Journal of Agricultural Technology 2011 Vol. 7(4):993-1003
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

# Statistical approach for kinetic study to optimize the reaction conditions for alkaline phosphatase produced by *Bacillus licheniformis* MTCC 1483

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Pandey, S.K., Singh, P. and Banik, R.M. (2011) Statistical approach for kinetic study to optimize the reaction conditions for alkaline phosphatase produced by *Bacillus licheniformis* MTCC 1483. Journal of Agricultural Technology 7(4):993-1003.

A statistical approach called Response Surface Methodology (RSM) was used to study the cumulative interactive effect of the assay conditions such as pH, temperature, reaction time and substrate (p-nitrophenylphosphate disodium salt) concentration and to optimize their effect to enhance the activity of alkaline phosphatase produced by *Bacillus licheniformis*. A factorial central composite experimental design was used to study the combined effect of the assay conditions. The P-value of the coefficient for linear effect, quadratic effect and interactive effect of pH, temperature, reaction time and substrate concentration were found to be less than 0.05. The optimal combinations of the assay conditions for alkaline phosphatase activity were pH 9.62, 48.87°C, reaction time 22.58 min and substrate concentration of 3.00 mg/ml. The experimental values were agreed with predicted values, and the correlation coefficient was 0.901. The maximum reaction rate ($V_{max}$) and Michaelis-Menten constant ($K_m$) of alkaline phosphatase were determined by using an optimized assay conditions. The value of $V_{max}$ and $K_m$ were found as 3146.34 U/ml and 10.77 mM respectively.

**Key words:** Alkaline phosphatase, *Bacillus licheniformis,* Response Surface Methodology, Central Composite Design

## Introduction

Alkaline phosphatase (E.C. 3.1.3.1) 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 (Mc Comb *et al*., 1979; Kobori *et al*., 1984). Alkaline phosphatase is widely distributed in nature that is found in both prokaryotes and eukaryotes (Wojciechowski and Kantrowitz, 2002; Guimaraes *et al*., 2007; Simao *et al*., 2007; Sasajima *et al*., 2010). *Escherichia coli* alkaline phosphatase, which has been studied in great detail, is a periplasmic enzyme consisting of two identical

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subunits, each containing two $Zn^{++}$ ions and one $Mg^{++}$ ion necessary for activity (Kim and Wyckoff, 1989; Coleman, 1992; Lu *et al*., 2010). Most bacterial strains have been used for production and properties of various phosphatases in a variety of gram-negative organisms (Prada *et al*., 1996; Angkawidjaja *et al*., 2006; Huang *et al*., 2009), as well as in gram-positive organisms (Ghosh *et al*., 1971; Okabayashi *et al*., 1974; Dhaked *et al*., 2005). Alkaline phosphatase is commonly used as a tool in molecular biology and clinical assays. In molecular biology, alkaline phosphatase is used for removing terminal phosphates from nucleic acids (Chen *et al*., 2006). The enzyme has been widely used in the diagnostics, immunology and molecular biology as biochemical markers in quantitative measurements of disease, linked enzymes in ELISA (Sun *et al*., 2007; Muginova *et al*., 2007) and used in non radioactive detection techniques, probing, blotting and sequencing systems (Baranov *et al*., 2008; Nilgiriwala *et al*., 2008). Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes (Banik and Pandey, 2009). RSM defines the effect of the independent variables, alone or in combination, on the process. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process (Ya-Hong *et al*., 2004). It has been successfully applied to optimizing conditions in food, chemical and biological process (Banik *et al*., 2007; Santhiagu and Banik, 2008; Pandey and Banik, 2010). In the present study, an attempt was made to optimize the reaction conditions for maximum alkaline phosphatase activity produced by *B. licheniformis* using central composite design. The $V_{max}$ and $K_m$ values for alkaline phosphatase were determined using substrate between the concentration of 0.25 to 4.0 mg/ml at optimized reaction parameters such as pH-9.62, temperature-48.87°C and reaction time-22.58 minutes.

## Materials and methods

### *Organism and culture condition*

A strain of *B. 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 2% glucose, 1% 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***

Fermentations were carried out by adding 5% inoculum to 50 ml medium in 250 ml Erlenmeyer flasks and incubating in an orbital shaker at 37±1°C and 175 rev/min for 72 hours. 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-nitro phenyl phosphate disodium salt (pNPP) at 415nm (Garen and Levinthal, 1960). A reaction mixture contained 0.1ml enzyme sample and 1.9 ml pNPP was used for determination of alkaline phosphatase activity by taking 1.5-4.0 mg/ml pNPP concentration in 1 M Tris-HCl buffer of different pH 7.0-11.0 and incubating at different temperature 30°C-60°C for 10-30 minutes. The reaction was terminated by adding 0.5 ml of NaOH solution (5M) and the absorbance of the product p-nitrophenol was measured at a wavelength of 415 nm using UV-Vis spectrophotometer (Shimadzu) (Pandey and Banik, 2011). One unit of enzyme activity is defined as the amount of the enzyme catalyzing the liberation of 1μmol of p-nitro phenol per minute.

***Optimization of reaction conditions using RSM***

Optimization of the reaction conditions for the maximum activity of alkaline phosphatase by *B. licheniformis* was done by central composite design (CCD), where $2^4$factorial design was employed with 31 experiments (Table 1). These 31 experiments performed with different combinations of four independent variables on the activity of alkaline phosphatase were studied at five experimental levels: -α, -1, 0, +1, +α, where $\alpha = 2^{n/4}$, here n was the number of variables and 0 corresponded to the central point. The experimental levels for these variables were selected from our preliminary experimental work, which indicated that an optimum could be found within the level of parameters studied. 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).

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

The experimental plan and levels of independent variables are shown in Table 1. Substrate concentration had a lower limit of 1.5 mg/ml and an upper limit of 4.0 mg/ml, pH was varied between 7.0 - 11.0 and temperature was varied between 30°C - 60°C. The lower and upper limits of reaction time were 10 - 30 minutes, respectively. The response surface methodology was used to analyze the experimental design. The response variable was fitted by a second order polynomial model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation is:

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

Where $Y$ is the predicted response, $x_i$, $x_j$ are input variables which influence the response variable $Y$; $\beta_0$ is the offset term; $\beta_i$ is the $i$th linear coefficient; $\beta_{ii}$ is the $i$th quadratic coefficient and $\beta_{ij}$ is the $ij$th interaction coefficient. The second order polynomial coefficients were calculated using the Minitab statistical software package (version-14).

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's $F$-test (overall model significance), its associated probability $p(F)$, correlation coefficient $R$, determination coefficient $R^2$ which measures the goodness of fit of regression model. It also includes the student's $t$-value for the estimated coefficients and the associated probabilities $p(t)$. For each variable, the quadratic models were represented as contour plots (2D). The optimal combination was determined from the contour plots.

### *Determination of enzyme kinetics*

Kinetic constant for alkaline phosphatase reaction were determined with the conventional method by using optimized reaction parameters such as pH, temperature and reaction time. The p-nitro phenyl phosphate disodium salt was used as a substrate between the concentrations of 0.5 to 4.0 mg/ml. The $V_{max}$ and $K_m$ values for alkaline phosphatase were determined using Lineweaver-Burk plot at optimized parameters.

## Results and discussion

### *Optimization of assay conditions using central composite design (CCD)*

In order to search for the optimum combination of reaction conditions, to enhance the alkaline phosphatase activity, experiments were performed according to the CCD experimental plan. Thirty one experiments were carried out from the design (Table 1) by applying multiple regression analysis on the experimental data, the following second-order polynomial equation was found to explain the activity of alkaline phosphatase produced by *B. licheniformis*.

$$Y = 2956.62 + 23.85X_1 + 179.11X_2 + 161.58X_3 + 166.24X_4 - 319.07X_1^2 - 368.33X_2^2 - 424.03X_3^2 - 335.83X_4^2 - 216.99X_1X_2 + 14.14X_1X_3 + 203.44X_1X_4 + 256.03X_2X_3 - 13.30X_2X_4 + 96.32X_3X_4 \quad (3)$$

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, substrate (p-nitrophenyl phosphatae disodium salt), pH, temperature and reaction time respectively. The significance of each coefficient in the equation (3) was determined by P-values (probability) (Montgomery, 2001). The small value of P (<0.05) indicated the significance of the model terms. The non-significant value of lack of fit (>0.05) showed that the quadratic model was valid for present study (Hamsaveni *et al*., 2001). Regression analysis of the experimental data (Table 2) showed that substrate, pH, temperature and reaction time had positive linear effect on alkaline phosphatase activity (P<0.05). The coefficients for quadratic effects of substrate, pH, temperature and reaction time are most significant due to very low probability value (P<0.05). The coefficients of interactions between substrate-pH, substrate-reaction time and pH-temperature were significant as shown by low P-values (<0.05) for interactive terms. However, the coefficients of interactive effect of substrate-temperature, pH-reaction time and temperature-reaction time were found to be insignificant as given by P value above 0.05. Hence, these terms were excluded from the regression equation (3) used for this model. Analysis of variance (ANOVA) for the alkaline phosphatase activity obtained from this design is given in table 3. ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in alkaline phosphatase assay with the designed parameters level. If the F-test for the model is significant at the 5% level (P<0.05), then the model is fit and can adequately explain the variation observed. The closer the value of R (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. Here the value of R (0.901) indicates a good agreement between the experimental and

predicted values. The P-value for lack of fit (0.063) indicated that the experimental data obtained fitted well with the model and explained the effect of reaction parameters on alkaline phosphatase activity produced by *B. licheniformis*. Fig. 1-6 shows the contour plots of alkaline phosphatase activity for each pair of reaction parameters by keeping the other parameters constant.

**Table 1.** Alkaline phosphatase activity by *Bacillus licheniformis* using assay parameters based on central composite design criterion.

| Substrates | pH | Temperature | Reaction time | Activity (U/ml) | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 4.00 | 7 | 30 | 30 | 1864.52 | 2138.05 |
| 2.75 | 9 | 45 | 20 | 3026.69 | 2956.62 |
| 2.75 | 9 | 15 | 20 | 1048.68 | 937.33 |
| 5.25 | 9 | 45 | 20 | 2486.84 | 2128.02 |
| 1.50 | 11 | 60 | 10 | 2306.45 | 2039.27 |
| 2.75 | 13 | 45 | 20 | 2196.58 | 1841.54 |
| 0.25 | 9 | 45 | 20 | 1000.25 | 1232.64 |
| 2.75 | 9 | 45 | 20 | 3016.97 | 2956.62 |
| 2.75 | 9 | 45 | 20 | 2685.94 | 2956.62 |
| 2.75 | 9 | 45 | 20 | 2794.86 | 2956.62 |
| 1.50 | 11 | 30 | 30 | 1037.76 | 1131.32 |
| 4.00 | 7 | 60 | 10 | 1298.68 | 1211.47 |
| 4.00 | 11 | 60 | 30 | 2630.65 | 2579.78 |
| 2.75 | 9 | 75 | 20 | 1598.73 | 1583.65 |
| 2.75 | 9 | 45 | 20 | 2875.65 | 2956.62 |
| 2.75 | 9 | 45 | 5 | 902.35 | 1280.83 |
| 1.50 | 7 | 60 | 30 | 986.65 | 853.24 |
| 1.50 | 11 | 60 | 30 | 1690.68 | 2130.91 |
| 4.00 | 11 | 60 | 10 | 1426.45 | 1674.39 |
| 4.00 | 11 | 30 | 10 | 863.76 | 1003.52 |
| 2.75 | 9 | 45 | 20 | 2905.68 | 2956.62 |
| 1.50 | 7 | 60 | 10 | 965.34 | 708.39 |
| 4.00 | 11 | 30 | 30 | 1146.59 | 1523.62 |
| 4.00 | 7 | 60 | 30 | 1906.03 | 2170.07 |
| 4.00 | 7 | 30 | 10 | 1884.89 | 1564.74 |
| 1.50 | 7 | 30 | 30 | 1005.64 | 877.78 |
| 2.75 | 9 | 45 | 20 | 2960.58 | 2956.62 |
| 1.50 | 7 | 30 | 10 | 1061.01 | 1118.23 |
| 1.50 | 11 | 30 | 10 | 1568.94 | 1424.98 |
| 2.75 | 9 | 45 | 40 | 2450.68 | 1945.77 |
| 2.75 | 6 | 45 | 20 | 896.48 | 1125.09 |

The optimal combination of the parameters of reaction conditions for alkaline phosphatase activity evaluated from the contour plots was as follows: substrate (p-nitro phenyl phosphate disodium salt) - 3.00 mg/ml, pH - 9.62; temperature - 48.87°C and reaction time - 22.58 minutes. The predicted optimum activity of

the alkaline phosphatase was 3066.95 U/ml and the experimental optimal activity was 3146.34 U/ml. Two fold increases in the alkaline phosphatase activity was observed after optimization with RSM. The kinetic constants $V_{max}$ and $K_m$ for alkaline phosphatase were determined with the conventional method by using optimized reaction parameters and found to be 3146.34 U/ml and 10.77 mM respectively.

**Table 2.** Regression analysis of central composite design criterion data for alkaline phosphatase activity by *Bacillus licheniformis*.

| Terms | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 2956.62 | 130.96 | 22.576 | 0.000 |
| Substrate | 223.85 | 70.73 | 3.165 | 0.006 |
| pH | 179.11 | 70.73 | 2.532 | 0.022 |
| Temperature | 161.58 | 70.73 | 2.285 | 0.036 |
| Reaction time | 166.24 | 70.73 | 2.350 | 0.032 |
| Substrate*Substrate | -319.07 | 64.80 | -4.924 | 0.000 |
| pH*pH | -368.33 | 64.80 | -5.684 | 0.000 |
| Temperature*Temperature | -424.03 | 64.80 | -6.544 | 0.000 |
| Reaction time*Reaction time | -335.83 | 64.80 | -5.183 | 0.000 |
| Substrate*pH | -216.99 | 86.62 | -2.505 | 0.023 |
| Substrate*Temperature | 14.14 | 86.62 | 0.163 | 0.872 |
| Substrate*Reaction time | 203.44 | 86.62 | 2.349 | 0.032 |
| pH*Temperature | 256.03 | 86.62 | 2.956 | 0.009 |
| pH*Reaction time | -13.30 | 86.62 | -0.154 | 0.880 |
| Temperature*Reaction time | 96.32 | 86.62 | 1.112 | 0.283 |

$R^2 = 90.1\%$

**Table 3.** Analysis of variance for alkaline phosphatase activity by *Bacillus licheniformis* using central composite design criterion.

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Regression | 14 | 17437775 | 17437775 | 1245555 | 10.37 | 0.000 |
| Linear | 4 | 3262335 | 262335 | 815584 | 6.79 | 0.002 |
| Square | 4 | 11556544 | 1556544 | 2889136 | 24.06 | 0.000 |
| Interaction | 6 | 2618897 | 2618897 | 436483 | 3.64 | 0.018 |
| Residual Error | 16 | 1920972 | 1920972 | 120061 | | |
| Lack-of-Fit | 10 | 1650364 | 1650364 | 165036 | 3.66 | 0.063 |
| Pure Error | 6 | 270608 | 270608 | 45101 | | |
| Total | 30 | 19358747 | | | | |

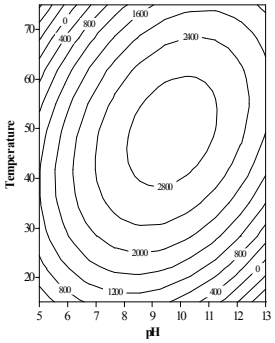


**Fig. 1.** Effect of pH and temperature on alkaline phosphatase activity (U/ml).

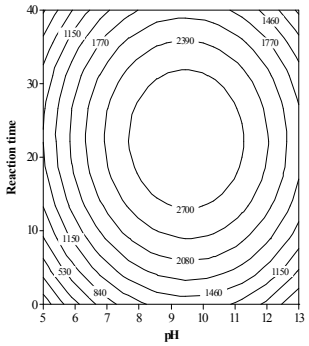


**Fig. 2.** Effect of pH and reaction time on alkaline phosphatase activity (U/ml).

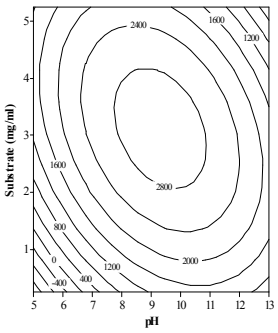


**Fig. 3.** Effect of pH and substrate on alkaline phosphatase activity (U/ml).

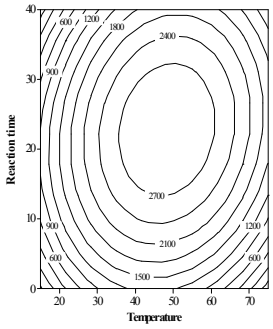


**Fig. 4.** Effect of temperature and reaction time on alkaline phosphatase activity (U/ml).

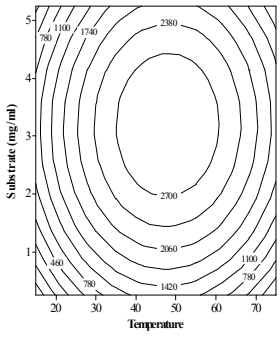


**Fig. 5.** Effect of temperature and substrate on alkaline phosphatase activity (U/ml).

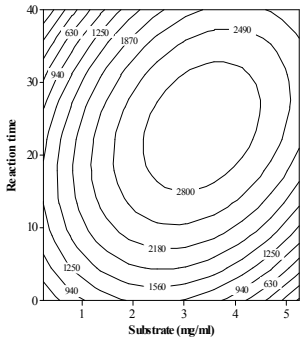


**Fig. 6.** Effect of substrate and reaction time on alkaline phosphatase activity (U/ml).

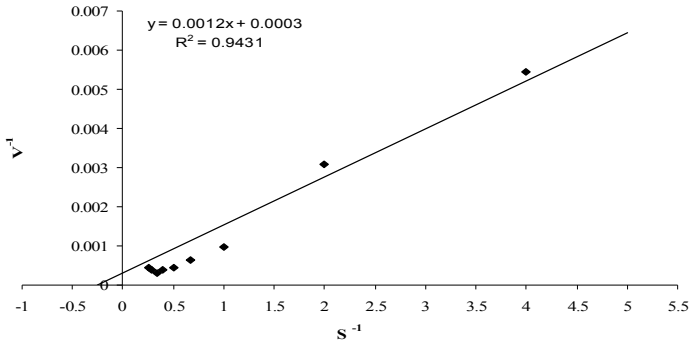


**Fig. 7.** Lineweaver-Burke plot for determination of $V_{max}$ and $K_m$ values of alkaline phosphatise.

To the best of our knowledge there is limited information available in open literature concerning optimization of reaction conditions for maximum alkaline phosphatase activity produced from *B. licheniformis* by response surface methodology. The reaction conditions was optimized by applying central composite design for alkaline phosphatase activity was substrate (p-nitro phenyl phosphate disodium salt) - 3.00 mg/ml, pH - 9.62, temperature - 48.87°C and reaction time - 22.58 minutes. The kinetic constants of alkaline phosphatase were determined with the conventional method by using optimized reaction parameters. The chosen method of optimization of an assay conditions was efficient, relatively simple, and time and material saving.

## Acknowledgements

The authors are thankful to the School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi, India for providing research facilities and financial support to Mr. Sanjeev Kumar Pandey in the form of SRF by UGC, India.

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(Received 5 March 2011; accepted 30 May 2011)