RESEARCH ARTICLE

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Response surface methodological approach for optimizing the enzyme activity and enzymatically mediated bioprecipitation of heavy metals by alkaline phosphatase

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ABSTRACT

This study investigates using an alkaline phosphatase enzyme isolated from *Bacillus cereus* to decontaminate heavy metals. The experiments were performed with several process parameters, including substrate concentration, pH, and temperature. To optimize the best experimental conditions, they were estimated by using a central composite experimental design combined with response surface methodology (RSM). Variables were concentration of substrate ((p-NPP 14 to 17 mM), pH 8 to 10.5, and temperature (35 to 45°C). Statistical analysis of variance (ANOVA) was performed to classify the competence of the developed model and revealed a good understanding between the experimental data and the proposed model. The highest enzymatic activity 25.73 units/ml was identified by the RSM with the following optimal set of parameters: concentration of substrate 15.5 mM, pH 9.25, and temperature 34oC. The accuracy of the predicted model optimum parameters was confirmed by experimenting under the same parameters. It was found that the experimental enzyme activity efficiency under optimum conditions was very close (less than a 3% error) to the model-predicted value. The removal efficiency of each heavy metal was found to be in the following order: Hg >Pb>As.

Key words: *Bacillus cereus*, Alkaline phosphates, Response surface methodology, Bio-precipitation

Introduction

Soil pollution by different pollutants, such as heavy metals, hydrocarbons, pesticides, and petroleum can lead to biological as well as serious environmental effects and also affects soil fertility (Kasassi et al., 2008; Smith, 2009). This pollutant not only harms human beings and animals, they can leach into soil and water and affect the microorganisms living in the soil as well (Sreedevi et al., 2022). The pollutants can directly influence the properties of important microbial enzymes found in the topsoil, which play major roles in the decomposition of organic matter. Heavy metal pollutants present in soil can cause harmful effects. The permissible limit of heavy metal in the soil (mg/kg) is Hg (30), Cd (6), Zn (600), As (3) Mn (20), Pb (500), Cu (270), Ni (75), Cr (50) (Kasassi et al., 2008). These heavy metals can go through redox cycling, which then causes oxidative stress in soil organisms and compete for metal-cofactor binding of metalloenzymes to their inactivation.

Current studies focus on the effect of heavy metal pollution on the soil enzymes that have been used for the following studies; dehydrogenase, cellulose, beta-glucosidase, phenol oxidase, amidase, urease, phosphatase, and arylsulphatase. All of these studies have been performed in heavy metal-treated soil (Khan et al., 2007).

Alkaline phosphatases are known in a broad variety of bacteria and mammals, which proficiently hydrolyze some mono and di-esters (Hoylaerts et al., 1997). The enzyme alkaline phosphatase precipitates metal ions as insoluble metal phosphate. In recent years, alkaline phosphates have been considered for application in the bioremediation of heavy metals from industrial wastes. A serine residue situated at the catalytic site of the enzyme initially gets phosphorylated and after hydrolysis either phosphor anhydride or phosphor ester, that intermediate undergoes the production of inorganic phosphate (Nayudu & Meis, 1989). This can bind with metal and successively precipitate it as metal-phosphate.

Response surface methodology is a modern sophisticated statistical technique, developed by Box and Wilson in 1951. RSM is commonly used for optimizing process variables and their interactions affect the response. This mathematical approach enables the designing of experiments; output variables are analyzed, which is influenced by input variables. RSM helps to reduce problems related to traditional optimization methods and helps to reduce the experimental trials (Tabssum et al., 2018). This mathematical approach has

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been extremely employed for the optimization of remediation and extraction processes (Wróbel et al., 2023).

The current study was designed to optimize the alkaline phosphate activity using response surface methodology to get the highest precipitation of heavy metals. The substrate concentration, pH, and temperature parameters were optimized through the CCD of RSM. We also examined the multiple heavy metals (As, Pb, and Hg) precipitation assays on alkaline phosphatase activity.

Materials and Methods

Enzyme and chemicals

The purified alkaline phosphatase from *Bacillus cereus* isolated from Gevra coal mine Korba, Chhattisgarh (22.336312, 82.545748) was used throughout the experiments. Heavy metals $HgCl_2$, $Pb(NO_3)_2$, $NaAsO_2$, and other chemicals were procured by Sigma- Aldrich Chemical Company, USA.

Screening of Bacillus cereus capable for production of alkaline phosphatase

Phosphatase agar plate was used for screening. The test microorganism was streaked onto the Phosphatase agar plate. The streaked plate was then incubated in a bacteriological incubator at 37°C for 24 hours. Positive results are indicated by the appearance of yellow coloration in the media around the streaked microorganism (Xian et al., 2015). The production of Alkaline phosphatase enzyme was done by submerged fermentation (Peptone- 13.5 gm/L, Sodium Chloride- 5gm/L, Beef extract- 1.5gm/L, yeast extract -3.0gm/L, Dextrose- 25gm/L, Magnesium sulphate ($MgSO_4$)– 0.8gm/L, Manganese sulphate ($MnSO_4$)- 0.015gm/L, Calcium Chloride ($CaCl_2$)- 1 gm/L, di-Potassium Hydrogen orthophosphate (K_2HPO_4)- 1.5gm/L, Distill water- 1000ml, pH-7.2±0.2.) (Holtz & Kantrowitz, 1999).

Purification of alkaline phosphatase enzyme

100mL of the crude supernatant enzyme was salt precipitated using 80% ammonium sulphate salt saturation. Salt precipitated crude supernatant was transferred to the dialysis membrane. The dialysis process was carried out on 0.001M dialysate buffer for at least 36-48 hours. Dialysed crude supernatant enzyme then purified through Ion exchange chromatography. DEAE cellulose was used as the stationary phase and six elutes were prepared with definite concentrations of Tris HCl and gradient concentrations of Sodium chloride (NaCl) such as Elutes such as **I** (25mM Tris HCl & 25mM NaCl), **II** (25mM Tris HCl & 50mM NaCl), **III** (25mM Tris HCl & 75mM NaCl), **IV** (25mM Tris HCl & 100mM NaCl), **V** (25mM Tris HCl & 125mM NaCl), and **VI** (25mM Tris HCl & 150 mM NaCl) (Cummins et al., 2017).

Enzymatic assay of alkaline phosphatase

The alkaline phosphatase assay was the standard assay based on the hydrolysis of the colorless p-nitrophenylphosphate (pNPP) to produce the yellow-colored phenolate anion (Brunel & Cathala, 1973). Total reaction mixture containing 0.5 ml 15.2 mM pNPP in Tris buffer as substrate and reaction started by adding 0.1 mL enzyme solution and incubated at 37°C for 10 minutes. After incubation 10 ml 20mM NaOH was added and the absorbance was measured at 410 nm using a spectrophotometer (Systronics, AU2701). Enzyme activity was calculated by following equation.

$$\text{Enzymatic activity } \left(\frac{\text{Units}}{\text{ml}}\right) = \\ = \frac{(\text{A410nm Test} - \text{A410nm Blank})(11.1)(\text{df})}{(18.3)\ (0.1)\ (10)}$$

Quantitative estimation of protein estimation

The quantitative estimation can be estimated by the Lowry method. For standard calibration curve, Bovine Serum Albumin (BSA) is universally used as a standard protein. The BSA Standard calibration curve was prepared by plotting Mean Absorbance at 660nm (y-axis) to standard BSA concentration (x-axis). The total protein content of samples was determined from the absorbance of the sample. The absorbance so obtained for the sample is plotted on the BSA Standard calibration curve to get the Total protein content (µg/ml equivalent of BSA). The slope equation for each calibration curve was established from the slope equation $y = mx + c$ (Sengupta & Chattopadhyay, 1993).

Characterization of alkaline phosphatase

Sodium Dodecyl sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE) method was used for the separation of fractions of protein based on the mass. The separating gel (10%) was first poured in gel castor and then poured stacking gel (4%), after that, a protein sample of 50 µL was loaded into the wells. The power supply was turned on from the Electrode powerpack and the supply of potential was continued upto 2 ½ hours continuously (Anand et al., 2017). After the protein sample runs in PAGE gel put into the staining buffer overnight. After incubation gel was transferred into the destaining solution, for 1 to 2 hours. The protein bands were then observed as the protein was stained by Commassie Brilliant Blue stain (Al-Tubuly, 2000; Battisti et al., 2024).

Optimization of enzyme activity using RSM

Response Surface Methodology is multivariate statistical software to scrutinize the bio-precipitation process of heavy metals and provides better results to optimize enzyme activity as well as bio-precipitation (Kumari & Gupta, 2019). The RSM procedure includes four stages: (1) a series of designed experiments to get the desired target response, (2) a mathematical response model, (3) the establishment of

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maximum and minimum response value for optimal parameters (4) the analysis and representation of process parameters with 2D and 3D plots (Behera et al., 2018).

Central composite design (CCD) is used to evaluate the quadratic response surface as well as second-order polynomial model development in RSM. In this study, CCD was developed using Design Expert (13.0; Stat Ease, USA) for optimizing three variables i.e. Substrate concentration, Temperature, and pH on enzyme activity. An analysis of variance (ANOVA) test was used to obtain the regression coefficients of linear, quadratic, and interaction terms (Sasidharan & Kumar, 2022). The linear, two-factorial interaction (2FI), quadratic and cubic functions are the most widely used mathematical models. The following equations could be used to express four models (Fertu et al., 2022).

Linear

$$Y = \beta_0 + \sum_{i=1}^{m} (\beta_i X_i + \varepsilon)$$

2Fi

$$Y = \beta_0 + \sum_{i=1}^{m} \beta_i X_i + \sum_{i<=2}^{m} \beta_i X_i X_j + \varepsilon$$

Quadratic

$$Y = \beta_0 + \sum_{i=1}^{m} \beta_i X_i + \sum_{i=1}^{m} \beta_{ii} X2_{ii} + \sum \sum_{i<=2}^{m} \beta_{ij} X_i X_j$$

Cubic

$$Y = \beta_0 + \sum_{i=1}^{m} \beta_i X_i + \sum_{i=1}^{m} \beta_{ii} X2_{ii} + \sum \sum_{i<=2}^{m} \beta_{ij} X_i X_j + + \sum \sum \sum_{i<j<k=3}^{m} \beta_{ijk} X_i X_j X_k$$

To optimize enzyme activity of alkaline phosphatase experiments were designed for pH (8 to 10.5), incubation temperature (28°C to 40°C) Substrate Concentration (p-NPP varying from -14.0 to 17.0 mM). Table 1 shows the actual values of the factors and their corresponding coded levels.

Heavy metal decontamination by Bio-precipitation

The determination of heavy metal decontamination by precipitation method evaluated the previously described process by Fu and Wang (2011) using atomic absorption spectroscopy (Agilent 240FS AA). In this method heavy metal 50 mg/L (Pb^{+2} and Hg^{+2}) and As^{+5} 25 mg/L, 200 U enzyme, 50 mM Ascorbic acid was incubated at 37 °C for 300 minutes incubation periods. The percentage of precipitation was calculated by the following equation:

$$\text{Percentage of Precipitation (x)} = \frac{a - b}{a} * 100$$

where:

x – Percentage of heavy metal precipitation,

a – Initial concentration of heavy metal in initial aliquot (at time = 0 minutes),

b – Final concentration of heavy metal in final aliquot/supernatant (sample drawn at different time intervals).

Model validation

The mathematical model generated by the RSM (Design Expert) approach was validated via performing experiments on specified optimal settings and statistical *t-tests* with different statistical parameters such as coefficient of determination (R2), adjusted R2 (R2adj), and predicted R2 (R2pred).

Table 1. *Independent variable and corresponding levels for enzyme activity.*

Independent variables	Coded levels				
	-α	**-1**	**0**	**+1**	**+α**
pH	6.21	8	9.25	10.5	11.36
Temperature (in centigrade)	26.31	28	34	40	41.61
Substrate Concentration (in mM)	12.45	14	15.35	17	18.45

Results and Discussion

The bacteria that produce the Alkaline Phosphatase enzyme, remove the phosphate group from substrate p-nitrophenol phosphate (colorless) to p-nitrophenolate (yellow color) that turns the color of the media to yellow color. In the screening process, Bacillus cereus had shown positive for phosphatase activity indicating the production of yellow coloration in the media (Figure 1). After screening of bacteria capable of production, the seed culture for Bacillus cereus was prepared. After a Bacterial suspension culture of 0.5 Macfarland unit (Cockerill & Franklin, 2012), with the absorbance of the bacterial suspension equivalent to 0.08-0.1 at 600nm, were selected for inoculation into the sterile production media broth. A 5% inoculum size was selected for the production media.

Figure 1. *Screening for production of Alkaline phosphatase enzyme by Phosphatase agar media.*

Purification of alkaline phosphatase

The alkaline phosphatase enzyme produced by Bacillus cereus was extra-cellular. So, the enzymes were produced outside of the cell in the production media. Then the media was centrifuged at 10,000 rpm for 15 minutes. The supernatant contained the crude enzyme fraction. The purification is mainly composed of three major steps: Salt precipitation, Dialysis, and ultra-purification by Ion Exchange chromatography. Salt precipitation was the first step for the purification of crude alkaline phosphatase enzyme. The Ammonium sulphate was added pinch by pinch to the supernatant to initially obtain 20% saturation and then for an hour the salts were added to finally obtain 80% of ammonium sulphate salt saturation. The salt-saturated sample was kept in the refrigerator overnight (~4°C). After overnight incubation, centrifuged at 10,000 rpm for 10 minutes at ~4°C. The pellet was collected and re-dissolved in the equivalent volume of 0.1M Tris-Glycine buffer (pH= 8.8). In the dialysis process, the protein samples prepared through salt precipitation were kept in dialysis bags. The dialysis bag containing the enzyme sample was placed in a low-concentration dialysate buffer. Due to the higher concentration of salts and contaminants in enzyme solution within the dialysis bag, they tend to come out to the dialysate buffer by diffusion through the pores in the bags. The enzyme molecule afterward elutes out of the stationary phase and is collected in the elution fraction and the enzymatic activity of each enzyme fraction was analyzed. The enzyme elutes having higher enzyme activity contains the Alkaline phosphatase enzyme. The highest enzyme activity recorded was at Elution no. 4 which contained 25.475 U/ml enzyme activity.

Enzymatic assay

Changes The enzymatic activity of the alkaline phosphatase enzyme is determined by the rate of conversion of p-nitrophenol phosphate to p-nitrophenol/p-nitrophenol in alkaline conditions. The enzyme activity of Crude extract was 39.426 U/ml. In the case of the purified sample salt precipitation and dialysis sample, the enzyme activity was 41.852 U/ml and 37.606 U/ml respectively. The ion exchange sample had 25.475 U/ml of enzyme activity.

Quantification of protein

The estimation of Total protein content for the different enzyme samples was done by Lowry's test. The standard calibration curve of Bovine Serum albumin (BSA) was first established. (Figure 2). The concentration of protein in the enzyme samples was determined by a standard calibration curve. The crude enzyme extract contains 265.86 µg/ml. The salt precipitation and dialysis samples contained 176.21µg/ml and 153.85µg/ml respectively. However, the Purified enzyme fraction (Ion exchange elution 4) was found to be 110.45µg/ml (Figure 3).

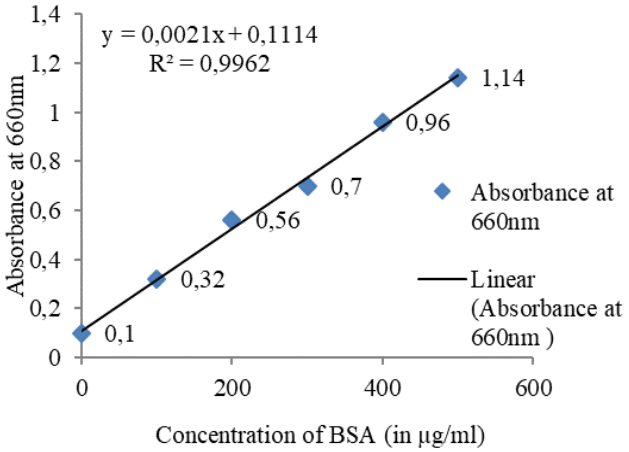

Figure 2. *Standard Calibration curve of BSA for Lowry's method.*

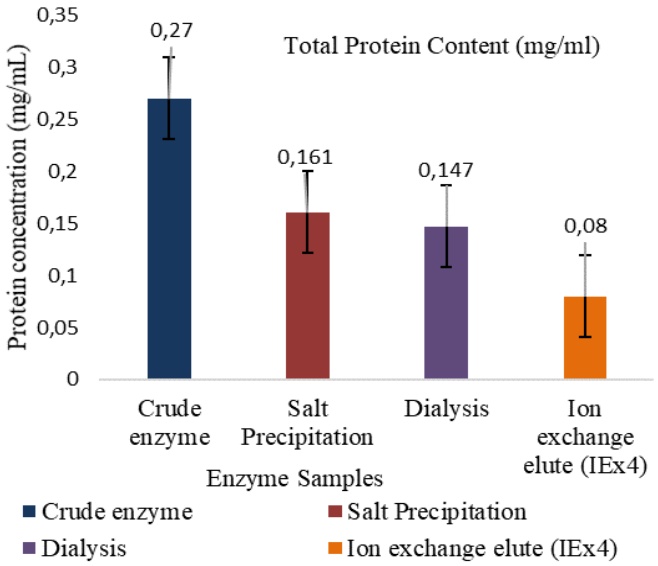

Figure 3. *Quantitative estimation of protein.*

Characterization of alkaline phosphatase enzyme

For the determination of the molecular weight of the protein, the protein sample has been run along with a ladder protein with a defined molecular weight. The molecular weight of the sample protein was determined from the migration distance. The retardation distance so obtained for the sample is plotted on the Retardation distance calibration curve of the Ladder protein to get the molecular weight of the sample protein (kDa) The slope equation for each calibration curve was established from the slope equation.

$$y = mx + c$$

where:

y – Retardation distance,

m – Slope,

x – Molecular weight of standard protein (kDa),

c – Intercept.

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From Standard ladder protein calibration curve (Figure 4), the molecular weight of unknown protein can be determined by putting the retardation distance (in mm) (y) of the unknown protein against the molecular weight of known standard ladder protein (Figure 5).

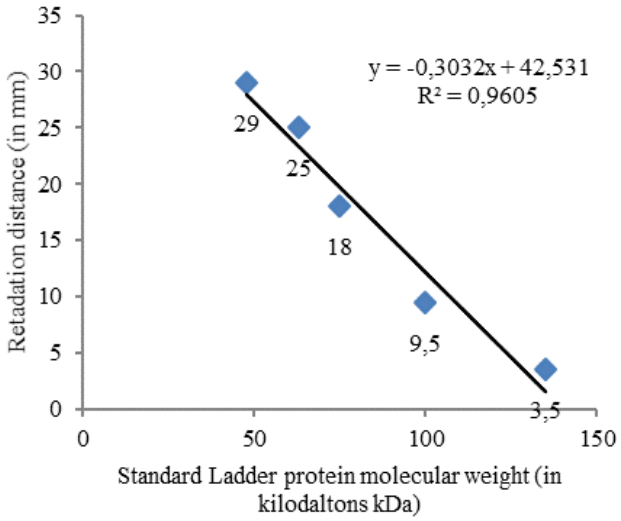

Figure 4. *Calibration curve of Standard Ladder protein for molecular weight determination.*

$$Unknown\ Molecular\ weight\ (x) = \frac{y - 42.53}{-0.303}$$

From the above equation, the molecular weight of the purified alkaline phosphatase was found to be ~ 61 kDa.

Optimization of experimental condition of enzyme activity

Experiments were performed for the enzyme activity using Design Expert model 13.0; Stat-Ease, USA. The optimization experiment was used to get higher enzyme activity, various numerical combinations were set to maximize the model

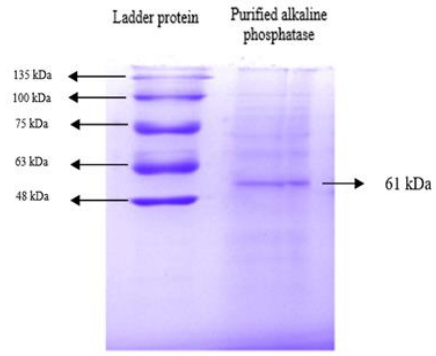

Figure 5. *Agarose gel electrophoresis image.*

functions. The optimized conditions of the highest responses for enzyme activity were obtained at pH - 9.25, temperature at 34°C, and substrate concentration at 15.5 mM (Figure 6). At optimized conditions, 25.73 unit/ml enzyme activity was predicted. The optimum conditions obtained via the program were further validated to confirm the enzyme activity through additional experiments. These additional confirmatory experiments reported 25.65 unit/ml enzyme activity, compared with the obtained model result validated the accuracy of the model.

Analysis of variance (ANOVA) results for enzyme activity models are given in Table 2. The "F-value" of the developed model was found to be 6.41 enzyme, illustrating that all these models are statistically significant. The values of "Prob > F"

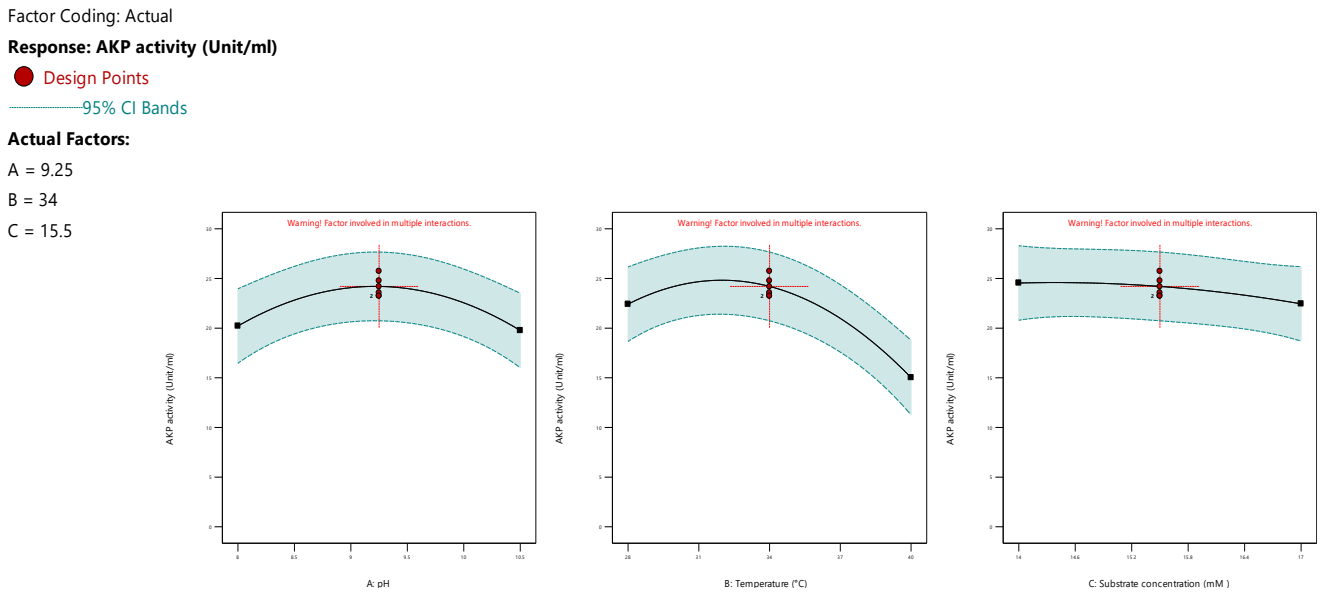

Figure 6. *Numerical optimization of process variables for enzyme activity.*

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Table 2. *ANOVA of quadratic response surface model for enzyme activity.*

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remark
Model	834.92	9	92.77	6.41	0.0038	significant
A-pH	0.6859	1	0.6859	0.0474	0.8321	
B-Temperature	186.49	1	186.49	12.88	0.0049	
C-Substrate concentration	15.06	1	15.06	1.04	0.3319	
AB	1.70	1	1.70	0.1175	0.7388	
AC	1.93	1	1.93	0.1333	0.7226	
BC	0.0010	1	0.0010	0.0001	0.9935	
A^2	255.60	1	255.60	17.65	0.0018	
B^2	432.89	1	432.89	29.89	0.0003	
C^2	7.18	1	7.18	0.4959	0.4974	
Residual	144.82	10	14.48			
Lack of Fit	139.96	5	27.99	28.76	0.0011	significant
Pure Error	4.87	5	0.9733			
Cor Total	979.74	19				

were obtained less than 0.0500, signifying that all the models. The model terms B, A2, and, B2 are found to be significant for the enzyme activity model are found significant, and p values for all the models were observed to be <0.001, representing the significance level for developed models. The model terms are insignificant if the p-values are higher than 0.10. In addition, other statistical parameters such as coefficient of determination (R2), adjusted R2 (R2 adj), predicted R2 (R2 pred), and coefficient of variation (CV%), were also needed to evaluate the proficiency of developed models.

Table 3. *Model statistics of the developed model.*

Statistical parameters	Values of developed model
R^2	Enzyme activity
Adjusted R^2	0.7191
predicted R^2	-0.0997
Adeq. Precision	8.0702
Std. dev.	3.81

R2 values and adjusted R2 for enzyme activity were found to be 0.8522 and 0.7191. The standard deviation for the model was also found to be 3.81. The R2 values close to the standard deviation values of the developed model present a better predicting response. Adeq Precision evaluates the signal-to-noise ratio, the model is desirable if the ratio is more than four. In this study, the obtained ratio for the model was found to be 8.0702, suggesting the existence of a satisfactory signal for navigating the design space. The statistics of the developed model are mentioned in Table 3. The actual values obtained by the CCD response are measured and the predicted response is evaluated by using the estimated function values for model estimation. A straight line was observed in normal percentage probability as opposed to externally studentized residual plots for all these models, consequently presenting a normal distribution of data (Figure 7).

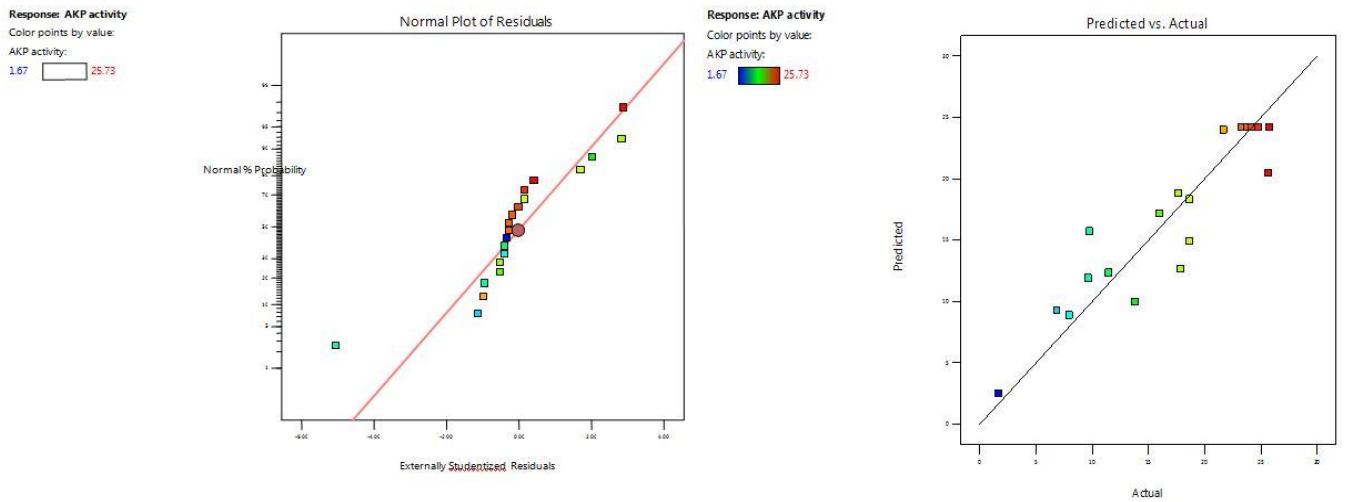

Figure 7. *Graphical plot of predicted Vs actual values of Enzyme activity.*

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2D contour graphs and 3-D surface plots analysis

To evaluate the combined outcome of the factors on enzyme activity, the graphical presentation was presented in 2D contour graphs and 3-D surface plots for regression equation. The 2D contour plot represents that the highest enzymatic activity was obtained in substrate concentration 15.5mM, temperature 34oC, and pH 9.25. But, when the concentration of substrate for enzyme activity was increased, enzyme activity was decreased. The enzymatic efficiency was obtained as proof from 3D surface plots (Figure 8).

Cube plot

The outcome of the developed experimental models is presented by cube plot. The axis represents all the experimental design factors, whereas the coordinate point shows the outcome. The values presented inside the cube represent the predicted highest enzyme activity by the variable used in this study. Minimum enzyme activity (18.81%) was achieved at pH 8.0, temperature of 28°C and, substrate concentration of 14mM, while maximum enzyme activity was achieved at pH - 9.25, the temperature at 34°C and substrate concentration at 15.5 mM (25.73 unit/ml) (Figure 9).

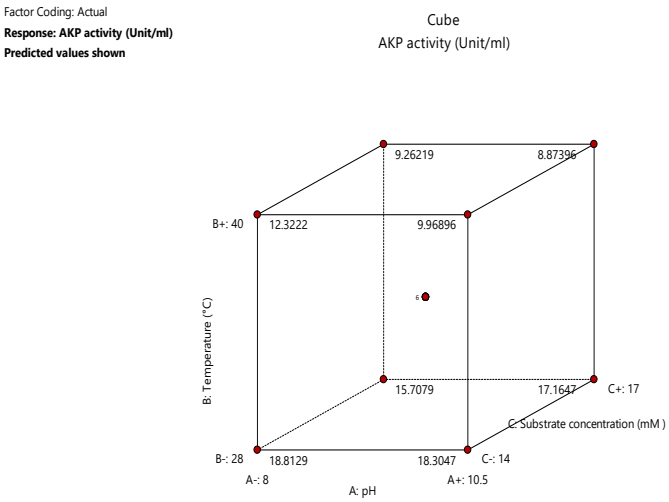

Figure 9. *Cube plot for enzyme activity.*

Heavy metal decontamination

The precipitation experimentation regarding bio remedial activity of alkaline phosphatase enzyme against different heavy metals was shown in Table 4.

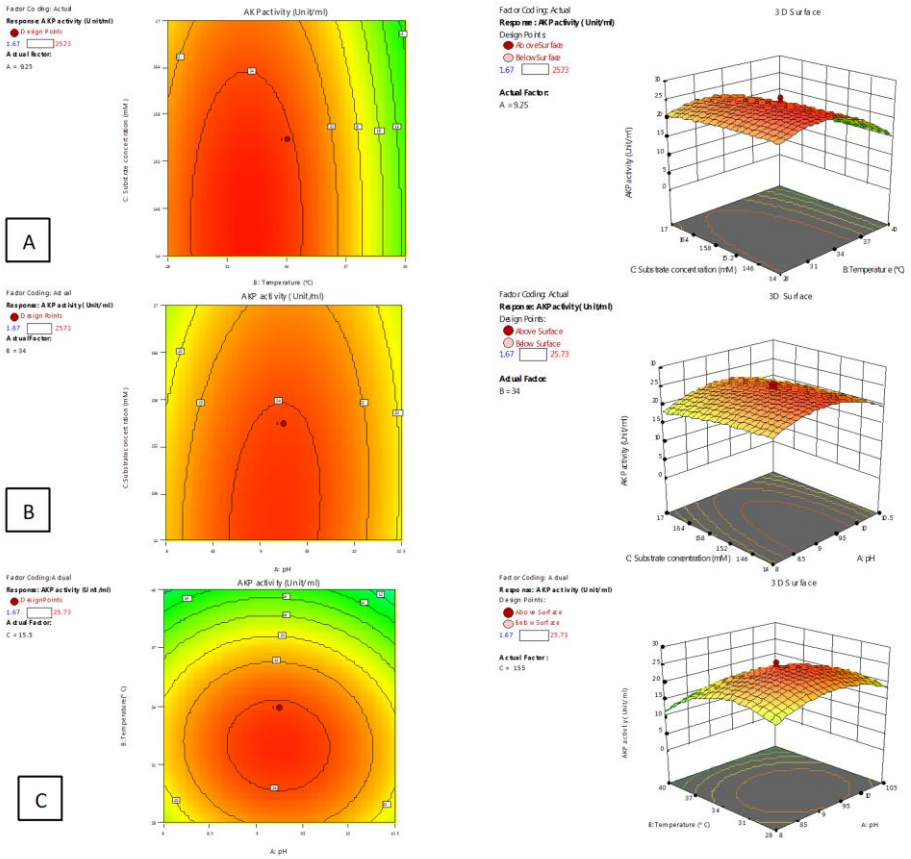

Figure 8. *2D Contour and 3D surface plots for enzyme activity (A) pH, (B) temperature, (C) substrate concentration.*

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Table 4. *Bio-precipitation of heavy metals by alkaline phosphatase.*

Sl. No	Initial concentration of heavy metal in initial aliquot (in mg/L) (a)	Final concentration of heavy metal in final aliquot/supernatant (in mg/L) (b)	Time interval (in minutes)	Percentage of heavy metal precipitation (%)
1.	50 (Pb)	05.00	300	80.00
2.	50 (Hg)	23.50	300	48.00
3.	25 (As)	18.00	300	28.00

Conclusion

Response surface methodology was adapted to optimize the enzyme activity parameters through alkaline phosphatase from *Bacillus cereus*. The parameters assessed included substrate concentration, Temperature and, pH. These were optimized using CCD of Response surface methodology and the parameters of best enzyme activity were found to be temperature at 34°C, at pH - 9.25 and, substrate concentration at 15.5 mM. Maximum enzyme activity under these conditions was found to be 25.73unit/ml, but a decrease in efficiencies was reported at higher concentration.

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