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Optimization of process parameters for alkaline phosphatase production by *Bacillus licheniformis* using response surface methodology

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Alkaline phosphatase production by *B. licheniformis* was investigated by batch fermentation. A two-step optimization procedure using central composite design with six factors (pH, temperature, fermentation time, orbital speed, age of inoculum and inoculum volume) were used to evaluate the effect of these parameters on alkaline phosphatase production. According to the results of Placket-Burman design methodology, pH, temperature, fermentation time and orbital speed were significant ($P<0.05$) on alkaline phosphatase production. The predicted maximum production of alkaline phosphatase was 792.043 U/ml with pH 8.0, temperature 36.7˚C, fermentation time 78 hours, and orbital speed 165 rpm. The validity of the response model was verified by a good agreement between predicted (792.043 U/ml) and experimental results (854.34 U/ml). A 1.5 fold increase in alkaline phosphatase production was achieved after optimization of the production parameters of alkaline phosphatase by response surface methodology.

Key words: alkaline phosphatase, *Bacillus licheniformis,* response surface methodology, placket-burman design, central composite design

Introduction

Alkaline phosphatase (E.C. 3.1.3.1) is widely distributed in nature, containing two Zn^{2+} ions and one Mg^{2+} ion (Janeway *et al*., 1993; Kim and Wyckoff, 1989). Alkaline phosphatase is a non-specific monophosphoester hydrolase that catalyzes the removal of phosphate groups from a variety of small organic molecules, as well as large biomolecules such as DNA and proteins (Kobori *et al*., 1984). The enzyme has been purified and characterized

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from many different organisms, ranging from a wide variety of bacteria to man (Guimaraes *et al*., 2007; Simao *et al*., 2007; Wojciechowski and Kantrowitz, 2002). Extracellular alkaline phosphatases have been reported from several bacteria (Angkawidjaja *et al*., 2006; Prada *et al*., 1996; Sharipova *et al*., 1998; Von Tigerstrom, 1984). Alkaline phosphatase is commonly used as a tool in molecular biology and clinical assays (Ausubel *et al*., 1994; Chen *et al*., 2006).

Response Surface Methodology (RSM) is advantageous over conventional methods available and it includes less experiment numbers, its suitability for multiple factor experiments and search for common relationship between various factors towards finding the most suitable production conditions for the bioprocess and forecast response (Kaur and Satyanarayana, 2005). To develop a bioprocess for industrial purpose, it was important to optimize highly significant factors. The aim of this paper was firstly to apply by one-variable-at-a-time approach to select the most significant fermentation parameters, then a 2^4 full factorial central composite design was optimized using the RSM for the maximum production of an extracellular alkaline phosphatase by *B. licheniformis* (Lee and Chen, 1997; Dey *et al*., 2001; Tari *et al*., 2006). The present work was an innovative step towards evaluating production parameters. In this linear or quadratic effects of experimental variables construct contour plots and a model equation fitting the experimental data. This facilitates the determination of optimum value of factors under investigation and prediction of response under optimized condition (Banik *et al*., 2007; Santhiagu and Banik, 2008).

The work described in this article deals with optimization of production parameters for alkaline phosphatase production by *B. licheniformis*. RSM was used here to optimize important production parameters screened by Placket-Burman design. Particularly in this work was applied RSM to evaluate the effect of physical variables on alkaline phosphatase production by *B. licheniformis* and search optimal condition to attain a higher alkaline phosphatase yield.

Materials and methods

Media and culture condition

A strain of *Bacillus licheniformis* (MTCC 1483) was used in this study. The organism was collected from Institute of Microbial Technology, Chandigarh, India. The culture was maintained in medium containing 0.1% beef extract, 0.2% yeast extract, 0.5% peptone and 0.5% NaCl. Alkaline phosphatase fermentation was carried out in a modified medium (Nomoto *et al*., 1988; Prada *et al*., 1996) containing 1% glucose, 0.5% peptone, 0.1% yeast

extract, 0.02% $MgSO_4.7H_2O$, 0.002% KH_2PO_4, 0.5% NaCl. Inoculum was developed by transferring one loop full of the organism from the slant culture to 50 ml production medium in 250 ml Erlenmeyer flask. The flask was incubated in an orbital shaker at 37±1˚C and 175 rev/min for 24 hours for inoculum development.

Production of alkaline phosphatase

Initially the effect of six parameters such as pH, temperature, fermentation time, orbital speed, age of inoculum and inoculum volume on the production of alkaline phosphatase were studied using Placket-Burman design criterion. Fermentations were carried out by using production parameters level given in Table 1. The fermentated medium was then centrifuged at 10,000g at 10˚C for 15 minutes and cell free supernatant was used for determination of alkaline phosphatase activity.

Analytical methods

Alkaline phosphatase activity was measured spectrophotometrically by monitoring the release of p-nitrophenol from p-nitrophenyl phosphate disodium salt (pNPP) at 415nm (Garen and Levinthal, 1960). The enzyme sample ($0.1cm^3$) was added to 1.9 cm^3 of p-nitro phenyl phosphate disodium salt solution (2 mg cm^{-3} in 1 mol dm^{-3} Tris-HCl buffer at pH 10.0) and the mixture was incubated at 50˚C for 20 minute. The reaction was terminated by adding 0.5 cm^3 of NaOH solution (5 mol dm^{-3}) and the absorbance of the product p-nitrophenol was measured at a wavelength of 415nm using UV-Vis spectrophotometer (Shimadzu) (Bansal-Mutalik and Gaikar, 2003).

One unit of enzyme activity is defined as the amount of the enzyme catalyzing the liberation of 1µmol of p-nitrophenol per minute.

Response surface methodology

RSM consist of a group of empirical technique was used for evaluation of relationship between cluster of controlled experimental factors and measured response. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach. Placket-Burman design was used to pick factors that influence alkaline phosphatase production significantly and insignificant ones were eliminated in order to obtain a smaller, manageable set of factors. Response surface methodology was applied in two stages, first to identify the significant factors for production of alkaline phosphatase using Placket-Burman design criterion and later the significant factors resulted from

Placket-Burman design were optimized by using a central composite design. The experimental design and statistical analysis of the data were done by using Minitab statistical software package (version-14).

Placket–Burman design

Each variable was tested at two levels namely a high level denoted by (+1) and a low level denoted by (-1) as listed in Table 1. Six variables were screened by conducting twelve experiments using Placket-Burman design. All experiments were conducted in triplicate and the average value of alkaline phosphatase yield was used for statistical analysis. The variables, which were significant at 5% level ($P < 0.05$) from the regression analysis were considered to have greater impact on alkaline phosphatase production and were further optimized using central composite design.

Central composite design

A central composite design (CCD) was applied to determine the optimum concentration of four significant production parameters screened from Placket–Burman design criterion. The effect of the parameters pH, temperature, fermentation time and orbital speed on the production of alkaline phosphatase was studied at five experimental levels: $-\alpha, -1, 0, +1, +\alpha$, where $\alpha = 2^{n/4}$, here n was the number of variables and 0 corresponded to the central point. The levels of factors used for experimental design are given in Table 1. The actual level of each factor was calculated by the following equation (Paul *et al*., 1992):

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

The experimental plan and levels of independent variables were obtained from central composite design; pH had a lower limit of 2.5 and an upper limit of 12.5. Temperature was varied between 10˚C and 50˚C. Fermentation time was varied between 24 and 120 hours. The lower and upper limits of orbital speed were 50 and 250 rpm, respectively.

The response variable was fitted by a second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation used in this study is:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad \ldots\ldots\ldots\ldots(2)$$

Where Y is the predicted response, x_i, x_j are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the

*i*th quadratic coefficient and β_{ij} is the *ij*th interaction coefficient. Analysis of variance (ANOVA), regression analysis was done and contour plots were drawn by using Minitab Statistical Software package.

Results and discussion

Screening of parameters using Placket-Burman design criterion

The parameters significantly affect alkaline phosphatase production by *B. licheniformis*, Placket-Burman design was determined. All six parameters such as pH, temperature, fermentation time, orbital speed, age of inoculum and inoculum volume were studied at two widely spaced levels. The levels of the parameters were selected based on the preliminary experiments and the information available in the literature as stated by Nilgiriwala *et al*. (2008) and Oh *et al*. (2007). The low level (-1) and high level (+1) of each factor are listed in Table 1.

Table 1. Level of parameters used for the production of alkaline phosphatase by *B. licheniformis* using Placket–Burman design criterion.

Code	Parameters	High level (+1)	Low level (-1)
A	pH	10.0	5.0
B	Temperature	40°C	20°C
C	Fermentation time	96 hrs	24 hrs
D	Inoculum volume	8%	4%
E	Age of inoculum	24 hrs	12 hrs
F	Orbital speed	200 rpm	100 rpm

Maximum production of alkaline phosphatase (768.46 U/ml) in the medium having higher level (+1) of pH, temperature, fermentation time and orbital speed was shown in Table 2. It is also showed that out of the six parameters studied, four parameters (pH, temperature, fermentation time and orbital speed) gave significant stimulatory effect on alkaline phosphatase production as evidenced by their P values (<0.05) obtained from regression analysis of Placket-Burman design (Table 3). The goodness of fit of model was checked by the determination of coefficient (R^2). The coefficient of determination (R^2) of the model was 0.906, which indicated that the model could explain up to 90.6% variation of the data. Alkaline phosphatase yield obtained from Placket-Burman design experiments showed wide variation which indicated that further optimization was necessary.

Table 2. Parameters for the production of alkaline phosphatase by *Bacillus licheniformis* using Placket-Burman design criterion.

Run	A	B	C	D	E	F	Activity (U/ml)	
							Experimental	Predicted
1	1	1	-1	1	-1	-1	358.68	404.990
2	1	-1	-1	-1	1	1	342.64	344.883
3	-1	-1	-1	-1	-1	-1	289.34	351.760
4	1	-1	1	-1	-1	-1	415.67	353.030
5	-1	1	1	-1	1	-1	527.64	539.733
6	1	1	-1	1	1	-1	426.58	380.270
7	1	-1	1	1	-1	1	545.86	559.060
8	1	1	1	-1	1	1	510.38	557.577
9	-1	-1	-1	1	1	1	536.42	533.070
10	-1	1	1	1	-1	1	768.46	770.483
11	-1	1	-1	-1	-1	1	642.34	581.027
12	-1	-1	1	1	1	-1	528.37	516.497

Table 3. Regression analysis of Placket-Burman design criterion data for prediction of significant parameters.

Term	Effect	Coef	SE Coef	T	P
Constant	-	491.03	17.60	27.91	0.000
A	-115.46	-57.73	17.60	-3.28	0.022
B	95.96	47.98	17.60	2.73	0.041
C	116.73	58.36	17.60	3.32	0.021
D	72.73	36.36	17.60	2.07	0.094
E	-24.72	-12.36	17.60	-0.70	0.514
F	133.30	66.65	17.60	3.79	0.013

$R^2 = 90.62\%$

Optimization of parameters level using CCD

Thirty one experiments were carried out according to the CCD as shown in Table 4. By applying multiple regression analysis on the application data, the following second order polynomial equation was found to explain the alkaline phosphatase production by *B. licheniformis*.

$$Y = 792.043 + 56.132X_1 + 59.136X_2 + 60.720X_3 + 45.768X_4 - 160.652X_1^2 - 74.27X_2^2 - 95.83X_3^2 - 79.56X_4^2 + 19.808X_1X_2 + 23.799X_1X_3 + 8.970X_1X_4 + 24.743X_2X_3 + 5.949X_2X_4 - 6.820X_3X_4 \quad \text{.....(3)}$$

Where Y is the predicted response variable, alkaline phosphatase activity (U/ml) and X_1, X_2, X_3 and X_4 the values of independent variables, pH, temperature, fermentation time and orbital speed respectively.

Table 4. Alkaline phosphatase production by *Bacillus licheniformis* using significant parameters based on central composite design criterion.

Run	pH	Temperature	Fermentation time	Orbital speed	Activity (U/ml)	
					Experimental	Predicted
1	5.0	40	48	100	248.24	253.696
2	10.0	40	96	100	594.67	572.201
3	7.5	30	72	150	847.62	792.043
4	10.0	20	48	200	378.67	354.752
5	7.5	10	72	150	396.78	376.690
6	7.5	30	24	150	361.56	287.282
7	5.0	40	96	100	345.38	390.664
8	7.5	30	72	150	854.34	792.043
9	7.5	30	72	150	742.14	792.043
10	5.0	40	48	200	320.52	352.829
11	10.0	20	96	100	387.67	376.727
12	5.0	40	96	200	382.24	462.516
13	5.0	20	48	200	214.13	311.760
14	10.0	40	48	200	417.67	475.051
15	5.0	20	96	100	256.64	274.420
16	7.5	30	72	150	810.54	792.043
17	10.0	20	48	100	248.65	243.535
18	12.5	30	72	150	271.34	261.702
19	7.5	30	120	150	552.41	530.162
20	7.5	30	72	50	389.67	382.267
21	7.5	30	72	150	814.45	792.043
22	2.5	30	72	150	43.06	37.172
23	5.0	20	96	200	356.68	322.477
24	7.5	30	72	250	654.46	565.337
25	7.5	30	72	150	758.86	792.043
26	10.0	20	96	200	390.96	460.665
27	10.0	40	96	200	684.34	679.934
28	7.5	30	72	150	716.35	792.043
29	10.0	40	48	100	284.47	340.039
30	5.0	20	48	100	210.65	236.422
31	7.5	50	72	150	689.67	613.233

Regression analysis of the experimental data showed that pH, temperature, fermentation time and orbital speed had positive effect on alkaline phosphatase production ($P<0.05$) (Table 5). All the four parameters pH, temperature, fermentation time and orbital speed had highest impact on alkaline phosphatase production as given by highest linear coefficient and most significant as shown by low P values (<0.05).

These parameters also showed significant quadratic effect on alkaline phosphatase production ($P<0.05$). The interaction between pH, temperature, fermentation time and orbital speed were found to be less significant as the P values are above 0.05 for interactive terms.

Table 5. Regression analysis of central composite design criterion data for alkaline phosphatase production by *Bacillus licheniformis*.

Term	Coef	SE Coef	T	P
Constant	792.043	26.52	29.867	0.000
pH	56.132	14.32	3.919	0.001
Temperature	59.136	14.32	4.129	0.001
Fermentation time	60.720	14.32	4.240	0.001
Orbital speed	45.768	14.32	3.196	0.006
pH*pH	-160.652	13.12	-12.244	0.000
Temperature*Temperature	-74.270	13.12	-5.661	0.000
Fermentation time*Fermentation time	-95.830	13.12	-7.304	0.000
Orbital speed*Orbital speed	-79.560	13.12	-6.064	0.000
pH*Temperature	19.808	17.54	1.129	0.275
pH*Fermentation time	23.799	17.54	1.357	0.194
pH*Orbital speed	8.970	17.54	0.511	0.616
Temperature*Fermentation time	24.743	17.54	1.411	0.178
Temperature*Orbital speed	5.949	17.54	0.339	0.739
Fermentation time*Orbital speed	-6.820	17.54	-0.389	0.703

$R^2 = 94.6\%$

The closure the value of R (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. In the present study the value of R (0.946) revealed that the model could explain up to 94.6% variation of alkaline phosphatase production. The P value for lack of fit (0.179) indicated that the experimental data obtained fitted well with the model and explained the effect of pH, temperature, fermentation time and orbital speed on alkaline phosphatase production by *B. licheniformis*. Figs. 1-6 shows the 2D contour plots of alkaline phosphatase production for each pair of parameters by keeping the other two parameters constant. The 2D contour plots are the graphical representation 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. The optimal combination of the parameters for alkaline phosphatase production was obtained from the contour plots are as follows: pH 8.0; Temperature 36.7˚C; Fermentation time 78 hours; and Orbital speed 165 rpm. At these optimum levels of parameters, alkaline phosphatase production of 854.34 U/ml was optimized. 1.5 fold increase in the alkaline phosphatase yield was observed after optimization with response surface methodology.

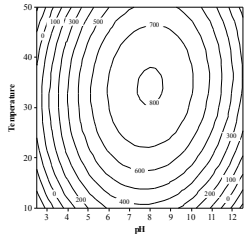

Fig. 1. Contour plot of alkaline phosphatase activity (U/ml): effect of pH and temperature on alkaline phosphatase production.

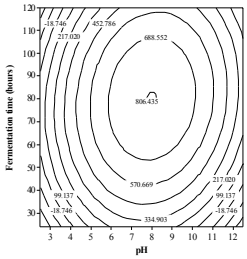

Fig. 2. Contour plot of alkaline phosphatase activity (U/ml): effect of pH and fermentation time on alkaline phosphatase production.

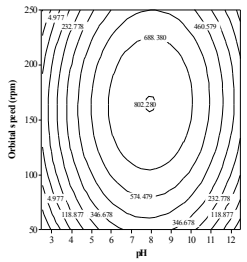

Fig. 3. Contour plot of alkaline phosphatase activity (U/ml): effect of pH and orbital speed on alkaline phosphatase production.

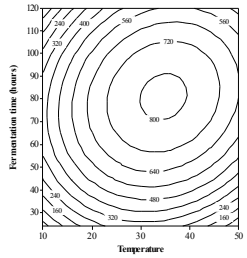

Fig. 4. Contour plot of alkaline phosphatase activity (U/ml): effect of temperature and fermentation time on alkaline phosphatase production.

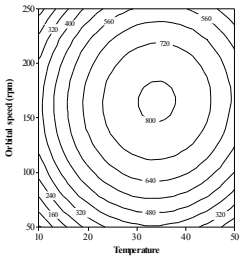

Fig. 5. Contour plot of alkaline phosphatase activity (U/ml): effect of temperature and orbital speed on alkaline phosphatase production.

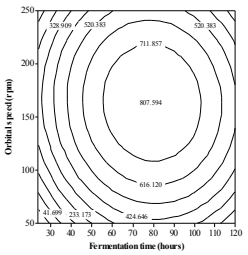

Fig. 6. Contour plot of alkaline phosphatase activity (U/ml): effect of fermentation time and orbital speed on alkaline phosphatase production.

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