Optimization of cultivation conditions for the production of phytate-degrading enzymes by *Enterobacter sakazakii* ASUIA279 isolated from Malaysian maize root

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ABSTRACT

The production of extracellular phytase by Enterobacter sakazakii ASUIA279 was optimized using response surface methodology with full-factorial faced centred central composite design. Two sets of experiments were carried out to optimize the five most profound factors of the cultivation conditions in order to maximize phytase production. Incubation temperature, initial pH of the media and percentage of rice bran supplemented into the media were optimized in Erlenmeyer flasks while agitation and aeration effect were controlled in a bioreactor. This design reduced the number of actual experiment performed to optimize phytase production and allowed the study of possible interactions among the factors. In the first set of experiments linear and quadratic effect of initial pH was determined to be the most significant factor affecting phytase production. In the bioreactor both linear effects of agitation and aeration, were identified to be highly significant (> 99 %) in respect to phytase yields. Optimal phytase production was observed at a incubation temperature of 39.7 ºC, an initial pH of 7.1, supplementation with 13.6 % rice bran , 320 rpm of agitation and 0 vvm of aeration.

Key-words: Bacterial phytase, statistical optimization, cultivation conditions

INTRODUCTION

Most cereals and legumes are rich in protein and fat but the presence of phytate discourages their use in food. Approximately 70% of total phosphorus in cereal grains and legume seeds exists as phytate. The phytate is poorly utilized by monogastric animals and acts as an anti-nutrient due to its chelating of various metals and binding of protein. This diminishes the bioavailability of proteins and nutritionally important minerals. In animal diets, the supply of phosphorus, an essential mineral salt 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. 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 drinking (Krishna and Nokes, 2001).

Phytate degrading enzymes or phytases convert phytate to partially phosphorylated *myo*-inositol and phosphate, making phosphorus available for absorption (Irving 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 (Wodzinski and Ullah, 1996). To obtain a good source of phytase, a variety of microorganism has been screened for the enzymes such as *Bacillus* sp. (Powar and Joganathan, 1982; Choi et al., 1999), *Klebsiella terrigena* (Greiner et al., 1997), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *Escherichia coli* (Greiner et al., 1993), *Myceliophthora thermophila* (Mitchell et al., 1997), *Aspergillus terreus* (Yamada et al., 1968), *Aspergillus niger* (Nagashima et al., 1999), *Aspergillus fumigatus* (Pasamontes et al., 1997) and ruminal bacteria

(Yanke et al., 1998). Partial 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 and Reardon, 1996). Preliminary experiments in our laboratory indicated that rice bran composition and physical parameter of cultivation conditions play a significant role in phytase production. Therefore an investigation was performed to statistically optimize the cultivation conditions for the production of the highly potential extracellular phytase from *Enterobacter sakazakii* ASUIA279. Response surface methodology (RSM) is now being routinely used for optimization studies in several biotechnological and industrial processes (Beg et al., 2002; De Cornick et al., 2000; Puri et al., 2002). RSM is used to explain the interaction among the possible influencing parameters with limited numbers of parameters.

In this paper, we determine the optimum composition of rice bran supplement in the media, initial pH of the media, incubation temperature, agitation and aeration during cultivation for phytase production by using full factorial Face Centred Central Composite Design in RSM.

MATERIALS AND METHODS
Microorganism and culture conditions
Enterobacter sakazakii ASUIA279 was isolated from the endophyte of Malaysian Maize root and identified using biochemical and phylogenetic analysis (Anis Shobirin, 2008). The strain was maintained on agar slopes at 4ºC. LB broth (Merck) with initial pH of 7.0 in universal bottle used as the standard inoculum medium and kept in incubating shaker at 37ºC and 200 rpm for 18 h.

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

Standard Phytase Assay
Phytate-degrading activity was determined at 50°C in 390 µl 100 mM sodium acetate buffer, pH 5.0 containing 1.03 mM sodium phytate. The enzymatic reaction was started by adding 10 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 was 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 mmol phosphate ($\varepsilon = 8.7$ cm^2/nmol). Activity (units) was expressed as 1 µmol phosphate liberated per minute. Blanks were run by addition the ammonium molybdate solution prior to adding the enzyme to the assay mixture.

Experimental design
After preliminary optimizing the values of various nutritional and physical factors in individually, the five most important factors in respect to phytase production by *Enterobacter sakazakii* ASUIA279, namely rice bran concentration (X_1), incubation temperature (X_2), initial pH (X_3), agitation speed (X_4) and aeration volume (X_5) were identified under batch fermentation conditions. Taking the above factors and constraint of time into consideration, a response surface methodology using a two-step experimental design was adopted for improving total phytate-degrading enzyme secretion from *Enterobacter sakazakii* ASUIA279. The experiments involved the phytate-degrading enzyme production for 20 run in Erlenmeyer flask (see table 2) and then scaling up in a bioreactor for 13 run (see table 3).

In step-one, 10 ml of inoculums (10 % v/v) were transferred into 250 ml Erlenmeyer flasks containing 100 ml production medium for optimization of the rice bran concentration, the initial pH of media and the incubating temperature. The flasks were then placed in an orbital shaker at 200 rpm. The experiments were done in triplicate. While in step-two, 2 L bioreactor (Biostat® B-DCU- B. Braun Biotech International, Sartorius Group) containing 1 L production medium was used for optimization of the agitation and the aeration. The production medium, the initial pH of the media and the incubation temperature was the optimized conditions derived from step-one. The samples of each one were analyzed three times. Samples of 1.5 ml were withdrawn from both steps after 5 days of incubation.

Full-factorial Central Composite Experimental Design with centred face was used to optimize the

cultivation conditions for phytate-degrading enzyme production by *Enterobacter sakazakii* ASUIA279. The experiments were designed by using the Minitab 14 Statistical Software. A 2^3 – factorial experimental design with four axial points (with α = 1 and six replicates at the centre point (n_0 = 6) leading to a total number of twenty experiments was employed for the optimization of rice bran composition (X_1), initial pH (X_2) of media and incubation temperature (X_3) in Erlenmeyer flasks and a 2^2 – factorial experimental design leading to thirteen trials for the optimization of agitation (X_4) and aeration (X_5) in a 2L bioreactor.

The design matrix and levels of the independent variables chosen for the study in encoded in a form shown in table 1. The range of the variable chosen is based on other studies (Vohra and Satyanarayana, 2003). The average of the maximum phytate-degrading enzyme activity of the duplicate values obtained was taken as dependent variable or response Yi (U). Regression analysis and isoresponse contour plots were performed on the data obtained using MINITAB 14. Microsoft Office Excel 2003 is used to calculate the predicted responses.

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

Variables	Symbol coded	Range and level		
		-1	0	+1
Temperature (°C)	X_1	29	37	45
Initial pH	X_2	5	7	9
Rice bran % (w/v)	X_3	5	10	15
Agitation (rpm)	X_4	200	300	400
Aeration (vvm)	X_5	0	1	2

The final validation of the statistical regression model for prediction of phytate-degrading enzyme production from *Enterobacter sakazakii* ASUIA279 was carried out in both steps. Samples were withdrawn for phytase assay after 5 days of cultivation.

RESULTS

From the preliminary study, the factors that have the major influence for the production of phytate-degrading enzymes by *Enterobacter sakazakii* ASUIA279 were the composition of rice bran, incubation temperature, initial pH, agitation and aeration. Due to apparatus and time constraints, the experiments were split into two sets where the first three factors were optimized in Erlenmeyer flask. With the optimized cultivation conditions derived from the first set od experiments, agitation and aeration were optimized in a 2 L bioreactor
The response was taken at the maximum phytate-degrading enzyme activity, which was observed after 5 days of fermentation. Table 2 and 3 summarize the phytate-degrading enzyme activity

(response) of the experiments for both steps. The coefficients of the regression equation were calculated using MINITAB 14 and the following regression equations were obtained.
Step-one;
$Y = - 44.4 + 1.15\ X_1 + 6.96\ X_2 - 0.165\ X_3 - 0.0212\ X_1^2 - 0.759\ X_2^2 - 0.0439\ X_3^2 + 0.0991\ X_1X_2 + 0.0299\ X_1X_3 + 0.226\ X_2X_3 - 0.00595\ X_1X_2X_3$ (1)
$R^2 = 97.4\%$

Step-two;
$Y = - 7.03 + 0.102\ X_4 - 3.93\ X_5 - 0.000156\ X_4^2 + 0.586\ X_5^2 - 0.00172\ X_4X_5.$(2)
$R^2 = 98.3\%$

Where *Y*= the response or phytate-degrading enzyme activity
X_1= temperature (°C)
X_2= pH
X_3= rice bran (% w/v)
X_4= agitation (rpm)
X_5= aeration (vvm)

Table 2. Experimental design of three independent variables in step-one showing experimental responses

Experiment run no.	Temperature (°C)	pH	% Rice Bran	Experimental Phytase Activity (U/ml)
1	37	7	10	12.009
2	45	5	5	6.360
3	45	5	15	7.227
4	37	7	10	12.408
5	29	5	5	5.030
6	37	7	10	12.058
7	29	7	10	8.708
8	37	7	5	9.393
9	37	7	15	10.506
10	45	7	10	10.673
11	45	9	5	8.696
12	37	9	10	8.118
13	29	5	15	5.883
14	37	7	10	11.209
15	37	5	10	7.905
16	37	7	10	11.839
17	37	7	10	12.197
18	29	9	5	2.931
19	29	9	15	5.903
20	45	9	15	7.871

Table 3. Experimental design of two independent variables in step-two showing experimental responses.

Experiment run no.	Agitation (rpm)	Aeration (vvm)	Experimental Phytase Activity (U/ml)
1	300	0	10.237
2	300	1	5.583
3	400	2	2.108
4	300	1	5.891
5	300	1	5.460
6	200	0	6.835
7	200	2	1.229
8	400	0	8.402
9	400	1	5.011
10	300	1	5.664
11	300	2	2.515
12	200	1	3.448
13	300	1	5.545

The significance of each coefficient was determined by T values and P values which are listed in table 4 and 5. From t-table, alpha value of 0.05 and degree of freedom of 19, the critical value of t is 1.73 (step-one) while for degree of freedom of 12, the critical value of t is 1.78 (step-two). Therefore, factors with t values greater than t critical are significant to the model. Based on the t-test results, it is observed from table 6 that the coefficient for temperature (X_1) and initial pH (X_2) are the most significant factors for step-one. Furthermore, the quadratic interactions X_1^2, X_2^2, X_3^2 and the factor of interactions X_1X_2 (temp, pH), X_2X_3 (pH, % rice bran), $X_1X_2X_3$ (temp, pH, % rice bran) were also found to be very significant.

Table 4. The least-squares fit and parameter estimates (significance of regression coefficient) for step-one.

Predictor	Coefficient	SE Coefficient	T	P
Constant	-44.44	11.140	-3.99	0.003
X_1	1.148	0.505	2.27*	0.049
X_2	6.960	1.815	3.84*	0.004
X_3	-0.165	0.843	-0.20	0.849
X_1^2	-0.021	0.006	-3.49*	0.007
X_2^2	-0.759	0.097	-7.82*	0.000
X_3^2	-0.044	0.016	-2.83*	0.020
$X_1 X_2$	0.099	0.032	3.11*	0.012
$X_1 X_3$	0.030	0.021	1.44	0.183
$X_2 X_3$	0.226	0.108	2.10*	0.066
$X_1 X_2 X_3$	-0.006	0.003	-2.09*	0.066

* indicates significance to the model.

Table 5 shows that the coefficient for both agitation speed (X_4) and aeration (X_5) as well the quadratic interaction X_4^2 and X_5^2 are significant factors for step-two. These significant factors are very important as they indicates the limiting factors in which the smallest variations in their values can alter the phytate-degrading enzyme production.

Table 5. The least-squares fit and parameter estimates (significance of regression coefficient) for step-two.

Predictor	Coefficient	SE Coefficient	T	P
Constant	-7.030	2.302	-3.05	0.018
X_4	0.102	0.016	6.53*	0.000
X_5	-3.927	0.837	-4.69*	0.002
X_4^2	-0.0001	0.000	-6.09*	0.000
X_5^2	0.586	0.256	2.29*	0.056
$X_4 X_5$	-0.002	0.002	-0.81	0.446

* indicates significance to the model.

The coefficients of the regression equation were re-analysed and calculated using MINITAB 14 and the following regression equations were obtained.

Step-one,

$$Y = -61.5 + 1.84 X_1 + 9.35 X_2 - 0.0266 X_1^2 - 0.845 X_2^2 - 0.00718 X_3^2 + 0.0596 X_1 X_2 + 0.107 X_2 X_3 - 0.00201 X_1 X_2 X_3 \dots (3)$$
$$R^2 = 94.7\%$$

Step-two,

$$Y = -6.51 + 0.100 X_4 - 4.44 X_5 - 0.000156 X_4^2 + 0.586 X_5^2 \quad (4)$$
$$R^2 = 98.1\%$$

Where Y= the response or phytate-degrading enzyme activity
X_1= temperature (ºC)
X_2= pH
X_3= rice bran (% w/v)
X_4= agitation (rpm)
X_5= aeration (vvm)

The regression equation and determination coefficient R^2 were evaluated to test the fit of the model. The model presented a high determination coefficient of 90.8 % for step-one and 98.1 % in step-two of the variability in the response.

Table 6 and 7 summarize the phytate-degrading enzyme activity (response) of the experiments for both steps with the predicted response. The predicted responses were calculated using the re-analysed regression equation. The high value of R^2 indicating the high significance of the model. It revealed a good agreement between the experimental and predicted values of the responses. From the predicted responses, the optimum operating conditions for the enzyme production were at temperature of 37ºC, initial pH of 7, rice bran of 15%, 300 rpm of agitation speed and 0 vvm of aeration.

Table 6. Experimental design of three independent variables in step-one showing experimental and predicted responses.

Experiment run no.	Temperature (ºC)	pH	% Rice Bran	Phytase Activity (U/ml)	
1	37	7	10	12.009	11.212
2	45	5	5	6.360	6.704
3	45	5	15	7.227	6.096
4	37	7	10	12.408	11.212
5	29	5	5	5.030	4.795
6	37	7	10	12.058	11.212
7	29	7	10	8.708	8.325
8	37	7	5	9.393	10.609
9	37	7	15	10.506	11.457
10	45	7	10	10.673	10.695
11	45	9	5	8.696	7.843
12	37	9	10	8.118	7.935
13	29	5	15	5.883	5.794
14	37	7	10	11.209	11.212
15	37	5	10	7.905	7.729
16	37	7	10	11.839	11.212
17	37	7	10	12.197	11.212
18	29	9	5	2.931	11.212
19	29	9	15	5.903	2.762
20	45	9	15	7.871	5.710
					7.897

Table 7. Experimental design of two independent variables in step-two showing experimental and predicted responses.

Experiment run no.	Agitation (rpm)	Aeration (vvm)	Phytase Activity (U/ml)	
			Experimental	Predicted
1	300	0	10.237	9.450
2	300	1	5.583	5.596
3	400	2	2.108	1.994
4	300	1	5.891	5.596
5	300	1	5.460	5.596
6	200	0	6.835	7.25
7	200	2	1.229	0.714
8	400	0	8.402	8.53
9	400	1	5.011	4.676
10	300	1	5.664	5.596
11	300	2	2.515	2.914
12	200	1	3.448	3.396
13	300	1	5.545	5.596

Figure 1 show the contour plot of the relative effects of two most significant factors when all the factors were kept at their central levels. The plots depicted interaction of incubation temperature and initial pH for phytase production in step-one. The contour plot clearly shows an ellipse where the optimum conditions can be easily determined. These indicate that there are major interactions among the independent variable corresponding to the response surface. Optimal production of phytate-degrading enzymes was observed at an incubation temperature of 39.7 ºC, an initial pH of

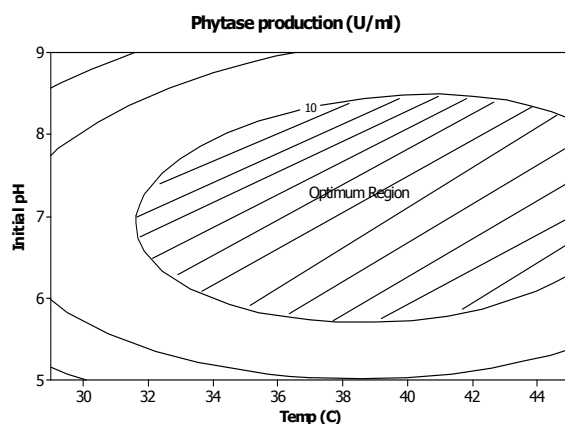the medium of 7.1 and a rice bran supplementation of 13.6%.

Figure 1 - Contour plot of phytate-degrading enzyme production from Enterobacter sakazakii ASUIA279 showing interaction between incubation temperature and initial pH.

The validation of model with experimental data as well the predicted values from response surface model under the optimized cultivation conditions (included only the most significant coefficient) are presented in table 8. Phytate-degrading enzyme productions under the optimized cultivation conditions for the validation model for both steps were within the range of the predicted value from the response surface model. The optimization of these parameters using RSM has increased the production of phytate-degrading enzyme by *Enterobacter sakazakii* ASUIA279. There are no known reports on statistically optimization of temperature, initial pH, rice bran concentration, agitation and aeration for production of phytase. Quan et al (2001) and Gautam et al (2002) had done an individual optimization (keeping other parameters as constant) on the effect of temperature and pH besides other parameters for phytase production. The effect of solid substrates (canola meal, corn meal, soy meal and wheat meal) has been optimized using Plackett- Burman Design by Bogar et al. (2003).

Table 8. Experimental (validation studies) and predicted results (from Response Surface Model) of phytate-degrading enzyme activity (U/ml) under optimized cultivation condition.

Phytase Activity in Erlenmeyer Flask (U/ml)		Phytase Activity in Bioreactor (U/ml)	
Experimental	Predicted	Experimental	Predicted
11.14 ± 0.12	11.511	9.56 ± 0.09	9.516

DISCUSSION

The conventional methods for multifactor experimental design are time-consuming and incapable of obtaining the true optimum, due especially to the interactions among the factors (Nagashima et al., 1999). In order to design a suitable bioprocess for optimized phytate-degrading enzyme production in *Enterobacter sakazakii* ASUIA279, the statistical design approach using response surface methodology with face centred central composite design (FCCCD) was used to study the interactive effects of various nutritional and physical factors on phytate-degrading enzyme production. According to Vohra and Satyanarayana (2003), the source and optimal concentration of carbon are important factors for the production of phytate-degrading enzyme and the most important physical parameters having profound influence on growth of organisms and production of metabolites from them are pH, temperature, agitation, dissolved oxygen, and pressure.

Student's t-test was used to determine the significant coefficient of the experimental run. If the T value observed from the regression coefficient data was higher or equal to critical t obtained from t-table, indicated that the coefficient was significant to the model. For step-one, at α = 0.05, degree of freedom = 19, and the critical value of t is 1.73, its shows that the most significant factor that affect the production of phytate-degrading enzymes were the incubation temperature and the initial pH of the medium. While for step-two, at α = 0.05, degree of freedom = 12, and the critical value of t is 1.78, both agitation speed and aeration were significant factors that affect production. The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is evident from the Fishers F-test with very low probability value (P > F = 0.0001).

The larger the magnitude of the T value and the smaller the P value the higher the significance of the corresponding coefficient (Karthikeyan et al.**,**

1996). It can be seen in step-one that the variable with the most effect was the quadratic effect of initial pH of the medium (X_2^2, T = 7.82, P = 0.000) followed by linear effect of pH (X_2, T = 3.84, P = 0.004) and than quadratic effect of temperature (X_1^2, T = 3.49, P = 0.007). The highest probability value of coefficient is the linear effect of rice bran supplementation (0.838), indicating that only 16 % of the model is affected by this variable. Since linear and quadratic effect of initial pH of the medium is the most significant, meaning that it can act as limiting factor and little variation will alter the product formation rate. In the bioreactor, it shows that both linear effects and quadratic effects of agitation and aeration are highly significant (> 99 %).

The values of determination coefficient R^2 (step-one) = 90.8%, R^2 (step-two) = 98.1% and the adjusted determination coefficient R^2 (step-one) = 87.6%, adjusted determination coefficient R^2 (step-two) = 97.2% of the significant factors 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 a good agreement between the experimental and predicted values of phytate-degrading enzyme production.

The contour plots are graphical representations of the regression equation. The main goal of response surface is to hunt efficiently 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 is the optimal region. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar et al., 2001).

From several studies (Park, and Reardon, 1996; Berka et al., 1998) the optimum temperature for the production of phytate-degrading enzymes of most of the micro-organisms lies in the range 25 to 37 ºC. Using the regression equation, the predicted most optimum level of cultivation conditions for higher phytate-degrading enzyme production was found to be at an incubation temperature of 39.7 ºC, pH 7.1 and 13.6 % of rice bran concentration with 11.511 U/ml of enzyme yield. And when it was scale up in bioreactor for the optimization of agitation speed and aeration, the predicted optimum level of both factors were at 320 rpm and at 0 vvm it was 9.516 U/ml. A study done by Segueilha et al. (1992), phytate-degrading enzyme production by *Schwannimyces castellii* was carried out continuously in a fermentor aerated at 1 vvm and agitation kept at 600 rpm. Phytate-degrading enzyme was produced maximally by *Bacillus* sp. DS11 by shaking at 230 rpm (Kim et al., 1998). And, phytate-degrading enzyme produced by *Aspergillus niger, Aspergillus ficuum* NRRL 3135 and *Aspergillus terreus* was carried out by shaking at 270 rpm (Shieh and Ware, 1968). This indicates that optimum aeration and agitation are important to keep the cultivation conditions in a capable state.

The response surface model is a valuable tool for predicting and optimizing the cultivation conditions to maximize the phytate-degrading enzyme production. The results show that phytate-degrading enzyme production by *Enterobacter sakazakii* ASUIA279 can be improved by controlling various factors simultaneously. The validation model for both sets of experiments was in close agreement with the predicted value from the response surface model for phytate-degrading enzyme productions under the optimized cultivation conditions. The production of phytate-degrading enzyme was increased about 6-fold (compared to during screening) when cultivated at optimized condition in Erlenmeyer flask. However, the phytate-degrading enzyme production was decreased to 5-fold when the volume was scale-up in bioreactor. This could be due to the different environments in term of temperature, and volume between the two apparatus.

ACKNOWLEDGEMENT

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