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Increased expression of recombinant cholesterol oxidase in *Escherichia coli* by optimization of culture condition using response surface methodology

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Aim. **The FAD-containing enzyme, cholesterol oxidase (EC 1.1.3.6) is an enzyme that catalyses sequential reactions which convert cholesterol (5-cholesten-3-b-ol) to 4-cholesten-3-one with concomitant reduction of oxygen to hydrogen peroxide. In fact, this enzyme catalyzes two reactions in one active site: oxidation and isomerization. Cholesterol oxidase is an industrially and commercially important enzyme.**

Methods. **Response surface methodology that uses quantitative data from appropriate experiments was applied to optimize the culture conditions for recombinant cholesterol oxidase production in *Escherichia coli* (*E. coli*). Parameters considered were concentrations of IPTG, induction time, fermentation temperature and incubation period. Design of optimal experimental conditions was performed by four-factor, three levels Box-Behnken model which yielded improved cholesterol oxidase production.**

Results. **Under optimal condition, productivity of cholesterol oxidase increased approximately 2-fold as compared to un-optimized medium.**

Conclusion. **These findings demonstrate that response surface methodology could efficiently be applied for optimization of recombinant cholesterol oxidase production in *E. coli* as the experimental data for productivity of cholesterol oxidase had a good correlation with predicted values.**

KEY WORDS: Cholesterol oxidase - Escherichia coli - Enzymes.

The FAD-containing enzyme, *cholesterol oxidase* (EC 1.1.3.6) is an enzyme that catalyses sequential reactions which convert cholesterol (5-cholesten-3-b-ol) to 4-cholesten-3-one with concomitant reduction of oxygen to hydrogen peroxide. In fact, this enzyme catalyzes two reactions in one active site: oxidation and isomerization.[1] Cholesterol oxidase is an industrially and commercially important enzyme. Over the decades, the amount of cholesterol in food and blood serum has been measured by cholesterol oxidase coupled with cholesterol esterase and peroxidase reaction.[2] It has been reported cholesterol oxidase may also be used in manufacturing of starting materials for the chemical synthesis of pharmaceutical steroids,[3] in regio-, stereo- and enantio-selective oxidation of non-steroidal compounds such as allylic alcohols [4, 5] and in association with lipid bilayers to study the role of enzyme in physical structure of lipid membranes.[6] Also, previous studies showed cholesterol oxidase has potent larvicidal activity against crop-damaging insects. This activity is very important and vital for pest control strategies in the case of transgenic crops.[7] Furthermore, some pathogenic bacteria (*e.g. Rhodococcus equi* and slow-growing *Mycobacteria*) require cholesterol oxidase for infecting their host macrophage and thus for their survival inside the infected cell. The membrane-perturbing effects of cholesterol oxidase is probably because of the ability of this enzyme

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to change the physical structure of the membrane by converting cholesterol to cholesten-4-en-3-one.[8] Due to the fact that cholesterol oxidase is specific for the species or strain of bacteria, inhibition of its activity in pathogenic bacteria can stop bacterial virulence. Targeting and blocking of bacterium enzyme may lead to emerging new class of antibiotics in future. More recently, it has been demonstrated that Alzheimer's disease β-amyloid, selectively oxidizes cholesterol at the C-3 hydroxyl group and catalytically produces 4-cholesten-3-one; therefore it mimics the activity of cholesterol oxidase.[9] Further studies may shed light on the etiological role of this activity in developing therapeutic targets for Alzheimer's disease.

Response surface methodology (RSM) is a powerful statistical technique for the optimization of process containing multivariable such as biomass cultivation,[10] spore generation,[11] enzymes production [12] and metabolite secretion.[13] Compared with conventional methods, "one factor at a time" approaches, which are time-consuming, this method can reduce the number of experiment runs. RSM allows modeling, analyzing and determining real optimal condition using important factors in process.

In our previous works, we cloned and characterized a cholesterol oxidase gene from *Rhodococcus equi* PTCC1635.[14-17] As far as known to the authors, no study has been conducted on optimization of the production of recombinant cholesterol oxidase using the response surface methodology (RSM) approach. This manuscript attempts to formulate a suitable production medium condition using statistical optimization that can increase the cholesterol oxidase production from recombinant *E. coli* by response surface methodology.

Materials and methods

Microorganism, media and cultivation conditions

Escherichia coli (*E. coli*) BL21 (DE3) pLysS harboring pET23a (choR) was constructed in our previous work.[14] *E. coli* strains were grown in Luria-Bertani (LB) medium consisting of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. For induction of the *lac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the LB medium with different concentration at the appropriate time according to table 1 and in previous study [14].

Cholesterol oxidase activity determination

To determine cholesterol oxidase activity, the absorbance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol, and peroxidase was measured at 505 nm by spectrophotometry. One unit of activity was defined as the formation of 1 mol of hydrogen peroxide (0.5 µmol of quinoneimine dye) per minute at 37 ºC and pH 7.0. The protein concentration was determined with the Bradford method using human albumin as a standard.

Experimental design and optimization by Box-Behnken design

Response surface methodology was used to predict and optimize the factors affect the production level of cholesterol oxidase. The major factors were optimized using a three-level (-1, 0, 1) four-factors Box-Behnken design (BBD). The following equation was generated based on the behavior of the system:

$$Y= b_0 + \sum b_i X_i + \sum b_{ii} X^2_{ii} + \sum b_i X_j$$

where Y, b_0, b_i, b_{ii}, b_{ij}, represents respectively, the predicted response in the form of cholesterol oxidase activity, the constant process effect in total, the linear, quadratic effect of X_i and the interaction effect between X_i and X_j on cholesterol oxidase activity.

Optimized conditions were as follows: concentrations of IPTG, induction time, fermentation temperature and incubation period in the medium. The four factors were designated as X_1, X_2, X_3 and X_4. A total of 27 experimental runs with different combinations of four factors were performed in triplicate fashion. The model prescription including coded and uncoded values of the variables at various levels are described in Table I. The levels of the variables for the BBD experiments were selected according to the results of the previous experiments (data not published).

Software for experimental design

MINITAB software 15 (Minitab, Inc.) was used to create experimental design and evaluate the results. The optimum set for cholesterol oxidase production was obtained by solving the regression equation and by analyzing the response surface plots using the same software.

TABLE I.—*The design of experiments and response of cholesterol oxidase activity.*

Run number	Induction time (X1)	Temperature (X2)	IPTG con. (X3)	Incubation (X4)	Activity (U/mL)	
					Actual	Predicted
1	0 (0.6)	-1 (15)	-1 (0.2)	0 (4)	0.630	0.660
2	0 (0.6)	1 (35)	1 (1)	0 (4)	1.197	1.298
3	0 (0.6)	0 (25)	0 (0.6)	0 (4)	1.815	1.826
4	0 (0.6)	0 (25)	-1(0.2)	-1 (2)	0.615	0.736
5	0 (0.6)	-1 (15)	1 (1)	0 (4)	1.128	1.251
6	1 (1)	-1 (15)	0 (0.6)	0 (4)	1.179	1.260
7	-1 (0.2)	0 (25)	0 (0.6)	-1 (2)	0.624	0.714
8	0 (0.6)	1 (35)	0 (0.6)	-1 (2)	1.656	1.560
9	-1 (0.2)	0 (25)	-1 (0.2)	0(4)	0.528	0.400
10	1 (1)	0 (25)	0 (0.6)	1 (6)	1.131	1.172
11	1 (1)	0 (25)	-1 (0.2)	0 (4)	0.678	0.583
12	0 (0.6)	-1 (15)	0 (0.6)	-1 (2)	1.017	0.875
13	1 (1)	0 (25)	0 (0.6)	-1 (2)	1.101	1.107
14	0 (0.6)	0 (25)	0 (0.6)	0 (4)	1.848	1.826
15	0 (0.6)	0 (25)	1 (1)	-1 (6)	0.903	0.925
16	0 (0.6)	0 (25)	0 (0.6)	0 (4)	1.815	1.826
17	-1 (0.2)	0 (25)	1 (1)	0 (4)	1.011	0.889
18	1 (1)	1 (35)	0 (0.6)	0 (4)	1.086	1.142
19	0 (0.6)	1 (35)	0 (0.6)	1 (6)	1.401	1.327
20	-1 (0.2)	0 (25)	0 (0.6)	1 (6)	1.101	1.226
21	1 (1)	0 (25)	1 (1)	0 (4)	1.134	1.045
22	0 (0.6)	-1 (15)	0 (0.6)	1 (6)	1.806	1.686
23	0 (0.6)	0 (25)	1 (1)	1 (6)	1.536	1.501
24	-1 (0.2)	-1 (15)	0 (0.6)	0 (4)	0.780	0.810
25	-1 (0.2)	1 (35)	0 (0.6)	0 (4)	1.248	1.253
26	0 (0.6)	1 (35)	-1 (0.2)	0 (4)	0.930	0.938
27	0 (0.6)	0 (25)	-1 (0.2)	1 (6)	0.675	0.739

Results and discussion

BBD was used to optimize cholesterol oxidase production by *E. coli* in shake-flask cultivation. Table I indicates preliminary results of twenty seven experiments which carried out according to 4-factor-3-levels BBD. A second-order polynomial function was fit to the experimental cholesterol oxidase yield, resulting in the following regression equation:

$$Y=0.608667 + \\ + [0.028250X1 + 0.027167X2 + 0.079250X3 + 0.048167X4] + \\ + 0.169500X_1^2 - 0.067125X_2^2 - 0.196000X_3^2 - 0,087625X_4^2] + \\ + [-0.046750X1,2 - 0.087000X2,4 + 0.047750X3,4]$$

According to prediction model shown in Table I, the maximum and minimum of cholesterol oxidase yields were in experiments no. 14 and 9 which are 1.848 and 0.528, respectively. As indicated in Table II, the regression analysis of the data showed coefficient of determination (R^2) value of 0.9573 and adjusted R^2 value of 0.9075. These values are in close agreement and it ensures a satisfactory adjustment of the proposed model with more than 90% predictability in response being explained by proposed model and only 9.25 of the total variance could not be explained by the model. The adjusted determination coefficient (Adj R^2=0. 9075) was also satisfactory to prove the significance of the model. This shows that calculated equation was an appropriate model to represent the response of the experiment pertaining to cholesterol oxidase production. The model coefficients calculated by regression analysis for each variable were given in Table II. t-Test and P-values were employed to evaluate the significance of each coefficient. The larger t-test value and the smaller P-value suggest higher significance of the corresponding coefficient. The analyses of predicted model vs. experimentally observed yield values suggested both positive and negative dispersion of observed values.

To determine the optimal condition of cholesterol oxidase production and the relationship between the response and the significant variables, F-test and ANOVA was performed through a joint test of four parameters with the minitab software package.

TABLE II.—*Estimated Regression Coefficients for activity.*

Term	Coef	SE Coef	T	P
Constant	0.608667	0.02362	25.764	0.000
X_1	0.028250	0.01181	2.392	0.034
X_2	0.027167	0.01181	2.300	0.040
X_3	0.079250	0.01181	6.709	0.000
X_4	0.048167	0.01181	4.078	0.002
X_1^2	-0.169500	0.01772	-9.566	0.000
X_2^2	-0.067125	0.01772	-3.788	0.003
X_3^2	-0.196000	0.01772	-11.062	0.000
X_4^2	-0.087625	0.01772	-4.945	0.000
$X_{1,2}$	-0.046750	0.02046	-2.285	0.041
$X_{1,3}$	-0.002250	0.02046	-0.110	0.914
$X_{1,4}$	-0.037250	0.02046	-1.821	0.094
$X_{2,3}$	-0.019250	0.02046	-0.941	0.365
$X_{2,4}$	-0.087000	0.02046	-4.252	0.001
$X_{3,4}$	0.047750	0.02046	2.334	0.038

R^2=95.73%, R^2 (adj)=90.75%.

TABLE III.—*Analysis of Variance for cholesterol oxidase production.*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	0.450593	0.450593	0.032185	19.22	0.000
Linear	4	0.121640	0.121640	0.030410	18.16	0.000
Square	4	0.273761	0.273761	0.068440	40.88	0.000
Interaction	6	0.055191	0.055191	0.009199	5.49	0.006
Residual error	12	0.020092	0.020092	0.001674		
Lack-of fit	10	0.020011	0.020011	0.002001	49.62	0.020
Pure error	2	0.000081	0.000081	0.000040		
Total	26	0.470685				

DF: degree of freedom; SS: sum of square; MS: mean square; RE: residual errors.

ANOVA for cholesterol oxidase production showed that fitted second-order response surface model was highly significant with F-test=19.22 (P=0.000) as shown in Table III. The Student's t-test was used to determine the significance of the regression coefficients of the variables. X_3 P-value is the indicator of the significance of the test, whose value below 0.05 indicates that test parameter is significant at 5% level of significance. The p-value is used for the evaluation of model significance. A very significant model has a P-value below 0.001, a significant model below 0.01 and an almost significant model below 0.05. Table II indicates that both induction time and fermentation temperature had significant effect on cholesterol oxidase production while the statistical model predicted IPTG concentration and incubation period had more linear effect on the response (P≤0.05).

Three dimensional response surface plots graphically represent regression equations and are generally used to demonstrate relationships between the response and experimental levels of each variable. These surface plots, therefore, allow for visualization of the optimum levels of each variable for the maximum production of microbial metabolites. Figure 1A-F represents the surface plots for the optimization of medium condition of cholesterol oxidase production. Each figure presents the effect pair-wise interaction of two factors on the production of cholesterol oxidase, while the other two factors maintained at their respective zero levels.

As shown in the surface plots, there was interaction between each pair of variables. Only the interaction between fermentation temperature (X2) and incubation period (X4) was highly significant (P=0.001). Interaction between induction time (X1) & fermentation temperature (X2) and IPTG concen-

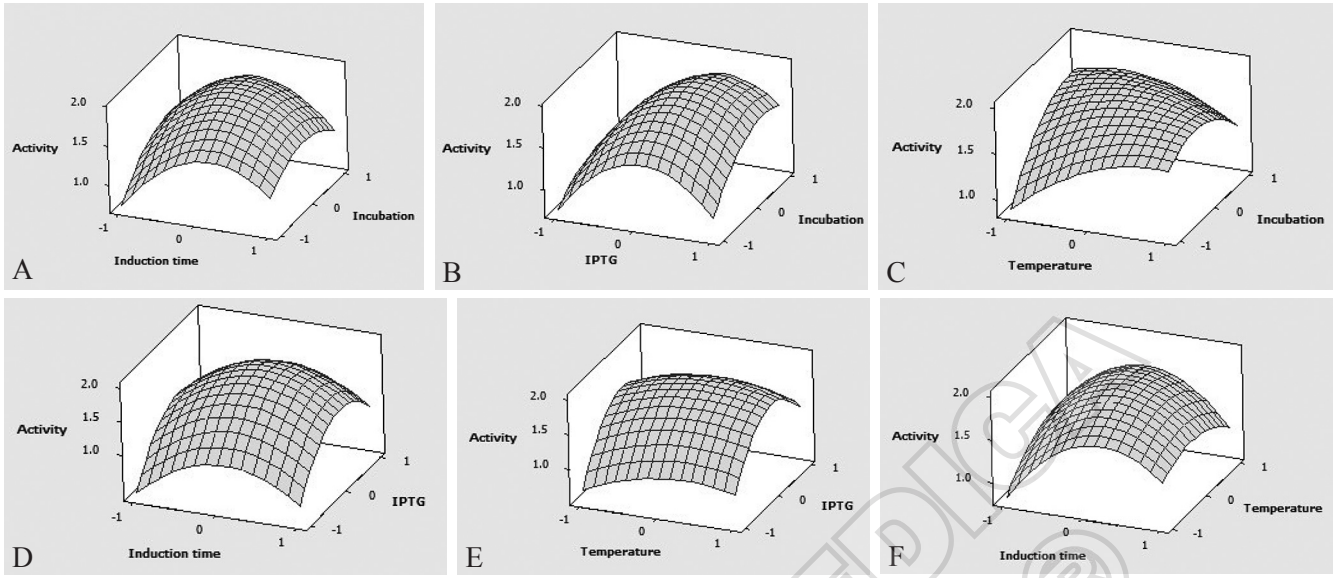

Figure1.—Surface plot of cholesterol oxidase production: effect of induction time and incubation period and their interaction on cholesterol oxidase production. Other variables are held at zero level. A) Surface plot of cholesterol oxidase production: effect of incubation period and IPTG concentration and their interaction on cholesterol oxidase production. Other variables are held at zero level. B) Surface plot of cholesterol oxidase production: effect of fermentation temperature and incubation period and their interaction on cholesterol oxidase production. Other variables are held at zero level. C) Surface plot of cholesterol oxidase production: effect of induction time and IPTG concentration and their interaction on cholesterol oxidase production. Other variables are held at zero level. D) Surface plot of cholesterol oxidase production: effect of fermentation temperature and IPTG concentration and their interaction on cholesterol oxidase production. Other variables are held at zero level. E) Surface plot of cholesterol oxidase production: effect of fermentation temperature and induction time and their interaction on cholesterol oxidase production. Other variables are held at zero level.

tration (X3) & incubation period (X4) had less effect on cholesterol oxidase production. But, the interaction between induction time (X1) & IPTG concentration (X3), induction time (X1) & incubation period (X4) and fermentation temperature (X2) & IPTG concentration (X3) was not significant (P=0.914, 0.094 and 0.365, respectively). High concentration of IPTG had an inhibitory effect on bacterial growth which caused decreased cholesterol oxidase production especially when the bacterial cells are in the last stage of logarithmic and plateau growth phase. Moreover, increasing the fermentation temperature enhances recombinant protein production but most of the expressed protein is formed as inclusion body and the amount of soluble protein will be lesser than optimum condition. In lower fermentation temperature, the folding machine of bacteria has enough time to process the protein appropriately and this yields more active protein production. An IPTG concentration of 0.6 mM, OD_{600} (induction time) of 0.6, fermentation temperature of 25 °C and incubation time of 4 hr were chosen as the optimal condition, the highest cholesterol oxidase activity of 1.848 was achieved, which reached 98.80% of the predicated value (1.82600) by the software. The outcome of experiments confirmed that this model founded by the software was successfully built and had excellent validity. There was about two-fold increase in yield of cholesterol oxidase as compared to the unoptimized media by using these statistical approaches.

To assess the validity of the quadratic model, the experimental conditions with the highest desirability were selected to be verified in additional series of trials in three replicates. Consequently, the highest level of cholesterol oxidase achieved from the trials was 1.785 (data not shown). This value is very close to the cholesterol oxidase production predicted by means of response surface methodology (RSM) under optimal conditions (1.82600). The verification revealed a high degree of accuracy of the model of more than 97.75%, which is an evidence for the model validation under the investigated conditions and demonstrates that the quadratic model is an adequate predictor of the experimental results.

Conclusions

The application of statistical design for screening and optimization of process parameters allows quick identification of important factors and interactions between them. To optimize the selected factors for maximal production and to check the effect of factors interaction, Box-Behnken design is an efficient method. In the present study, Box-Behnken design was useful in studying the factors that supported the enhanced production of cholesterol oxidase by *E. coli*. The results of this study have clearly indicates that RSM is a powerful and useful tool for enhancing cholesterol oxidase production by *E. coli*. Having studied the effect of independent variables on the cholesterol oxidase production, an almost 2-fold increase in yield was obtained when the strain was cultivated in the optimized medium developed by BBD, as compared to the original medium.

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