Running Head: Novel Recombinant Phytate-degrading Enzyme Production

Statistical Optimization of the Induction of Phytase Production by Arabinose in a recombinant *E. coli* using Response Surface Methodology

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Abstract

The production of phytase in a recombinant *E.coli* using the pBAD expression system was optimized using response surface methodology with full-factorial faced centered central composite design. The ampicilin and arabinose concentration in the cultivation media and the incubation temperature were optimized in order to maximize phytase production using 2^3 central composite experimental design. With this design the number of actual experiment performed could be reduced while allowing eludidation of possible interactions among these factors. The most significant parameter was shown to be the linear and quadratic effect of the incubation temperature. Optimal conditions for phytase production were determined to be 100 µg/ml ampicilin, 0.2 % arabinose and an incubation temperature of 37ºC. The production of phytase in the recombinant *E. coli* was scaled up to 100 ml and 1000 ml.

Keywords: recombinant phytase, statistical optimization, cultivation conditions,

1. Introduction

Most cereals and legumes are rich in protein and fat but the presence of phytate discourages their use in food and feed. Approximately 70% of total phosphorus in cereal grains and legume seeds occurs in form of phytate. Phytate is poorly utilized by monogastric animals and acts as an anti-nutrient due to the chelation of various metals and the binding to protein. This diminishes the bioavailability of proteins and nutritionally important minerals. In animal diets, the supply of phosphorus, an essential mineral for animal growth and development, comes from either the feedstuffs or from inorganic phosphate added to the diets (Poulsen, 2000). These nutritional impediments result in the release of undigested phytate phosphorus in the feces and urine. Phosphorus in the environment accelerates eutrophication of fresh waters and is the main problem in surface water quality, resulting in restricted water use for fisheries, recreation, industry and domestic use (Krishna and Nokes, 2001). The phytate degrading enzymes or phytases convert phytate to partially phosphorylated *myo*-inositol phosphates and phosphate, making phosphorus available for bioabsorption (Irvin and Cosgrove, 1972). Supplementation of microbial phytase to animal diets alters the phytate complexes and also increases the bioavailability of proteins and essential minerals, providing growth performance equivalent or better than those with phosphate supplementation, and also reduces the amount of phosphorus in animal manure (Wodzinki and Ullah, 1996).

Escherichia coli is being used as the most important host organism for recombinant protein production. Expression of a novel bacterial phytase has been achieved in *E. coli* BL21 by arabinose induction under the control of the pBAD expression system (unpublished data). Optimization of the cultivation conditions for the productivity is becoming one of the major goals in biotechnology today. The productivity of any cultivation is affected by process parameters and media composition (Park, K.M. and Reardon, 1996). Preliminary experiments in our laboratory indicated that antibiotic concentration, arabinose percentage and incubation temperature of cultivation media play significant roles in recombinant phytase production. Therefore an investigation was performed to statistically optimize the cultivation media for the phytase production. Response surface methodology (RSM) is now being routinely used for optimization studies in several biotechnological and industrial processes (Beg et al., 2002; De Cornink et al., 2000; Puri et al., 2002). RSM is used to explain the interactions among the possible influencing parameters with limited numbers of experiment.

In this paper, we determine the optimum concentration of ampicilin supplemented in the media, concentration of arabinose for induction purpose, and incubation temperature during cultivation for maximum recombinant phytase production by using full factorial Face Centered Central Composite Design in RSM. The production of phytase was then scaled up to 100 ml and 1000 ml using the optimized cultivation conditions.

2. Materials and methods

2.1. Microorganism and culture conditions

The phytase gene was isolated from a Malaysian waster-water bacterium and transformed into *E.coli* LMG194 using arabinose inducible *E.coli* system, a pBAD TOPO TA Expression system (Greiner and Farouk, 2007) and phytase was expressed as an intracellular enzyme.

E.coli cells were transformed with appropriate plasmids and were grown overnight at 37ºC on Luria Bertani (LB) agar containing 100 µg/ml ampicilin. The overnight colonies were transferred to a shake flask containing LB broth with 100 µg/ml ampicilin and were grown for 18h on an incubating shaker with 200 rpm agitation at 37ºC. This culture was used as an inoculum at 1:6 (v/v).

2.2. Experimental design

After preliminary optimizing, three factors, namely antibiotic concentration (X_1), arabinose concentration (X_2), and incubation temperature (X_3), were observed, to mainly controll phytase production by recombinant *E. coli* under batch fermentation. Taking in consideration the above factors and time constraint, response surface methodology was adopted for improving total phytase production in the recombinant *E. coli*. Inoculum (1:6 v/v) was transferred into 3 units of 250 ml Erlenmeyer flasks containing 120 ml LB broth with different concentrations of ampicilin (50, 100 and 150 µg/ml) for optimization of antibiotic concentration. The flasks were placed in an orbital shaker at 200 rpm and 37ºC. When the exponentially growing culture reached an optical density (OD) of 0.6 at 600 nm, the cultivated media were distributed aseptically into universal bottles (10 ml each) and arabinose induction was carried out at different concentrations (1, 2 and 3 µg/ml) and then the flasks were incubated at different temperatures (30, 37 and 44ºC) in an orbital shaker at 200 rpm. Sampling was done after 6 hours of incubation. The experiments were done in triplicates.

Full-factorial Central Composite Experimental Design with centered face was used to optimize the cultivation conditions for phytase production by the recombinant *E. coli*. The experiments were designed by using the Minitab 14 Statistical Software. A 2^3 – factorial experimental design with four axial points (with $\alpha = 1$ and six replicates at the center point ($n_0 = 6$)) leading to a total number of twenty experiments being employed for the optimization of antibiotic concentration (X_1), arabinose percentage (X_2) and incubating temperature (X_3).

The design matrix and levels of the independent variables chosen for the study in encoded form are given in Table 1. The average of maximum phytase activity of the duplicate values obtained was taken as dependent variable or response Yi (U). Regression analysis was performed on the data obtained using MINITAB 14. Microsoft Office Excel 2003 was used to calculate the predicted responses. The final validation of the statistical approach to phytase production by recombinant *E. coli* was carried out in triplicates. Samples were withdrawn after 6 hours of incubation.

2.3. Scale up of Phytase Production

The production of phytase in recombinant *E. coli* at optimized conditions (conc. of ampicilin: 100 µg/ml; conc. of arabinose: 2 µg/ml; incubation temperature: 37ºC) was carried out in universal bottles containing 10 ml LB broth, in 250 ml Erlenmeyer Flasks containing 100 ml LB broth and in a 2 L Bioreactor (Biostat- Sartorius) containing 1000 ml LB broth for scale up purpose. The wild type bacterial phytase was used as control (without ampicilin). Experiments were done in triplicates.

2.4. Analytical Method

2.4.1. Chemicals

Phytic acid, dodecasodium salt, was purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals and media were of analytical grade.

2.4.2. Sample preparation

The cultivated media was centrifuged at 13,000 rpm for 10 min (Idriss et al., 2002). The cell-free supernatant was separated and the bacterial pellet was re-suspended in 100 µl of 0.1 M sodium acetate buffer (pH 4.5). Cells were disrupted by 20 sec sonication at 0.5 cycles, 20 % amplitude setting (Labsonic P, B. Braun Biotech International, Germany, using ~3 mm diameter x 100 mm long probe), and placed in an ice-bath. The supernatant and disrupted cells were tested for phytase activity.

2.4.3. Standard phytase assay

Phytate-degrading activity was determined at 60°C in 399 µl 100 mM sodium acetate buffer, pH 4.5 containing 1.03 mM sodium phytate. The enzymatic reaction was started by adding 1 µl of enzyme solution to the assay mixture. After incubating for 30 min at 50°C, the liberated phosphate was measured according to ammonium molybdate method (Heinonen and Lahti, 1981) with some modifications. Added to the assay mixture were 1.5 ml of a freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v) and 100 µl citric acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5-600 µmol phosphate (ε = 8.7 $cm^2/\mu mol$). Activity (units) was expressed as 1 µmol phosphate liberated per minute. Blanks were run by the addition of ammonium molybdate solution prior to adding the enzyme to the assay mixture.

3. Results and discussion

The conventional methods for multifactor experimental design are time-consuming and incapable of showing the true optimum, due especially to the interactions among the factors (Liu and Tzeng, 1998). In order to design a suitable bioprocess for maximum phytase production by a recombinant *E. coli*, the statistical design approach using response surface methodology by face centered central composite design (FCCCD) was used to study the interactive effects of the nutritional and physical factors on phytase production. The factors that have influence on the final response of the system, obtained from a preliminary study carried out individually, were the antibiotic concentration, arabinose percentage and incubation temperature. The response was taken at the maximum phytase activity, which was observed after 6 hours of arabinose induction. Table 2 summarizes the phytase activity (response) of the experiments with the predicted response. The results were analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used. The coefficients of the regression equation were calculated using MINITAB 14 and the following regression equations were obtained.

$$Y = - 905 + 2.35\ X_1 + 146\ X_2 + 41.3\ X_3 - 0.00577\ X_1^2 - 18.1\ X_2^2 - 0.482 X_3^2 - 0.439\ X_1X_2 - 0.0389\ X_1X_3 - 1.98\ X_2X_3 + 0.0129\ X_1X_2X_3$$

Where Y is the response in phytase activity and X_1, X_2 and X_3 represent the test variables (antibiotic concentration supplemented in the media, arabinose concentration for induction and incubation temperature after the arabinose induction).

The significance of each coefficient was determined by T values and P values which are listed in Table 3. The larger the magnitude of the T value and the smaller the P value indicate the high significance of the corresponding coefficient (Karthikeyan et al., 1996). It can be seen that the variable with the most effect was the linear effect of incubation temperature (X_3) followed by the quadratic effect of incubation temperature (X_3^2) compared to the quadratic effect of arabinose concentration (X_2^2). The highest probability value of coefficient is the interaction effect of antibiotic concentration and arabinose concentration (0.231), indicating that about 76.9 % of the model is affected by these variables. Since linear and quadratic effects of incubation temperature are the most significant, it means that it can act as a limiting factor, where even small variations will alter the product formation rate.

The regression equation and determination coefficient R^2 were evaluated to test the fit of the model. The model presented a high determination coefficient with(?) 94 % of the variability in the response (Table 3). The values of determination coefficient and the adjusted determination coefficient are very high indicating a high significance of the model (Akhnazarova and Kefarov, 1982; Khuri and Cornell, 1987), which is more suited for comparing models with different numbers of independent variables (Beg et al**,** 2003). This reveals that there is a good agreement between the experimental and predicted values of phytase production (Table 2). The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is the evident from the Fishers F-test, with a very low probability value (P>F=0.0001).

Figures 1a-c show the contour plots of the relative effects of two factors when all the factors were kept at their central levels. The plots depict the interaction of antibiotic and arabinose concentration (Fig.1a), antibiotic concentration and incubation temperature (Fig. 1b) and arabinose concentration and incubation temperature (Fig.1c), respectively. The contour plots clearly show elliptical shapes where the optimum conditions can be easily determined. These indicate that there are major interactions among the independent variables corresponding to the response surface. In the investigated area, it shows that the optimum level of cultivation conditions that produce the highest phytase were in the LB broth with 100 µg/ml of ampicilin, induction with 2 µg/ml arabinose and incubated at 37ºC after induction. The contour plots are the graphical representations of the regression equation. The main goal of response surface is to efficiently hunt for the optimum values of the variables such that the response is maximized. Each contour curve represents an infinitive number of combination of two test variables with the other one maintained at their respective zero level. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar et al., 2001).

From several studies (Pasamontes et al, 1997; Berka et al., 1998; Ghosh**,** 1997) the optimum temperature for the production of phytases from most microorganisms was in the range of 25 to 37ºC. Using the regression equation, the predicted optimum level of cultivation media for higher phytase production are assumed to be at 87 µg/ml of antibiotic, 2.1 µg/ml of arabinose and incubated at 37.4ºC.

The response surface model is a valuable tool for predicting and optimizing the cultivation media to maximize phytase production. From the findings, it can be concluded that phytase production by the recombinant *E. coli* can be improved by controlling various factors simultaneously.

The validation model with experimental data as well the predicted values from response surface model under the optimized cultivation conditions are presented in Table 5. A phytase production under the optimized cultivation media for the validation model was within the range of the predicted value from the response surface model.

The scale-up production of phytase by the recombinant *E. coli* in different volume of cultivation media is shown in Table 6. Enzyme activity was shown to decrease with the increment of cultivation volume.

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Table 1: Experimental range and levels of the independent variables for phytate-degrading enzyme production

Variables	Symbol coded	Range and level		
		-1	0	+1
Antibiotic Concentration (µg/ml)	X_1	50	100	150
Arabinose Concentration (µg/ml)	X_2	1	2	3
Incubation Temperature (ºC)	X_3	30	37	44

Table 2: Experimental design of three independent variables showing experimental and predicted response

Antibiotic Concentration (µg/ml)	Arabinose Concentration (µg/ml)	Incubation Temperature (ºC)	Phytase Activity (U/ml)	
			Experimental	Predicted
50	3	44	29.41	29.57
50	2	37	66.18	71.26
150	3	30	22.31	21.33
100	3	37	55.15	63.42
100	2	44	44.12	56.27
50	1	30	10.42	10.83
100	2	37	84.57	77.35
50	3	30	33.09	34.03
100	1	37	44.12	55.08
100	2	37	80.40	77.35
100	2	37	88.24	77.35
100	2	30	44.12	51.20
150	2	37	40.44	54.59
100	2	37	77.21	77.35
150	1	44	7.35	5.05
50	1	44	44.12	43.75
100	2	37	84.57	77.35
150	1	30	10.05	8.52
150	3	44	18.38	16.59
100	2	37	73.54	77.35

Table 3: The least-squares fit and parameter estimates (significance of regression coefficient)

Predictor	Coefficient	SE Coefficient	T	P
Constant	-904.7	171.1	-5.29	0.001
X_1	2.3468	0.9401	2.50	0.34
X_2	146.03	47.00	3.11	0.13
X_3	41.250	9.087	4.54	0.001
X_1^2	-0.005765	0.00232	-2.49	0.035
X_2^2	-18.089	5.800	-3.12	0.012
X_3^2	-0.4817	0.1184	-4.07	0.003
X_1X_2	-0.4394	0.3659	-1.20	0.260
X_1X_3	-0.03894	0.02173	-1.79	0.107
X_2X_3	-1.981	1.0896	-1.82	0.102
$X_1X_2X_3$	0.012911	0.009716	1.33	0.217

Table 4: ANOVA of the experiment

Source	DF	SS	MS	F	P
Regression	10	13125.5	1312.5	14.19	0.0001
Residual Error	9	832.7	92.5		
Total	19	13958.1			

S = 9.61859 R^2 = 94.0% R^2 (adj) = 87.4%

Table 5: Experimental (validation studies) and Predicted Results (from Response Surface Model) of Phytase Activity (U/ml) under Optimized Induction Conditions

Experimental	Predicted
69.96 ± 1.93	77.35

Table 6: Scale up of phytase production by the recombinant *E. coli*

Volume of production (ml)	Phytase Activity (U/ml)	Relative Activity (%)
10 (Control)*	21.66 ± 3.91	22.3
10	97.18 ± 7.21	100
100	92.28 ± 6.45	95.0
1000	49.51 ± 8.49	51.0

*Wild type bacterial phytase (Greiner and Farouk, 2007) strain

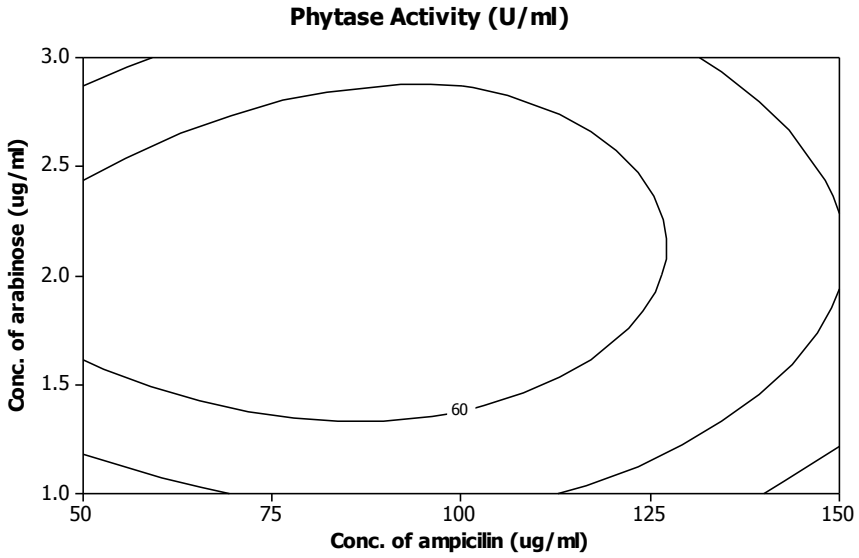

Figure 1a: Contour plot of phytase production by the recombinant *E. coli* showing interaction between ampicilin and arabinose concentration

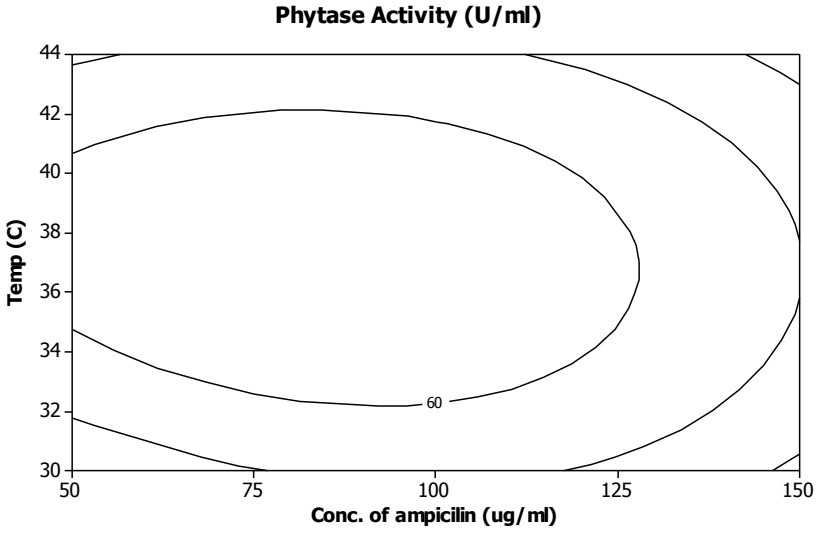

Figure 1b: Contour plot of phytase production by the recombinant *E. coli* showing interaction between ampicilin concentration and incubation temperature

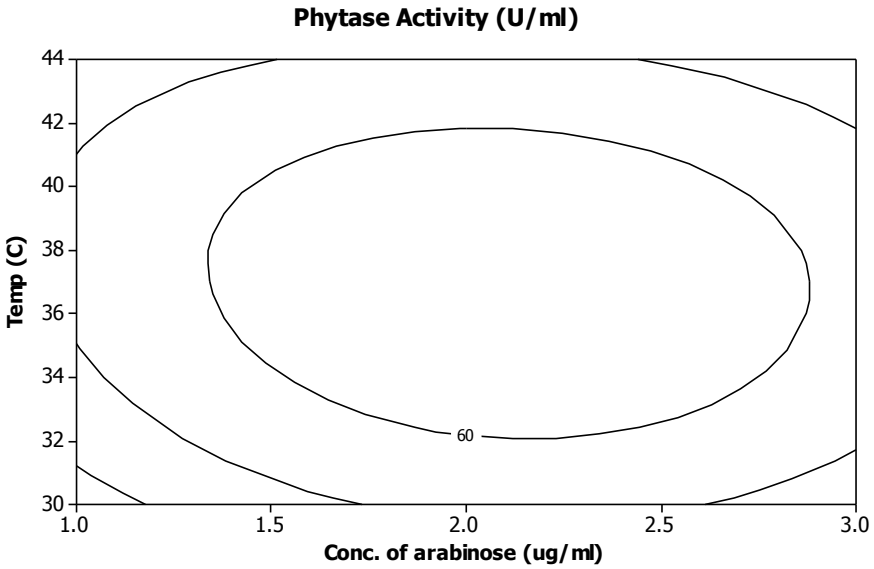

Figure 1c: Contour plot of Phytase Production by the recombinant *E. coli* showing interaction between arabinose concentration and incubation temperature