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TOXICITY OF BINARY AND TERNARY METAL MIXTURES ON DEHYDROGENASE ACTIVITY OF *Pseudomonas fluorescens*: AN ASSESSMENT USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Toxicity of cadmium, cobalt, lead and nickel as individual chemicals and their binary, ternary mixtures effect on *Pseudomonas fluorescens* was determined using response surface methodology. The mixture ratios were designed using central composite response surface design and implemented using minitab statistical software. The toxicity assay showed Cadmium was the most toxic while lead was the least toxic where increase in dehydrogenase enzyme inhibition was observed with corresponding increase in concentration of these metals. The concentration of the individual metals in the mixture was designed not to exceed EC_{45}, EC_{30}, EC_{25} or EC_{20} for binary and ternary mixtures respectively. The dose response relationships obtained were fitted into a logistic dose-response model to determine the median inhibitory concentration (EC_{50}). The EC_{50} values deduced are cadmium 0.023 ± 0.003, lead 0.135 ± 0.007, cobalt 0.099 ± 0.006 and nickel 0.080 ± 0.006. The concentration of the metals alone with their calculated percentage inhibition was used to derive the residual plots, contour plots and surface plots for the binary and ternary mixtures respectively. The surface plots generated were seen to be curved which is as a result of the quadratic nature of the models having the highest values of inhibition (%) found at the top right corner of the plot while the lowest values were seen at the bottom left corner of the surface plot. The contour plots showed different shapes which indicated different interactions between the variables where an elliptical contour plot indicated the interactions between the variables were significant while a circular contour plot meant otherwise. The contour plots showed contour lines which connected points having the same response values during the course of the analysis. Residual plots generated showed data points closer to the regression line without outliners thus statistically inferring a good fit for the linear model. Coefficients of determinations derived during course of this work were relatively good with the lead-nickel binary mixture observed to have the highest coefficient of determination (R^2) of 99.47% and adjusted determination coefficient (R^2_{Adj}) value of 99.39% which means that the calculated model was able to explain 99.47% of the results. The adjusted determination coefficient (R^2_{Adj}) of the lead-nickel binary model was 0.9939, which indicated only 0.61% of the total variations were not explained by the model.

INTRODUCTION

Pollution is one menace the world is suffering today where it is considered a critical global environmental problem. This global environmental problem caused by pollution of the environment is as a result of unrestrained industrialization and urbanization without proper emission controls and pollution abatement[1] Pollution of the environment can be by several ways of which pollution by Heavy metals such as Nickel, Cadmium, Cobalt and lead is not left out. These heavy metals find their way to pollute the environment through several activities such as mining, soil erosion, natural weathering of the earth's crust, industrial effluents discharge, urban runoff, sewage discharge, insect or disease control agents applied to crops, and many others.[2] At certain low doses, these heavy metals have been found to be of beneficial value in the environment[3] while at relatively high doses they tend to affect proper functioning of microorganisms where a typical case can be seen in inhibition of dehydrogenase activity of *Pseudomonas fluorescens.* Heavy metals are natural elements which are characterized by high atomic mass and high density.[4]

The toxic nature of some of these heavy metals gave rise to unset of toxicity assessment. Toxicity assessment being a major component of risk assessment is a tool used to investigate the potential of a substance to cause harm and how much of the toxicant causes harm.[5] Several methods have been found to be used for toxicity assessment of these metals of which response surface methodology is a typical example of such methods.

Response surface methodology is a collection of mathematical and statistical techniques used for empirical model building.[6] The objective of the response surface methodology is to optimize a response (output variable) which is influenced by several independent variables (input variables). Metals have been found to rarely occur in the environment in singles but rather in mixtures thus making their toxicity assessment not to be straight forwardly inferred. This drove the aim of this study which is to assess the toxicity of binary and ternary mixtures of these heavy metals on dehydrogenase activity of *Pseudomonas fluorescens* using response surface model.

MATERIALS AND METHODS

Test Chemical and Reagents: The compounds $CdSO_4.1/7H_2O$, $PbNO_3$, $NiCl_2$ and $CoCl_2$ were used as the sources of the metal ions Cd^{+2}, Pb^{+2}, Ni+ and Co^{+2}. 2-iodophenyl, 3-nitrophenyl, 5-phenyl tetrazolium chloride (INT) was used as the enzyme substrate and was obtained from Sigma (USA). Analytical grade of Butanol was used for the formazan extraction.

Preparation of stock solution: 10mM stock solution of each test chemical was prepared by dissolving known amount of each metal in requisite volume of distilled water. Working stock concentrations of 0.1mM, 1mM and 2mM were prepared by further diluting the 10mM solutions of the chemicals with desired volume of distilled water.

Confirmation of Test Organism: Pure culture of *Pseudomonas fluorescens* that was previously isolated and characterized was obtained from Prof C.O. Nweke of the department of microbiology federal university of technology Owerri. Identity of test organism was further confirmed by adopting methods as described by.[8]

Preparation of Test Inoculum for Toxicity Assay: Preparation of the test inoculum was done as described by Nweke *et al*., 2018. The test organism was grown in nutrient broth and placed on a rotary shaker (150rpm) at room temperature (28 ± 2^{0}C) for 24 hours. After 24 hours of incubation, cells were harvested and washed by centrifugation at 4000rpm for 10 minutes.[7] The washed cells were suspended in sterile distilled water and Optical density (OD) adjusted to 0.1 at 540nm wavelength.

Design of experiment: Toxicity assessment of binary and ternary mixtures of these heavy metals on dehydrogenase activity of *Pseudomonas fluorescens* was carried out using central composite response surface design. Minitab software was used as a tool for generation of the working protocols with which the toxicity assessments were done. The heavy metals were assessed in a lead-nickel mixture, cadmium-cobalt mixture, Cadmium-nickel binary mixture pattern as well as orders for the ternary mixtures. A 2^2 factorial full central composite design for two test variables, each at five levels with four star points and six replicate at the center indicating 14 experimental runs was used for the binary mixture of metals. The alpha value used was 1.414. A 2^3 factorial full central composite design for three test variables, each at five levels with six star points and six replicate at the center indicating 20 experimental runs was used for the ternary mixture of metals. The alpha value used was 1.682. In each case, duplicate of control experiment without any added metal ion was also included and the mean used to compute inhibition (%) of dehydrogenase activity. The test variables were coded according to equation 1.

$$X_i = \frac{X_i - X_0}{\Delta X_i} \qquad \text{.. (1)}$$

Where X_i and x_i are the actual and coded values respectively of the independent variable i. X_0 is the actual value of the independent variable i at central point and ΔX_i is the step change of X_i corresponding to a unit variation of the coded value. The coded and actual values for this design are shown in tables.

Table 1: Coded and actual values of heavy metals for binary mixture toxicity assay.

Factors	-1.414	-1	0	+1	+1.414	ΔXi
Cd	0.011	0.012	0.015	0.018	0.019	0.003
Pb	0.053	0.055	0.06	0.065	0.067	0.005
Co	0.012	0.02	0.04	0.06	0.068	0.02
Ni	0.007	0.015	0.035	0.055	0.063	0.02

Table 2: Coded and actual values of heavy metals for Ternary mixture toxicity assay.

Factors	-1.682	-1	0	+1	+1.682	ΔXi
Pb	0.052	0.055	0.06	0.065	0.068	0.005
Cd	0.005	0.007	0.01	0.013	0.015	0.003
Co	0.013	0.02	0.03	0.04	0.047	0.01
Pb	0.052	0.055	0.06	0.065	0.068	0.005
Cd	0.005	0.007	0.01	0.013	0.015	0.003
Ni	0.017	0.02	0.025	0.03	0.033	0.005

Table 3: Central composite design matrix of ternary mixtures in coded values.

Test runs	Coded level		
	X1	X2	X3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Table 4: Central composite design matrix of binary mixtures in coded and actual values.

Test runs	Coded values		Actual values		Logit	Actual values		Logit			Logit
	X1	X2	Pb	Ni		Cd	Co		Cd	Ni	
1	-1	-1	0.055	0.015	-0.166	0.012	0.020	2.135	0.012	0.015	0.414
2	1	-1	0.065	0.015	-0.110	0.018	0.020	1.706	0.018	0.015	0.700
3	-1	1	0.055	0.055	0.915	0.012	0.060	2.905	0.012	0.055	0.750
4	1	1	0.065	0.055	1.198	0.018	0.060	2.589	0.018	0.055	1.035
5	0	0	0.060	0.035	0.712	0.015	0.040	2.156	0.015	0.035	0.906
6	0	0	0.060	0.035	0.902	0.015	0.040	1.957	0.015	0.035	0.694
7	0	0	0.060	0.035	-0.735	0.015	0.040	1.122	0.015	0.035	0.930
8	-1.414	0	0.053	0.035	1.027	0.011	0.040	2.719	0.011	0.035	1.016
9	1.414	0	0.067	0.035	0.590	0.019	0.040	1.995	0.019	0.035	1.256
10	0	-1.414	0.060	0.007	0.459	0.015	0.012	1.785	0.015	0.007	1.404
11	0	1.414	0.060	0.063	0.465	0.015	0.068	1.546	0.015	0.063	1.389
12	0	0	0.060	0.035	0.542	0.015	0.040	1.602	0.015	0.035	1.146
13	0	0	0.060	0.035	0.494	0.015	0.040	1.466	0.015	0.035	1.242
14	0	0	0.060	0.035	-0.227	0.015	0.040	2.134	0.015	0.035	0.388

Dehyrogenase enzyme activity Assay: Method described by Nweke *et al.*,[7,15] was adopted in determination of the bacterial dehydrogenase activity (DHA) inhibition by test metals. 2-iodophenyl, 3-nitrophenyl, 5-phenyl tetrazolium chloride (INT) was used as electron acceptor which was reduced to red color formazan. The intensity of the red coloration of the formazan formed was directly related to the viability of the cells in the chemical environment. X4- strength nutrient broth was prepared after which 0.5ml of the X4-strength nutrient broth and requisite volumes of sterile distilled water and stock solution of the respective metals were added to glass test tube. Into each tube 0.1ml each of INT and bacterial suspensions were added. The final volume of the solution in each tube was 2ml. Controls were prepared which consisted of the medium, test organism, INT and distilled water but without the chemicals. The cultures were incubated at room temperature (28±2^{0}C) for 24 h. After incubation, there was extraction of formazan produced with 4ml of butanol. Absorbance of the extract was determined spectrophotometrically at 540 nm wavelength.

Percentage inhibition determination: *Pseudomonas fluorescens* inhibition of dehydrogenase activity at varying concentrations of the metals as individual and binary mixtures were calculated using the equation below.

$$\textbf{Inhibition (\%)} = \frac{C_A - T_A}{C_A} \text{ X } 100 \quad \ldots\ldots\ldots\ldots \ (2)$$

C_A = The absorbance of Tri-phenyl formazan (TPF) extract in the control, T_A = The absorbance of Tri-phenyl formazan (TPF) extract in the test tubes

$$\textbf{Logit} = \ln\left(\frac{\text{Inhibition (\%)}}{100 - \text{Inhibition (\%)}}\right) \quad \ldots\ldots\ldots \ (3)$$

Toxicity threshold determination: The dose-response data from the assessment of toxic effects of the chemicals as singles and binary mixtures to dehydrogenase activity of *Pseudomonas fluorescens* were tested with 3-parameter logistic model to obtain their respective toxicity threshold (IC_{50}).

$$\textbf{Inhibition (\%)} = \frac{100}{1 + \left(\frac{x}{IC_{50}}\right)^{b}} \quad \ldots\ldots\ldots\ldots \ (3)$$

x = Concentration of the chemical, IC_{50} = Concentration that caused 50% inhibition, b = Parameter determining the relative slope at IC_{50}.

The mathematical relationships between the independent variables were approximated by the second order polynomial model.

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4}\sum_{j=1}^{4} \beta_{ij} x_i x_j + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \varepsilon$$

Where y is the predicted response, x_i and x_j are the independent variables, β_0, β_i, β_{ii} and β_{ij} are the model constant, linear coefficients, the quadratic coefficients and the cross-product coefficients respectively, ε denotes the random error.

RESULTS AND DISCUSSIONS

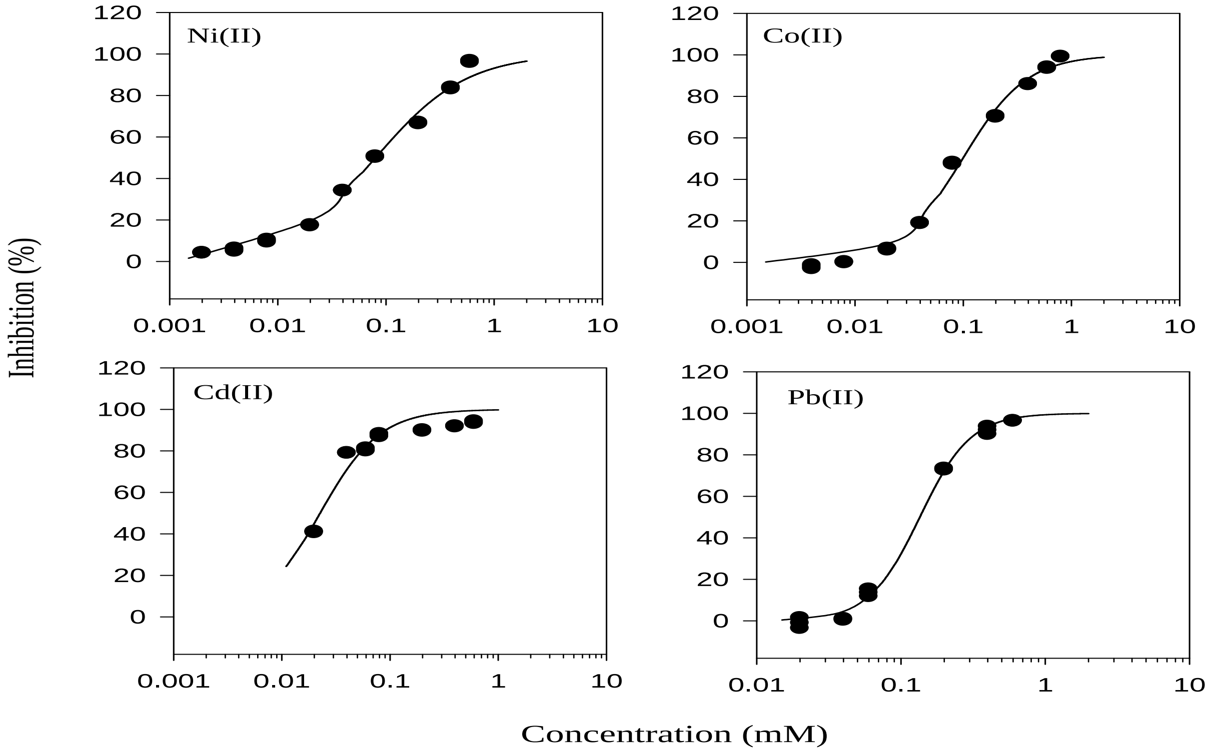

Figure 1: Effect of cadmium, nickel, cobalt & lead on dehydrogenase activity of *Pseudomonas fluorescens*.

Table 5: EC_{50} values for individual heavy metals (singles).

Chemicals	EC_{50} (mM)
Cd(II)	0.023 ± 0.003
Pb(II)	0.135 ± 0.007
Co(II)	0.099 ± 0.006
Ni(II)	0.080 ± 0.006

Table 6: Inhibition (%) and logit values for Cd+Co, Pb+Ni and Cd+Ni binary mixtures.

	PB+NI mixture		CD+CO mixture		CD+NI Mixture	
S/N	**% Inhibition**	**Logit**	**% Inhibition**	**Logit**	**% Inhibition**	**Logit**
1	45.871	-0.166	89.421	2.135	60.196	0.414
2	47.259	-0.110	84.631	1.706	66.810	0.700
3	71.409	0.915	94.810	2.905	67.912	0.750
4	76.822	1.198	93.014	2.589	73.791	1.035
5	67.106	0.712	89.621	2.156	71.219	0.906
6	71.131	0.902	87.625	1.957	66.687	0.694
7	32.408	-0.735	75.449	1.122	71.709	0.930
8	73.629	1.027	93.812	2.719	73.423	1.016
9	64.330	0.590	88.024	1.995	77.832	1.256
10	61.277	0.459	85.629	1.785	80.282	1.404
11	61.416	0.465	82.435	1.546	80.037	1.389
12	63.220	0.542	83.234	1.602	75.873	1.146
13	62.110	0.494	81.238	1.466	77.587	1.242

Table 7: Analysis of variance for; logit versus Cd(II) [mM], Co(II) [mM] binary mixture.

Source	**DF**	**F-Value**	**P-Value**
Model	5	40.40	0.000
Linear	2	20.92	0.000
Cd(II) [mM]	1	41.75	0.000
Co(II) [mM]	1	4.48	0.042
Square	2	32.11	0.000
Cd(II) [mM]*Cd(II) [mM]	1	42.25	0.000
Co(II) [mM]*Co(II) [mM]	1	27.89	0.000
2-Way Interaction	1	0.07	0.789
Cd(II) [mM]*Co(II) [mM]	1	0.07	0.789
Error	33		
Lack-of-Fit	3	9.52	0.000
Pure Error	30		
Total	38		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.215623	85.96%	83.83%	80.43%

Regression Equation in Uncoded Units

Logit = 11.01 - 1160 Cd(II) [mM] - 41.8 Co(II) [mM] + 37087 Cd(II) [mM]*Cd(II) [mM] + 630 Co(II) [mM]*Co(II) [mM] + 280 Cd(II) [mM]*Co(II) [mM]

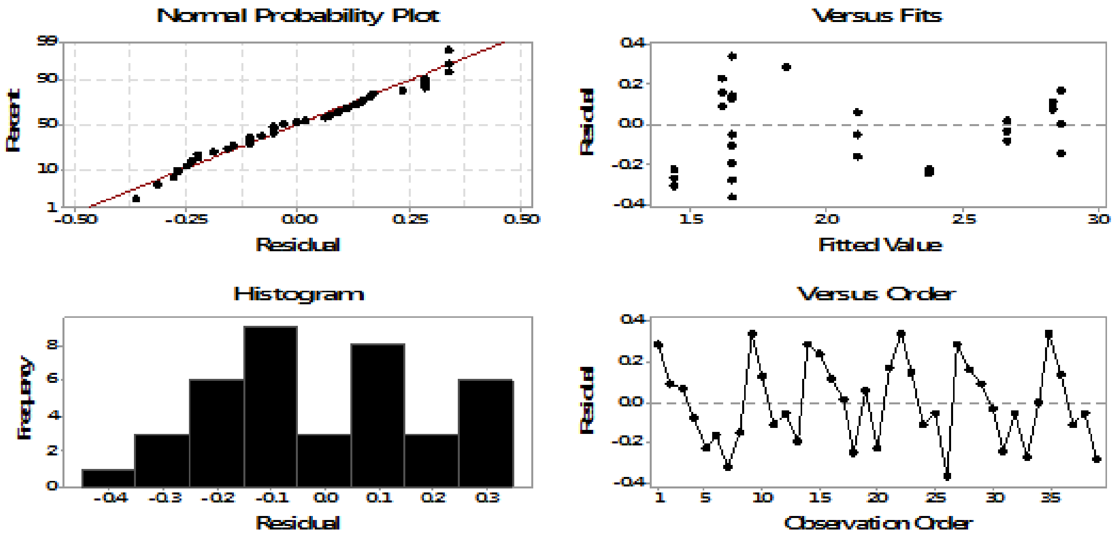

Figure 2: Residual plot for logit transformation of cadmium [mM] and cobalt [mM] binary mixture.

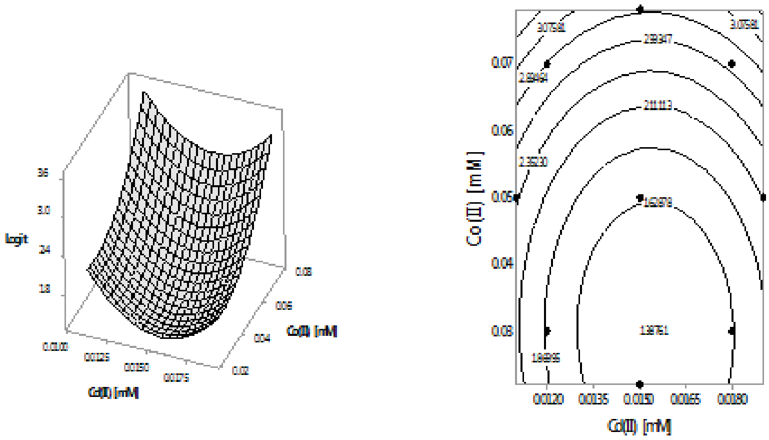

Figure 3: Surface and contour plot of logit versus cobalt [mM] and cadmium [mM].

Table 8: Analysis of variance for logit versus Pb(II) [mM], Ni(II) [mM] binary mixture.

Source	DF	F-Value	P-Value
Model	5	1241.71	0.000
Linear	2	148.18	0.000
Pb(II) [mM]	1	260.83	0.000
Ni(II) [mM]	1	16.10	0.000
Square	2	421.63	0.000
Pb(II) [mM]*Pb(II) [mM]	1	259.27	0.000
Ni(II) [mM]*Ni(II) [mM]	1	486.35	0.000
2-Way Interaction	1	26.23	0.000
Pb(II) [mM]*Ni(II) [mM]	1	26.23	0.000
Error	33		
Lack-of-Fit	3	2.53	0.076
Pure Error	30		
Total	38		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0418269	99.47%	99.39%	99.28%

Regression Equation in Uncoded Units

Logit = 20.70 - 723.8 Pb(II) [mM] + 29.84 Ni(II) [mM] + 5983 Pb(II) [mM]*Pb(II) [mM] - 512.1 Ni(II) [mM]*Ni(II) [mM] + 618 Pb(II) [mM]*Ni(II) [mM]

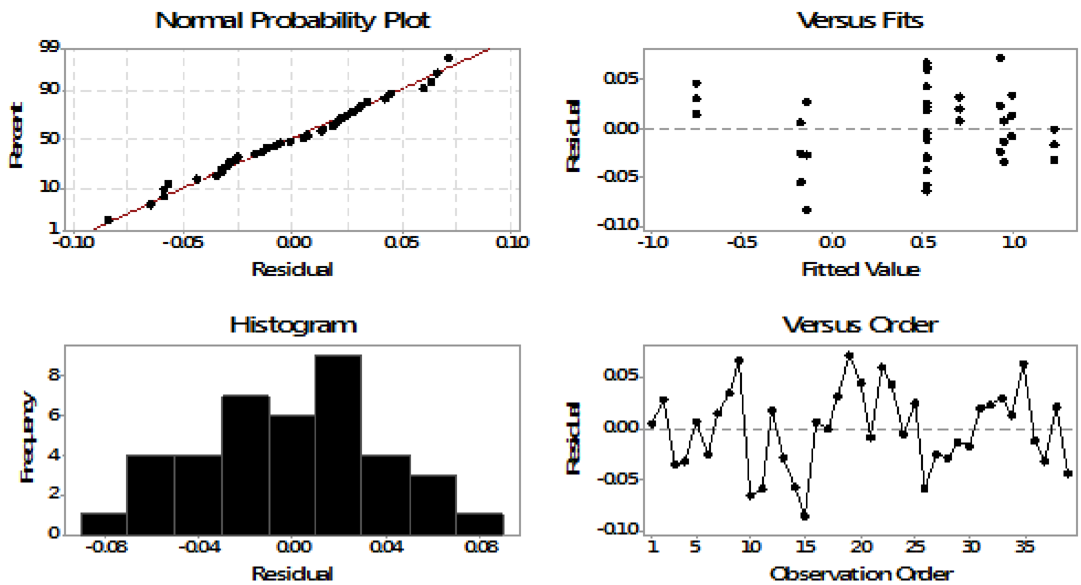

Figure 4: Residual plot for logit transformation of lead [mM] and nickel [mM] binary mixture.

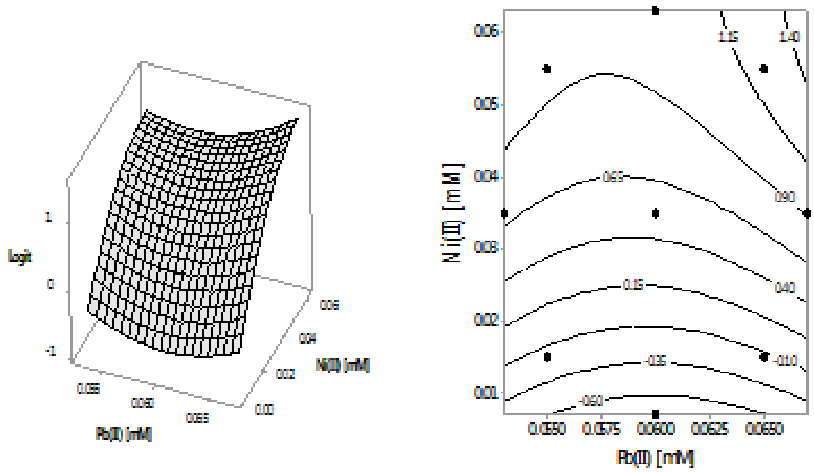

Figure 5: Surface and contour plot of logit versus lead [mM] and nickel [mM].

Table 9: Analysis of variance for logit versus Cd(II) [mM], Ni(II) [mM] binary mixture.

Source	DF	F-Value	P-Value
Model	5	35.64	0.000
Linear	2	59.03	0.000
Cd(II) [mM]	1	113.14	0.000
Ni(II) [mM]	1	20.64	0.000
Square	2	76.47	0.000
Cd(II) [mM]*Cd(II) [mM]	1	114.30	0.000
Ni(II) [mM]*Ni(II) [mM]	1	51.82	0.000
2-Way Interaction	1	0.41	0.528
Cd(II) [mM]*Ni(II) [mM]	1	0.41	0.528
Error	33		
Lack-of-Fit	3	23.65	0.000
Pure Error	30		
Total	38		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.118011	84.37%	82.01%	76.61%

Regression Equation in Uncoded Units

Logit = -7.362 + 1022.5 Cd(II) [mM] + 44.26 Ni(II) [mM] -33386 Cd(II) [mM]*Cd(II) [mM] - 470.3 Ni(II) [mM]*Ni(II) [mM] – 362 Cd(II) [mM]*Ni(II) [mM]

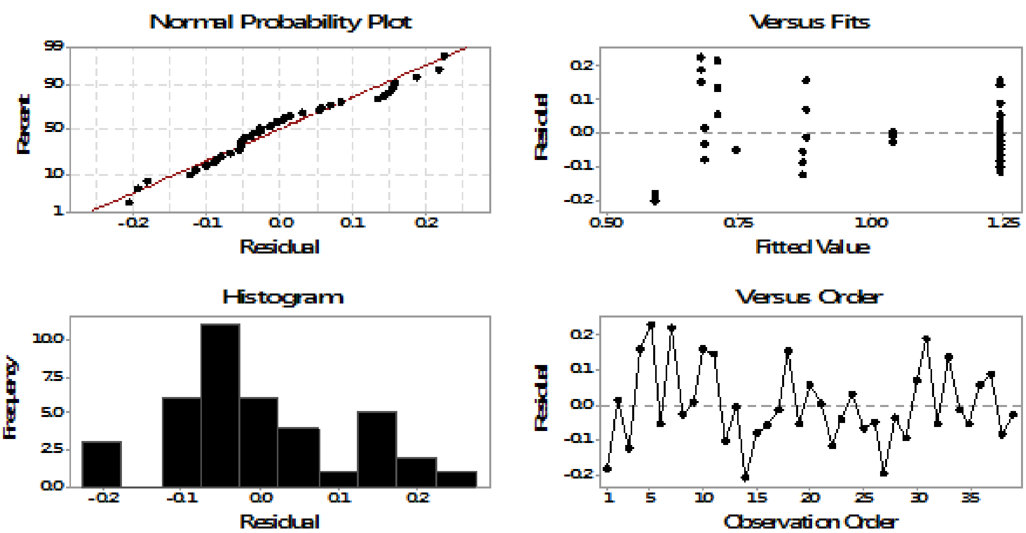

Figure 6: Residual plot for logit transformation of cadmium [mM] and nickel [mM] binary mixture.

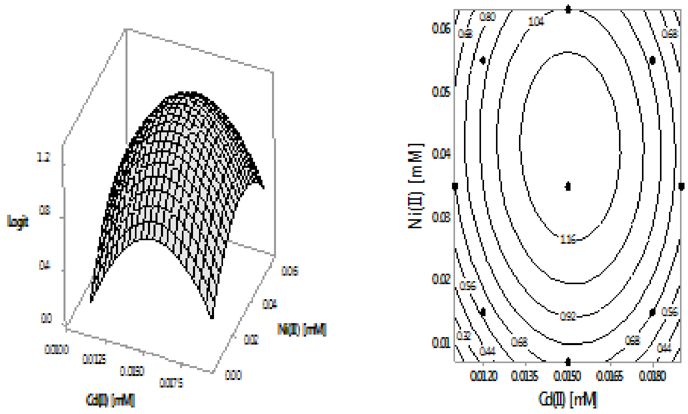

Figure 7: Surface and contour plots of logit versus cadmium [mM] and nickel [mM] binary mixture.

Table 10: Inhibition (%) and Absorbance values for Pb+Cd+Co, Pb+Cd+Ni Ternary mixtures.

	PB+CD+CO		PB+CD+NI	
S/N	**ABSORBANCE**	**% INHIBITION**	**ABSORBANCE**	**% INHIBITION**
1	0.343	54.206	0.344	54.072
2	0.301	59.813	0.330	55.941
3	0.34	54.606	0.400	46.595
4	0.278	62.883	0.273	63.551
5	0.280	62.617	0.476	36.449
6	0.239	68.091	0.399	46.729
7	0.163	78.238	0.475	36.582
8	0.275	63.284	0.175	76.636
9	0.364	51.402	0.421	43.792
10	0.266	64.486	0.243	67.557
11	0.319	57.410	0.359	52.070
12	0.310	58.611	0.404	46.061
13	0.309	58.745	0.401	46.462
14	0.210	71.963	0.258	65.554
15	0.270	63.952	0.352	53.004
16	0.256	65.821	0.359	52.070
17	0.262	65.020	0.350	53.271
18	0.258	65.554	0.359	52.070
19	0.254	66.088	0.340	54.606
20	0.278	62.884	0.351	53.138

Table 11: Analysis of variance for Inhibition (%) versus Pb + Cd + Co ternary mixture.

Source	**DF**	**F-Value**	**P-Value**
Model	9	19.35	0.000
Linear	3	17.81	0.000
Pb(II) [mM]	1	32.31	0.000
Cd(II) [mM]	1	17.72	0.000
Co(II) [mM]	1	18.17	0.000
Square	3	11.63	0.000
Pb(II) [mM]*Pb(II) [mM]	1	20.50	0.000
Cd(II) [mM]*Cd(II) [mM]	1	13.66	0.001
Co(II) [mM]*Co(II) [mM]	1	1.11	0.298
2-Way Interaction	3	9.70	0.000
Pb(II) [mM]*Cd(II) [mM]	1	8.99	0.004
Pb(II) [mM]*Co(II) [mM]	1	20.10	0.000
Cd(II) [mM]*Co(II) [mM]	1	0.01	0.920
Error	50		
Lack-of-Fit	5	41.84	0.000
Pure Error	45		
Total	59		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.24571	77.69%	73.68%	64.67%

Regression Equation in Uncoded Units

Inhibition (%) = -518.5 + 14940 Pb(II) [mM] + 12434 Cd(II) [mM] + 3735 Co(II) [mM] - 96539 Pb(II) [mM]*Pb(II) [mM] - 205320 Cd(II) [mM]*Cd(II) [mM] + 5091 Co(II) [mM]*Co(II) [mM] - 132399 Pb(II) [mM]*Cd(II) [mM] - 59413 Pb(II) [mM]*Co(II) [mM] + 2225 Cd(II) [mM]*Co(II) [mM

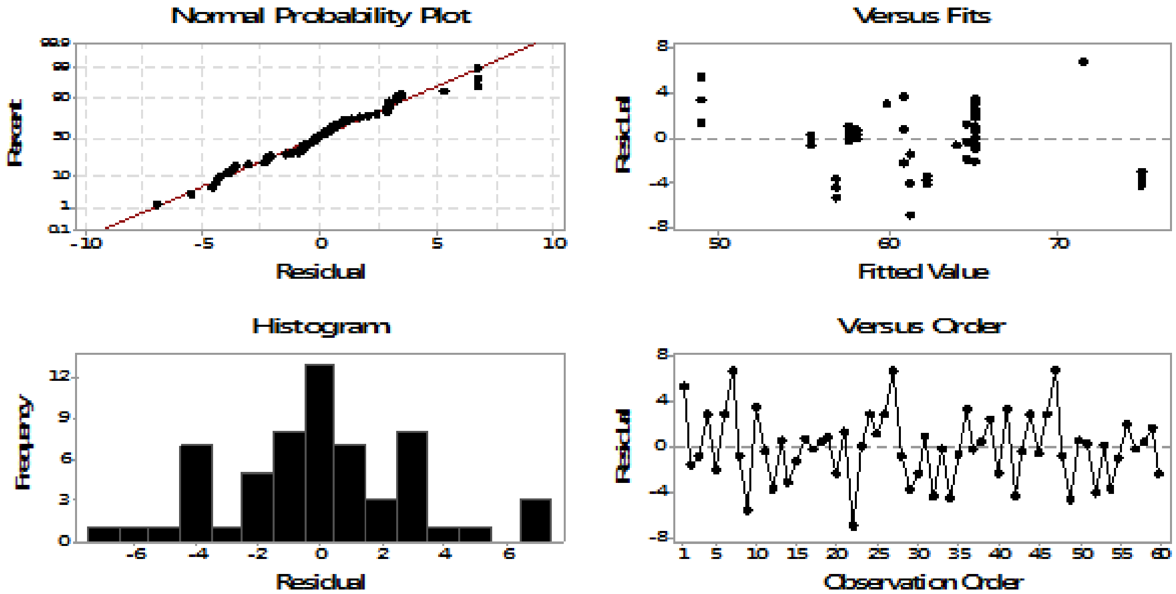

Figure 8: Residual plots for Inhibition (%) of Lead, Cadmium [mM] and Cobalt [mM] ternary mixture.

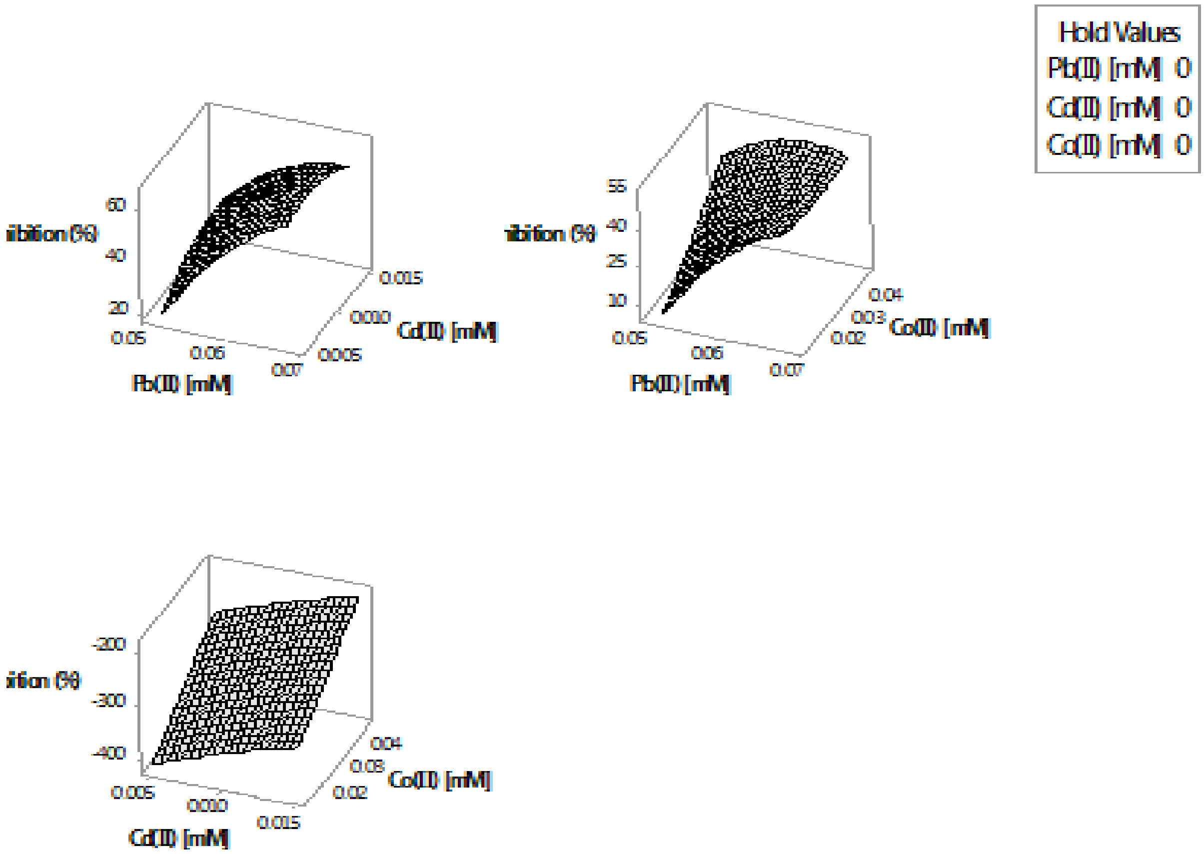

Figure 9: Surface plots of Inhibition (%) for Lead [mM], Cadmium [mM] and Cobalt [mM] ternary mixture.

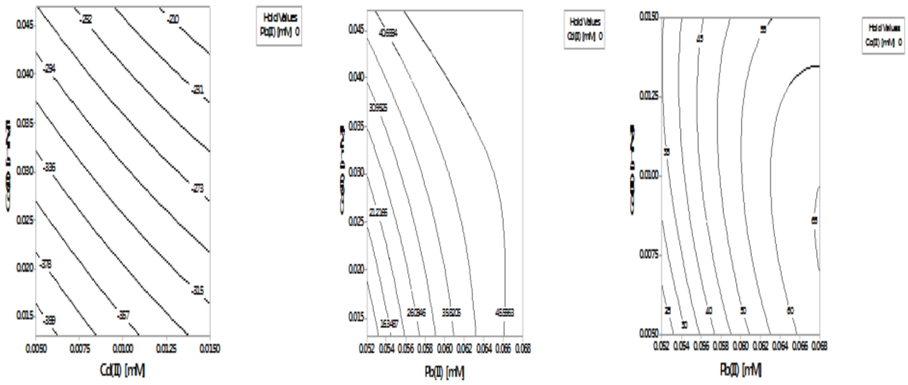

Figure 10: Contour plots of Inhibition (%) for Lead [mM], Cadmium [mM] and Cobalt [mM] ternary mixture.

Table 12: Analysis of variance for Inhibition (%) versus Pb + Cd + Ni ternary mixture.

Source	DF	F-Value	P-Value
Model	9	16.45	0.000
Linear	3	6.16	0.001
Pb(II) [mM]	1	2.46	0.123
Cd(II) [mM]	1	8.75	0.005
Ni(II) [mM]	1	11.25	0.002
Square	3	2.62	0.061
Pb(II) [mM]*Pb(II) [mM]	1	0.26	0.610
Cd(II) [mM]*Cd(II) [mM]	1	6.30	0.015
Ni(II) [mM]*Ni(II) [mM]	1	0.79	0.379
2-Way Interaction	3	9.71	0.000
Pb(II) [mM]*Cd(II) [mM]	1	16.52	0.000
Pb(II) [mM]*Ni(II) [mM]	1	10.17	0.002
Cd(II) [mM]*Ni(II) [mM]	1	2.43	0.125
Error	50		
Lack-of-Fit	5	29.23	0.000
Pure Error	45		
Total	59		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5.32017	74.75%	70.20%	59.14%

Regression Equation in Uncoded Units

Inhibition (%) = 412 - 6877 Pb(II) [mM] - 14946 Cd(II) [mM] - 10812 Ni(II) [mM] + 17900 Pb(II) [mM]*Pb(II) [mM] – 228131 Cd(II) [mM]*Cd(II) [mM] + 30938 Ni(II) [mM]*Ni(II) [mM] + 294281 Pb(II) [mM]*Cd(II) [mM] + 138518 Pb(II) [mM]*Ni(II) [mM] + 112928 Cd(II) [mM]*Ni(II) [mM]

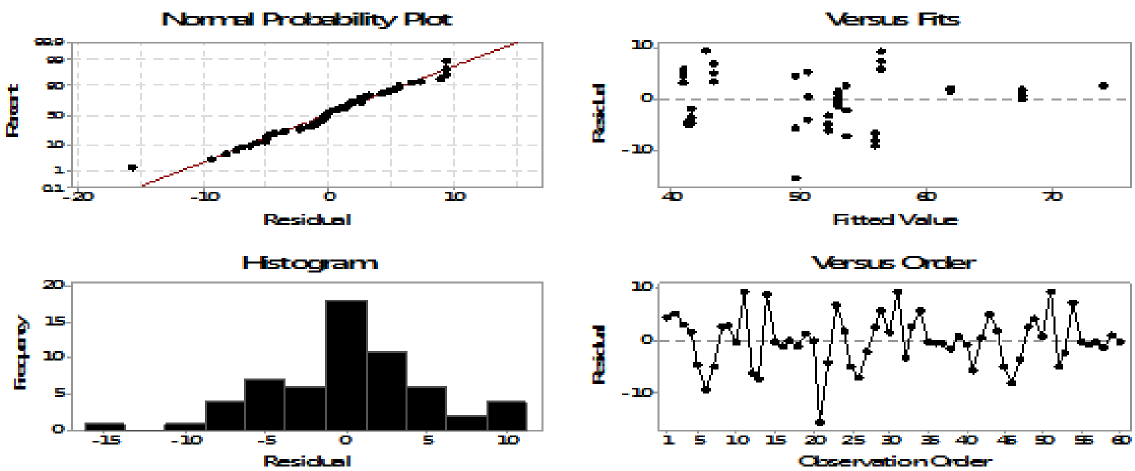

Figure 11: Residual plots for Inhibition (%) of Lead [mM], Cadmium [mM] and Nickel [mM] ternary mixture.

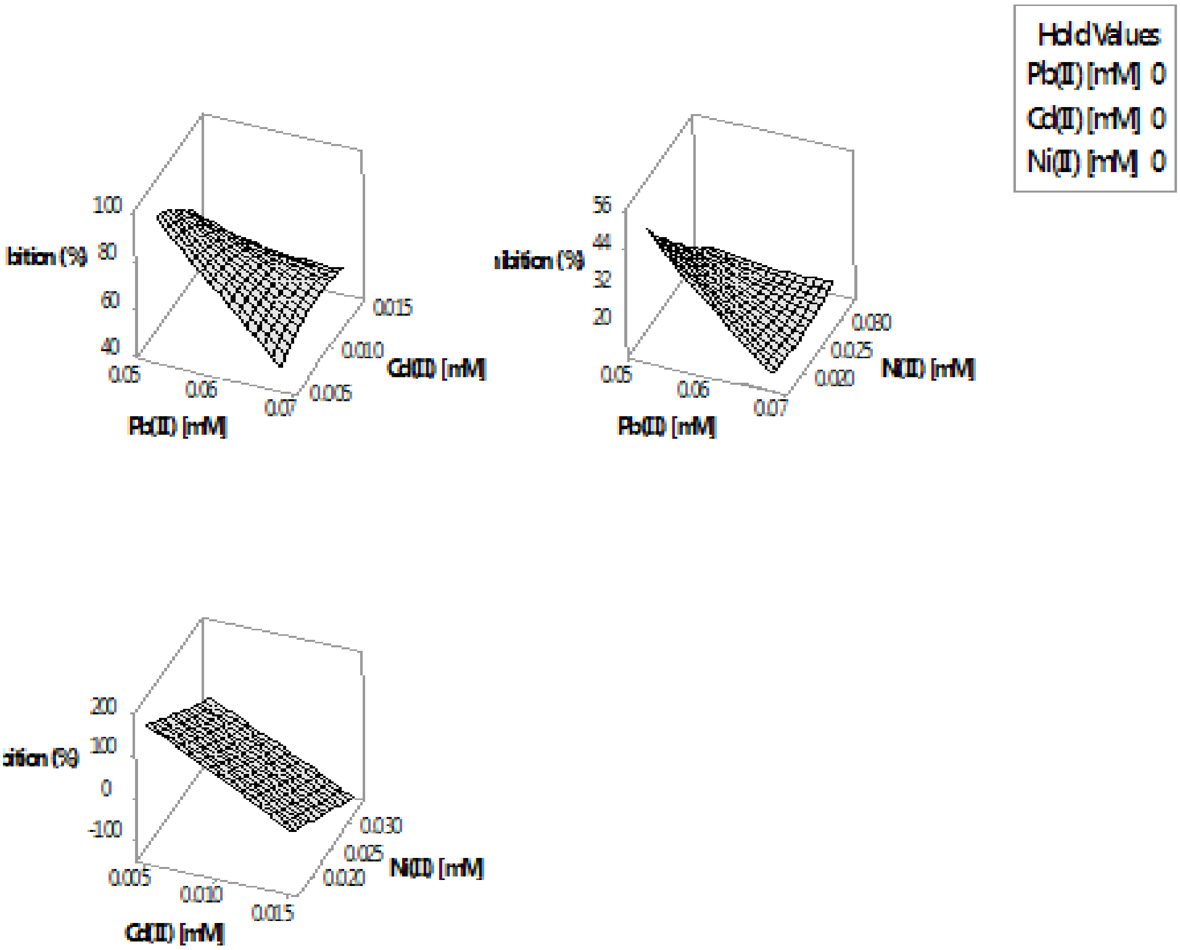

Figure 12: Surface plots of Inhibition (%) for Lead [mM], Cadmium [mM] and Nickel [mM] ternary mixture.

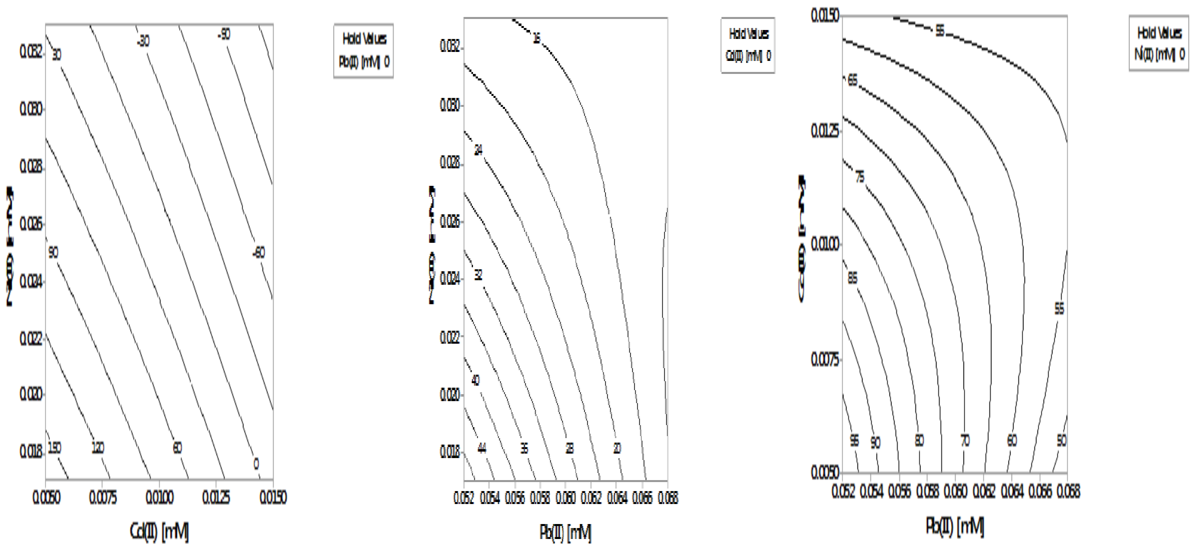

Figure 13: Contour plots of Inhibition (%) for Lead [mM], Cadmium [mM] and Nickel [mM] ternary mixture.

This study investigated the individual, binary and ternary mixture toxic effects of lead, cadmium, cobalt and nickel on the activity of the dehydrogenase enzyme of *Pseudomonas fluorescens*. Inhibiton of dehydrogenase enzymes by heavy metals have been reported.[9, 10,11] Response surface method was adopted for this toxicity assessment which is different from the uniform design method used by Nweke *et al.*,[7] Nweke *et al.*,[15] Cadmium showed to be most toxic during the assessment with lead being the least toxic which is just as reported.[13,15] Exposure of the test organism to individual metals showed progressive inhibition of dehydrogenase enzyme activity with increase in the concentration of the metals as seen in figure 1 that which has also been reported.[14,15] The toxicity of the single chemical is ranked as Cadmium > nickel >cobalt >lead having Cadmium to be the most toxic and Lead the least toxic among the metals that which is similar to pattern gotten by Nweke *et al*.[15] The more toxic nature of cadmium can be related to the fact that it has no physiological function. Lead was observed to be less toxic than trace elements nickel and cobalt that which could be attributed to lead tolerance shown by *Pseudomonas fluorescens* which has been reported.[9,10,11]

Sigma plot was used to statistically represent percentage inhibition against concentration of the individual metals which resulted into a sigmoid curve and also brought about the EC_{50} values as seen in table 1 which are not far from values gotten by other researchers.[12,15] Binary and ternary mixtures graphical plots and statistical analysis was done using minitab where the residual plots, surface plots and contour plots were generated. Statistical analysis done resulted in the generation of all statistical parameters as can be seen in table 7, table 8, table 9, table 11 and table 12. The surface plots and contour plots generated explained the relationship between the independent and the dependent variables. The statistical analysis brought about generation of linear regression equations and other parameters having good coefficient of determination R^2 which is similar to those by.[15] The coefficient of determination indicated the percentage of the variance in the dependent variable that the independent variable explains collectively.

Table 8 contains statistical analysis results of lead-nickel binary mixture which showed percentage R^2 value of 99.47% and percentage R^2 adjusted value of 99.39%. The quadratic regression model for lead-nickel binary mixture showed the value of determination coefficient (R^2) of 0.9947 which is similar to the R^2 of 0.9987 and R^2_{Adj} of 0.9925 gotten.[17] The observed determination coefficient of 0.9947 indicates that the calculated model was able to explain 99.47% of the results. The results also indicated that the model used to fit response variable was significant ($p < 0.0001$) and adequate to represent the relationship between the response and the independent variables. The significance of the model was also judged by F-test, which showed that model had a very high model F-value ($F = 1241.71$). R^2 $_{adj}$ (adjusted determination coefficient) is the correlation measure for testing the goodness-of-fit of the regression equation. The $R_{2\ adj}$ value of this model is 0.9939, which indicated only 0.61% of the total variations were not explained by the model this was close to result gotten.[17] The lead-nickel mixture showed a standard deviation of 0.0418 which shows that the data points tend to be very close to the mean. Figure 4 shows the residual plot for the lead-nickel binary mixture having no visible outliner and all data points appearing closer to the regression line. The surface and contour plots for the lead-nickel mixture are found in figure 5. Figure 5 shows a graphical representation of 3-dimensional surface plot and a 2-dimensional contour plot of lead-nickel binary mixture. It shows the simultaneous effect of lead and nickel on logit. From figure 5, increase in nickel concentration was seen to result to a continuous increase in logit. Increase in lead concentration brought about a slight decrease in logit until it got to a point after which further increase in lead concentration resulted to an increase in logit. The contour plots showed contour lines which were found to join points with the same response value.

Table 7 showed the end results of the cadmium-cobalt analysis having a standard deviation of 0.216 and coefficient of determination R^2 percentage of 85.96% which indicated that the model was able to explain 85.96% of the results and having 14.04% of the total variation not explained by the model. The adjusted determination coefficient percentage R^2_{Adj} was 83.83% with an F-value of 40.40 and a relatively low P-value ($P<0.0001$). Figure 2 shows the residual plot of the cadmium-cobalt analysis where inhibition % was plotted against the residuals resulting to data points closer to the regression line generated which indicated a good fit for the linear model. Figure 3 shows the surface and contour plots for the cadmium-cobalt binary mixture. It showed the effect of cadmium and cobalt on logit where it was observed that logit continuously increased with an increase in cobalt concentration. On the other hand, there was a decrease in logit with an initial rise in cadmium concentration until it got to a point after which further increase in cadmium concentration resulted to an increase in logit. The contour plot was just a 2-dimensional representation of what is seen in the surface plot with the presence of contour lines.

The cadmium-nickel binary mixture had statistical parameters duely fitted in table 9 with standard deviation of 0.118 and percentage R^2 of 84.37% with percentage adjusted determination coefficient R^2_{Adj} of 82.01%. The contour and surface plots are seen in figure 7 where the surface plot showed that an initial increase in nickel concentration brought about an increase in logit after which further increase in nickel concentration resulted to a decrease in logit. On the other hand, cadmium concentration increase brought about an increase in logit until further increase in cadmium concentration resulted to decrease in logit.

Table 11contains all statistical results of the lead-cadmium-cobalt ternary mixture analysis. The statistical analysis gave rise to percentage coefficient of determination (R^2) of 77.69% and having the percentage adjusted determination coefficient (R^2_{Adj}) of 73.68%. The analyzed model showed a standard deviation of 3.246 and a p-value of $p< 0.0001$. The residual plot is seen in figure 8 with regression line having data points closely arranged around the regression line. Three (3) surface plots were generated as seen in figure 9. Because the surface plot shows only two (2) continuous variables at a time, Any extra variables are held at a constant level that which is clearly seen in the case of the lead-cadmium-cobalt ternary mixture surface plots. The first surface plot showed the effect of lead and cadmium on percentage inhibition while holding cobalt at a constant value of zero. The plot showed that increase in lead concentration resulted to an increase in percentage inhibition that which was also the case with regards cadmium effect. The second plot had cadmium held at zero value will the effect of lead and cobalt on percentage inhibition was observed. This second plot showed that percentage inhibition increased with an increase in lead concentration until further increase in lead concentration brought about a decrease in percentage inhibition. On the other hand, continuous increase in cobalt concentration resulted in a continuous increase in inhibition percentage. The third surface plot had lead held at a constant zero value. In this plot, continuous increase in cadmium and cobalt concentration resulted to an increase in percentage inhibition. The contour plots are as displayed in figure 10. The contour plots showed contour lines connecting points with same response value. The contour plots generated showed only two variables with the third being held at zero value.

Figure 12 shows surface plots for the cadmium-lead-nickel ternary mixture with resulting (3) surface plots. The first surface plot had nickel held at zero value. The first plot showed that an increase in lead concentration resulted to a decrease in percentage inhibition while on the other hand, An increase in cadmium concentration resulted into a slight increase in percentage inhibition. The second plot has cadmium held at a constant value of zero thus having percentage inhibition decreased with increase in lead concentration while increase in nickel concentration showed no visible increase in percentage inhibition. Lead was held at a zero constant value in the third plot. This third plot showed that an increase in cadmium concentration resulted to a corresponding decrease in percentage inhibition while increasing nickel concentration resulted to a decrease in inhibition percentage. The contour plots are as seen in figure 13. There are three (3) generated contour plots with each containing only two continuous variables at a time while the third variable was held at a zero constant value. The contour plots are just a 2-dimensional representation of what is on the surface plots with contour lines that connects points with the same response value.

CONCLUSION

This research was aimed at investigating toxicity of heavy metals on dehydrogenase enzyme of *Pseudomonas fluorescens* using response surface method. These heavy metals effectively inhibited the activity of the dehydrogenase enzyme as individual toxicants and in mixtures of which cadmium and Lead were the most toxic and the least toxic respectively after individual toxicity assessment of these metals. Analytical results generated from this study will tend to provide good knowledge of the effect of heavy metals in the likes of cadmium, cobalt, lead and nickel on inhibition of dehydrogenase activity. Understanding the basis of mixture toxicity was also made possible by this study since studying the combined action of heavy metals is becoming increasingly important in various branches of biomedical research of which toxicology is not left out.

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