOD for extracellular SOD (Marklund et al., 1982). MnSOD is located in the mitochondrial matrix as well as in bacteria, (Huber, 1994) while FeSOD is present in many aerobic bacteria. FeSOD has a molecular weight (MW) of 39 000 contains on Fe^{3+} per molecule, and appears to be composed of two subunits of equal size and on the other hands, MnSOD has a MW of 40 000 with two subunits. It is generally considered that FeSOD is a constitutive enzyme, while MnSOD is an inducible enzyme (Fridovich, 1975). Besides being antioxidants, SOD is also acts as anticarcinogens, inhibitors at the initiation stage of mutation in mammalian cell, and protectors against oxidative damage (Yi Sun, 1990).

Since the SOD is a potential biological antioxidant, production of SOD will be possible by using recombinant strain of *E. coli BL21* (DE3) pLysS in batch and fed-batch fermentation. Genetic modification by Tan, 2003 was successfully insert a full length of SOD gene from locally isolated *Lactoccocus lactis* M4 by Polymerase Chain Reaction (PCR) technique and cloned into pRSET A which is an *E. coli* expression vector.

To achieve high product yields, it is a perquisite to design a proper production medium in an

efficient fermentation process. Little information, however, is available in the scientific literature on complete optimization of culture media and pH for SOD production; it is well known that medium optimization is approached either empirically or by statistical methods. But either the classical or empirical method has several problems for complete optimization. The traditional one-factor at a time approach to optimization is time consuming and incapable of reaching the true optimum due especially to interaction among factors. Moreover, it assumes that the various fermentation parameters do not interact and that the process response is a direct function of the single varied parameter.

Response Surface Methodology is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such technique. Another advantage of RSM is the interaction among possible influencing parameters can be evaluated with limited number of experiments. In this study, we applied RSM, especially FFD and CCD, to further attempt to enhance the maximum growth of *E. coli* carrying the SOD gene using the optimized pH, carbon and nitrogen sources in shake flask culture. Another advantage of RSM is the interaction among possible influencing parameters can be evaluated with limited number of experiments. Recently, Tan (2003) was successfully insert a full length of SOD gene from locally isolated *Lactoccocus lactis* M4 into pRSET A, which is an *E. coli* expression vector using Polymerase Chain Reaction technique. Hence, in this study, we highlighted an optimization study on the growth of the strain, especially using FFD and CCD to enhance the growth of *E. coli* using the optimized initial pH, carbon and nitrogen sources in shake flask cultures.

Materials and Methods

Strain and Culture Conditions

E. coli BL21 (DE3) pLysS, carrying SOD gene (ESOD) from local isolated *Lactoccocus lactis* M4 was used in this study (Tan, 2003). All optimization experiments were carried out using 250ml Erlenmeyer flasks, containing 50ml of modified Terrific Broth medium with various concentrations of glucose and nitrogen sources according to the experiment design, K_2HPO_4, 9.4g/L and KH_2PO_4 2.2 g/L. Initial pH of the medium were varies accordingly using 1M of HCl and 1M NaOH. All media were autoclave separately as stock solution at 121°C for 15 min and cooled to room temperature prior to use. Filtered sterile antibiotics, Chramphenicol and Ampicillin with concentration 35 μg/ml and 50 μg/ml respectively were added to each flask. The flasks were incubated at 37°C for 24 hours, and agitated at 250 rpm in rotary incubator shaker. 1ml of sample was withdrawn at time interval for dry cell weight determination.

Experimental Design

Full Factorial Design (FFD)

Full Factorial Design (FFD) was applied as a screening method to the independent variables that were selected in this study. The variables were carbon to nitrogen ratio and initial pH of the medium. The level was determined as high and low value for each of the variables. The design was characterized by Design Expert version 6.0.6 (State Ease Inc., Minneapolis, MN, USA). Table 1 indicates the FFD experiments with different C/N ratio and initial pH conditions. Glucose concentration used was ranged between 0g/L to 25g/L, while yeast extract (5g/L) to tryptone (2.5g/L) ratio was 2:1 as a nitrogen source. 10g/L of yeast extract and 5g/L of tryptone (2:1) were used in the run 1 and 7 experiments. The experiments based on the FFD were done in duplicate.

Table 1: Full Factorial Design

Run	Carbon/Nitrogen	pH
1	0	9
2	7	7.5
3	0	6
4	7	7.5
5	0	9
6	14	6
7	0	6
8	14	9
9	14	9
10	14	6

Central Composite Design (CCD)

The experiment was designed using Design Expert version 6.0.6 (State Ease Inc., Minneapolis,MN,USA). The statistical software was used for regression and graphical analysis of the data obtained. In developing the regression equation the test factors were fitted with a second order polynomial equation by a multiple regression technique (Lu *et al.* 2004).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i x_j + \sum \sum_{i<j} \beta_{ij} x_i x_j \quad (1)$$

Y is the predicted response; $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ are constant coefficients and the x_i, x_j are the coded independent variables or factors. The quality of fit of the second order model equation expressed by the coefficient of determination of R^2, and its statistical significance was determined by an F test.

The major factor that were considered to influence the ESOD growth are glucose (X_1), yeast extract and tryptone (X_2) and pH (X_3). Table 2 indicate CCD study of 3 experimental factors and 5 levels in coded unit and actual unit with maximized within boundary ($\alpha = \pm 1.68$) .

Table 2: Experimental Factors and Levels

Code Unit/Level	Actual Value		
	Glucose g/L	Yeast Extract and Trytone g/L (2:1)	pH
1.682	30.11	37.80	7.84
1.0	25.00	32.00	7.50
0	17.50	23.50	7.0
-1.0	10.00	15.00	6.50
-1.682	4.89	9.20	6.16

Analysis Samples

Dry cell weight were estimated using a calibration curve between dcw and optical density at 600nm (OD_{600nm}). The dcw values of the calibration curve samples were obtained from aliquots of culture broth washed twice with 0.9% (w/v) NaCl isotonic solution, filtered and dried at 100°C

until constant weight. One optical density at 600nm is equal to 0.541 gl^{-1} dcw.

Determination and quantification of glucose and others organic acids were done by using High Performance Liquid Chromatography (HPLC) (column SUPELCOGEL C-610H) , 0.1% H_3PO_4 and 1 ml/min of mobile phase and flow rate respectively. Separation was done in room temperature and detection was by using Refractive Index detector (RI). pH was measured with pH meter, Metler Toledo MP320U.

Results and Discussion

Full Factorial Design

Table 3 shows the results obtained from FFD experiments on the effect of C/N ratio and initial pH on the maximum cell concentration of ESOD. From the table, highest cell concentration of ESOD obtained was 3.59 g/L at a combination of 7 C/N ratios and initial pH of 7.5. While about 3.25 g/L of maximum cell concentration was achieved when using C/N ratio of 14 at initial pH of 9. Lower cell concentrations were obtained at 0 C/N ratios, which are 0.79 g/L and 1.35 g/L at initial pH of 9 and 6 respectively. From the observation, addition of nitrogen gave significant increased of maximum cell concentration obtained at the same initial pH. Higher maximum cell concentration (1.89g/L) was obtained at run 1 as compared to run 5 (0.79g/L). Similar results were obtained at run 3 and 7 where addition of nitrogen improved maximum cell concentration achieved.

Table 3: Full Factorial Design Results

Shake flasks	Carbon/nitrogen	Initial pH	Final pH*	X_{max} (g/L) of ESOD
1	0	9	8.67	1.89
2	7	7.5	4.94	3.59
3	0	6	6.58	1.35
4	7	7.5	4.93	3.58
5	0	9	8.04	0.79
6	14	6	4.53	2.22
7	0	6	6.95	2.47
8	14	9	4.86	3.25
9	14	9	4.95	3.24
10	14	6	3.90	2.61

*pH obtained after 24 hours cultivation

Based on the results obtained, it can be suggested that C/N ratio and pH gave significant effect on the growth of ESOD. Higher C/N ratio value above 7 and pH ranging from 7 to 9 would promote better growth and higher cell concentration achieved. Higher initial pH (9) was favor for better growth of ESOD rather than low initial pH (6). Generally, pH of all cultures was observed to be decreased greatly to acidic conditions after 24h of cultivation to about pH 4 to 5. The reduction of pH in the culture is due to the presence of acetate and formation of several acids such as acetic and lactic acid (data not shown).

Acetate formation in the medium cultivation of *E. coli* was lavishly discussed by Lee, (1996), Roe *et al.* (2002), Suarez & Kilikian (2000), Luli & Strohl (1990) and Riesenberg (1991). There also reported that the high concentration of acetate (*i.e.* above $5gl^{-1}$ at pH 7) reduces growth rate, biomass yield and maximum attainable cell densities in High Cell Density Cultivation (HCDC) of *E. coli* (Luli & Strohl, 1990). A report has detailed the formation of acetate during HCDC of *E. coli* TG1 with glycerol as carbon source (Riesenberg, 1991). It is also noted that glycerol is more

expensive than glucose and that cells grow more slowly on glycerol than on glucose.

Robbins & Taylor, 1989 said that during aerobic growth of *Escherichia coli* (recombinant K-12 and strain B) on protein hydrolysate (L-broth) and a carbon source (glucose), acetic acid is produced via glucose metabolism until the late log phase. At this point, the culture pH starts to increase and the growth rate decreases. In cultures without further glucose supplementation, these changes are associated with the accumulation of ammonia, the utilization of acetic acid, the depletion of amino acids, and the complete depletion of glucose. They again hypothesize that, after depletion of the glucose, the bacteria catabolize amino acids for energy and carbon and give off the nitrogen as ammonia. Also contributing to the overall increase in pH is the depletion of the acetic acid produced earlier as it is metabolized upon exhaustion of glucose. However, there is a lag time of about 1 hour after the initial pH increase before the sustained accumulation of ammonia begins. This lag indicates that an unidentified factor, in addition to the increase in ammonia, contributes to the increase in pH.

Central Composite Design

The results from the FFD in this study signify that all the factors implemented were important components in optimizing the culture medium by using CCD. The results of the second order response surface model fitting in the form of analysis of variables (ANOVA) are given in Table 4. Based on the Table 4, the fisher F-test with a very low probability value (P_{model} >F = <0.0001) demonstrate a very high significance for the regression model. As reportted by Adinarayana & Ellaiah (2002), high significance for the regression mode could be demonstrated by very low probability value. The perfection of fit of the model was checked by the determination coefficient (R^2). In this study, the value of the determination coefficient (R^2 = 0.963) indicates that only 3.7% of the total variations are not explained by the model. The value of the adjusted determination coefficient (Adj R^2 =0.9297) is also very high, which indicates a high significance of the model. At the same time, a relatively lower value of the coefficient of variation (C.V= 6.35) indicates improved precision and reliability of the conducted experiments (Adinarayana & Ellaiah, 2002). Values of "Prob > F" less than 0.0500 indicate model terms are significant, therefore all the model terms in the Table 4 are significants. This was included first order, second order and interaction between variables.

The application of RSM yielded the folowing regression equation which empirical relationship between the concentration values of dry cell weight of ESOD and test variables in coded unit. The experimental results were subjected to multiple regression analysis and the dry cell weight was described in terms of the following second order polynomial equation for equation (1) ;

$$Y\ g/L = +3.31 - 0.26\ x_1 - 0.28\ x_2 + 0.14\ x_3 + 0.33\ x_1 2 - 0.34\ x_2 2 + 0.14\ x_3 2 - 0.35\ x_1 x_2 - 0.72\ x_1 x_3 - 0.33\ x_2 x_3 \quad (2)$$

Where Y is the response, that is the dry cell weight, g/L and x_1,x_2 and x_3 are the coded values of the test variables (glucose, yeast extract and tryptone (2:1) and pH respectively).

Table 4 : Analysis of Variables of ESOD

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	12.12	9	1.35	28.91	< 0.0001	***significant***
A	*0.95*	*1*	*0.95*	*20.30*	*0.0011*	
B	*1.06*	*1*	*1.06*	*22.68*	*0.0008*	
C	*0.25*	*1*	*0.25*	*5.38*	*0.0428*	
A2	*1.53*	*1*	*1.53*	*32.80*	*0.0002*	
B2	*1.65*	*1*	*1.65*	*35.36*	*0.0001*	
C2	*0.30*	*1*	*0.30*	*6.41*	*0.0298*	
AB	*0.96*	*1*	*0.96*	*20.67*	*0.0011*	
AC	*4.18*	*1*	*4.18*	*89.74*	*< 0.0001*	
BC	*0.89*	*1*	*0.89*	*19.09*	*0.0014*	
Residual	0.47	10	0.047			
Lack of Fit	*0.31*	*5*	*0.062*	*2.02*	*0.2294*	***not significant***
Pure Error	*0.15*	*5*	*0.031*			
Cor Total	12.59	19				
Std. Dev.		0.22		R-Squared		0.963
Mean		3.40		Adj R-Squared		0.9297
C.V.		6.35		Pred R-Squared		0.7745
PRESS		2.84		Adeq Precision		20.936

DF : Degree of Freedom
Std. Dev. : Standard Deviation
C.V. : Coefficient of Variance
PRESS : Predicted Residual Error Sum of Square

Adj R-Square : Adjusted R Square
Pred R-Square : Predisted R-Square
Adeq Precision : Adequate Precision

The statistical analysis of the experimental results shows that pH was more important variables for the growth of ESOD. It was evident from the values of coefficients in the equation (1) that pH ($\beta_3 = 0.14$). The response of square coefficient of glucose (x_1^2) and pH (x_3^2) have an effect on the ESOD growth rather than yeast extract and tyrptone (2:1) (x_2^2). Three dimensional graphs were generated for the pair-wise combination of the three factors, while keeping the other one at its medium levels for ESOD growth. Figure 1 is given to highlight the roles played by various factors and comparison between factors. All figure are saddle shaped that showed probability of the variables could be maximum or minimum point and the system of the contours is called a saddle or minimax system (Myers & Montgomery, 2002).

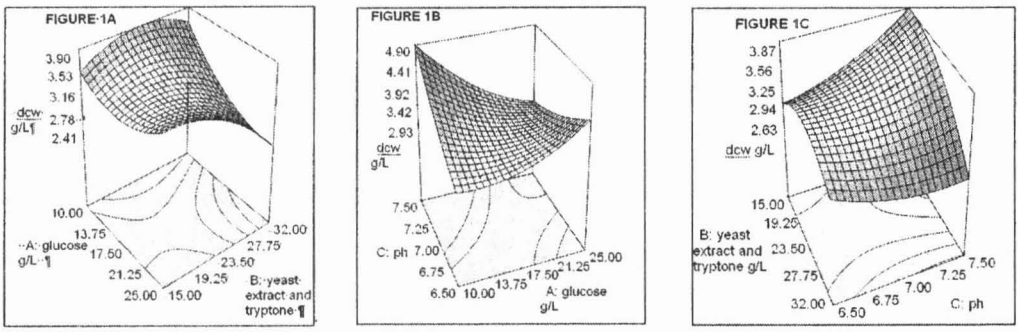

Figure 1: Response Surface plots for the effects of initial pH (A), yeast extract and tryptone, g/L (B) and glucose, g/L (C)

The optimised medium composition and pH allowed a maximum cell concentration of 7.39 g/L, which compared very well with the maximum predicted value of 7.385 g/L. Optimum conditions of the factors for the growth of ESOD were as follows; glucose (4.89 g/L), yeast extract and tryptone (21.86 g/L) and initial pH (7.84). The optimization of the medium composition and cultural conditions is very important for the cultivation of ESOD in order to design for HCDC, especially on the large scale cultivation.

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