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Lead Ion Detection in Challawa Water: Development and Application of Response Surface Methodology

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ABSTRACT

Agricultural areas in Nigeria depend upon unpolluted water sources and hence need continuous monitoring. River Challawa is among the water sources for domestic and agricultural purposes. It is located in an industrialized area of Kano, Nigeria. A cucumber protease-based assay for the detection of lead metal ion has been developed to determine the efficiency of the monitoring method. Cucumber protease is assayed by using casein as a substrate with Coomassie brilliant blue to track the complete hydrolysis of the substrate. In the presence of inhibitors such as metal ions, casein is not hydrolyzed to completion, and the solution is dark blue. The cucumber protease assay was optimized using One-Factor-at-a-Time (OFAT) and Response Surface Methodology (RSM). The RSM-optimized method demonstrated superior sensitivity, reducing the IC_{50} value for lead ion detection from 0.078 mg/L (OFAT) to 0.028 mg/L. The findings show a marked improvement in concentration, causing 50% inhibition (IC_{50}) for lead metal ion in River Challawa. The results of the comparison between one factor at a time (OFAT) optimization and RSM provide an improvement of IC_{50} for lead ion from 0.078 (95% CI,0.057 to 0.27) to 0.028 (95% CI, 0.022 to 0.037). The LOD for lead Pb^{2+} is 0.015 and 0.003 for OFAT and RSM, respectively. The results of this study indicate that cucumber-protease assay has the potential efficiency of being used in the determination of lead metal in water samples.

INTRODUCTION

Water, second only to air in importance, is vital for human survival, yet its quality is increasingly compromised by pollution [1]. In Nigeria, rivers which is a key sources of domestic water are heavily polluted by industrial waste and agricultural runoff [2]. Unlike organic pollutants, heavy metals are non-degradable and persist in ecosystems, bio-accumulating through the food chain and posing severe health risks, including DNA damage and carcinogenesis [3]. Human activities have significantly disrupted the natural balance of these metals, amplifying their toxicity. Rapid detection and monitoring of heavy metals are crucial, given their potential to contaminate water through various pathways, including industrial discharge and acidic rain [4]. While traditional analytical methods like atomic absorption spectroscopy offer accuracy, they require complex instrumentation, making them less suitable for on-site monitoring. Lead is widely used in a number of industries, which include battery manufacturing, construction, and pigments. Despite its usefulness since thousands of years, lead is in fact an environmental poison that pose serious consequences for the environment and public health. It is documented that civilians in the low- and middle-income countries are found to be likely exposed to lead as a result of pollution, contaminated consumer goods, water, soil, and air [5].

Even at low levels, lead has an effect on the neurological, renal, and reproductive systems, interfering with their normal function [6–8]. The use of leaded gasoline and paints, which previously contaminated soil and air, continues to pollute the environment on a global scale. Soil lead contamination in agricultural areas is primarily caused by phosphate fertilizers [9]. Plants can bioaccumulate lead, posing a threat to ecosystems and food safety. Lead poisoning remains a major issue in the world

today, particularly among children and those working in hazardous occupations. Long-term strategies for removing lead from the environment and detoxifying affected areas are currently being investigated [8,10,11]. Lead detection using a marriage between instruments and bioassay is increasingly being carried out due to cost and time issues [12,13].

The toxic effects of polluted water can lead to severe health issues, including organ damage, gastrointestinal disorders, and neurological impairments. Studies in Nigeria have confirmed elevated heavy metal levels in water sources, agricultural soils, and food products, often exceeding safe limits. This underscores the urgent need for improved water management policies and pollution control measures to safeguard public health and the environment [14]. In analytical chemistry, the optimization process is commonly carried out using the one-factor-at-a-time (OFAT) method, where only one parameter is adjusted while the others remain constant [15]. However, this method has notable drawbacks, including its inability to capture interactions between variables, which results in an incomplete understanding of their combined effects [16]. Additionally, OFAT requires a large number of experiments, leading to increased time, costs, and higher consumption of reagents and materials [17]. To overcome these limitations, response surface methodology (RSM) has become a valuable alternative.

RSM is a multivariate statistical technique that uses experimental data to fit a polynomial equation, enabling the simultaneous evaluation of multiple factors and their interactions [18]. This approach efficiently predicts efficiency of the optimal conditions and has been widely used in analytical chemistry and biochemistry to improve analyte detection in samples. Significantly, the application of RSM to enhance the efficiency and sensitivity of toxic metal detection in protease-based inhibitive assays has not been extensible explored before. This study attempts to apply RSM in this context, aiming to optimize the assay's efficiency, performance and sensitivity.

MATERIALS AND METHODS

Preparation of buffers

Citrate phosphate buffer from pH 5.0 to 6.0, sodium phosphate buffer from pH 6.0 to pH 7.5 and Tris buffer from pH 7.2 to pH 8.0. were prepared according to the methods of [19]. The final pH minor adjustment was carried out using NaOH (1 M) or HCl (1 M).

Preparation of dye-binding Bradford reagent

Approximately 0.1 g of Coomassie Brilliant Blue G-250 (Solarbio, China) was dissolved in a solution containing 50 mL of 95% ethanol and 100 mL of 85% phosphoric acid. The mixture was stirred overnight to ensure complete dissolution of the dye, and the final volume was adjusted to 1 L with distilled water. The resulting solution was then filtered using Whatman Filter Paper No. 1 and stored in a dark bottle to prevent degradation. Alternatively, a commercially available Bradford reagent (Solarbio, Beijing Solarbio Science & Technology Co., Ltd., China) was used following the manufacturer's instructions.

Preparation of Enzyme substrate

Two grams of casein (SORLARBIO) were dissolved in 100 mL of deionized water, and the pH was adjusted to 8.0. The mixture was stirred overnight at 60°C. The resulting casein stock solution was centrifuged at 10,000×g for 15 minutes. The protein concentration in the clear supernatant was determined using the Bradford dye-binding assay, with crystalline BSA (SORLARBIO) as the standard. Fresh working solutions of casein (0.3 mg mL^{-1}) were prepared daily.

Sample preparation

Cucumis sativus was purchased from the local market in Dutse, Jigawa state, Nigeria. *Cucumis sativus* was washed, cut into pieces using a knife and blended with a mechanical blender model 002 Panasonic. The juice obtained was screened for high protease activity prior to inhibitive studies. The juice was stored in plastic container and kept refrigerated until use.

Preparation of heavy metals solutions

Fresh working solutions of heavy metals, including mercury (II), arsenic (V), cadmium (II), lead (II), copper (II), cobalt (II), iron (II), chromium (VI), and silver (I), were prepared at concentrations ranging from 0.1 to 10 mg/L. These solutions were sourced from MERCK (Merck, Darmstadt, Germany) Atomic Absorption Spectrometry standards and stored in acid-washed polypropylene containers.

Optimization of Cucumber protease activity

The study investigated different concentrations of cucumber protease and casein incubated at room temperature. The casein working solution was mixed with the Bradford dye-binding reagent and incubated before measuring the absorbance at 595 nm. For optimization studies, the concentrations of casein and protease were maintained at 0.1 mg/L, while temperature and pH were optimized at 0.3 mg/L and 0.1 mg/L, respectively. One unit of protease activity was defined as the amount of casein hydrolyzed per minute.

RSM optimization

Central Composite Design (CCD) Experiments

The central composite design (CCD) was used to optimize three important experimental parameters namely; time of enzyme-substrate incubation, casein concentration, and cucumber protease concentration. A $2(^3)$ factorial CCD was used which gave a total of 20 experimental runs, which included factorial points, axial points, and center points to increase the precision and robustness of the model. These runs were systematically designed to optimize the detection of metal ions at a concentration of 0.035 mg/L to get precise and reproducible results. The response variable was the difference in absorbance values from the Bradford dye-binding assay which was used as an indirect measurement of protein hydrolysis.

Absorbance was measured at 595 nm after the reaction was carried out for 20 min at 30°C. The objective was to determine the optimal conditions that would result in the highest absorbance difference, indicating optimum proteolytic activity and optimal casein hydrolysis. In order to increase the reliability of the optimization process, all the experimental runs were carried out in triplicate and statistical analysis was done using response surface methodology (RSM). To check the significance of individual factors and their interactions, analysis of variance (ANOVA) was used for the model fitness. The optimized conditions were also verified by performing further confirmatory experiments.

Assay for heavy metals

Briefly, the assay was performed using a reaction mixture consisting of enzyme, heavy metal ions and deionized water. The mixture was left to stand at room temperature for 20 minutes in order to allow the metal ions to react with the enzyme. Afterwards, 30 µL of casein and Bradford dye-binding reagent were added to initiate the proteolytic reaction. The initial absorbance (time zero absorbance) was also taken at 595 nm,

after another 5 min incubation. In order to determine the level of proteolysis, the reaction was carried out for 60 minutes and the remaining mixture was processed to measure the final absorbance.vIndirect measure of enzyme activity and protein hydrolysis was by the difference between initial and final absorbance values under different metal ion concentrations. LOD and IC_{50} values (the concentration of metal ions that inhibits 50% of enzyme activity) were determined using non-linear regression analysis software, PRISM. All experiments were done in triplicate and the data were presented as means ± SE to ensure accuracy and reproducibility. The results helped in the understanding of the inhibitory effects of heavy metals on enzymatic activity to improve the detection and bioremediation strategies.

RESULTS AND DISCUSSION

The inability of the Bradford reagent to stain polypeptides with molecular weights smaller than 2 kDa is used as a principle in a protein assay with casein as a substrate. This property enables the distinction between hydrolyzed and intact casein in enzymatic reactions. When casein is completely hydrolyzed, the Bradford reagent is added to the reaction mixture, which remains brown as no intact protein is present. However, the solution turns blue when undigested casein is present because larger protein fragments can be complex with the Coomassie dye. Some other protein quantification methods are available, but these include the Biuret-Lowry assay, the Folin-Ciocalteu assay, and the BCA assay [20,21], and these methods are based on the copper-biuret reaction. This reaction employs copper ions as the main reagent, which can lead to the incorporation of unwanted impurities and thereby increase the complexity of the assay.

The Bradford assay is relatively newer than the copper-based methods and has several advantages, including a faster, simpler and more sensitive method. In addition, it gives a more direct and rapid determination of protein concentration without waiting for prolonged incubation or using more than one reagent. These features make the Bradford dye-binding assay suitable for many enzymatic studies and protein quantification experiments.

Recent progress in testing for metals using the Shukor inhibitory enzyme assay has emphasized the use of protease-based tests as a sensitive and cost-efficient alternative to traditional analytical methods. These assays function on the principle that heavy metals interact with enzyme active sites, leading to a measurable reduction in enzymatic activity. Among the proteases studied for this application, papain—a cysteine protease derived from the papaya plant—has been recognized as a viable option for detecting harmful metals in environmental samples [22].

One significant advancement in this area involves the casein hydrolysis-driven bromelain assay. In this method, casein serves as the substrate, and its enzymatic breakdown generates peptide fragments that react with Coomassie dye to produce a color-based response. Heavy metals such as mercury, cadmium, and lead inhibit the proteolytic activity of bromelain, resulting in a decrease in color intensity that can be accurately measured. This method offers several advantages, including simplicity, affordability, and suitability for on-site environmental monitoring due to its practicality. A major benefit of utilizing proteases such as bromelain in enzyme inhibition assays is their high sensitivity to metal ions, allowing for the detection of heavy metals even at minimal concentrations.

This provides a cost-effective alternative to conventional instrumental techniques such as atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS). While AAS and ICP-MS are known for their precision, they require expensive equipment and highly trained personnel for operation [23–27]. Recent studies have focused on optimizing assay conditions to enhance accuracy and precision. Factors such as pH, temperature, enzyme concentration, and incubation time have been systematically analyzed to improve assay performance.

The application of the Shukor inhibitory enzyme assay in real-world scenarios has yielded promising results. It has been tested on water samples from industrial effluents and polluted river systems, demonstrating its effectiveness in detecting significant levels of heavy metals [28]. Future advancements may involve integrating this assay into biosensor devices, enabling real-time metal contamination monitoring. Overall, the use of protease-based inhibition assays, particularly involving bromelain, represents a significant advancement in environmental monitoring. It offers a practical, reliable, and user-friendly method for assessing heavy metal pollution across different environments.

Optimization Using OFAT

The Cucumber-protease assay is an inhibitive assay used to assess enzyme activity in the presence of heavy metals. Under normal conditions without lead or mercury Cucumber protease hydrolyzes the substrate casein into oligopeptides smaller than 2 kDa. These smaller fragments do not react with the Bradford reagent, resulting in a brown-colored solution.

However, when mercury is present, it inhibits Cucumber protease activity, preventing casein hydrolysis. The intact casein then reacts with the Bradford reagent, producing an intense blue solution. Optimization of Cucumber protease activity using the One-Factor-at-a-Time (OFAT) method determined the following optimal conditions:

1. Enzyme concentration: 0.35 mg/mL cucumber protease (**Fig. 1**)
2. Substrate concentration: 1.0 mg/mL casein (**Fig. 2**)
3. Temperature: 40 °C (**Fig. 3**)

For comparison, the papain inhibitive assay for mercury was optimized at 0.1 mg/mL for both papain and casein, while the bromelain assay was optimized at 0.11 mg/mL bromelain and 0.25 mg/mL casein. The inhibition of Cucumber protease is intrinsically linked to its active site, as it belongs to the cysteine protease family. This enzyme relies on the sulfhydryl (SH) group within its active site for proper functionality.

Without toxic metals, the enzyme efficiently hydrolyzes casein, producing a light brownish solution when treated with the Bradford reagent. However, when lead is present, it binds to the sulfhydryl group of the active site, disrupting the enzyme's functionality. This interaction induces conformational changes that prevent casein from binding to the enzyme, thus inhibiting hydrolysis. As a result, the unhydrolyzed casein remains intact and reacts with the dye-binding reagent, producing a dark-blue coloration.
[22,29].

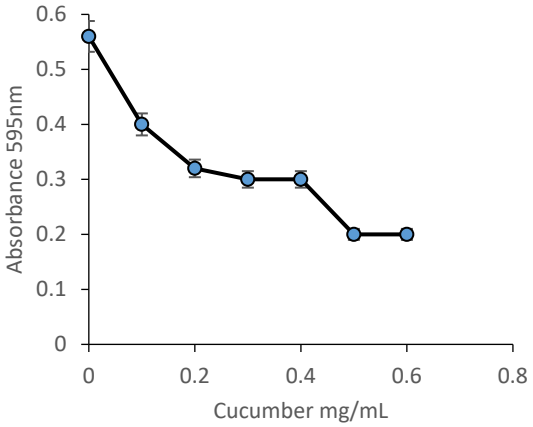

Fig. 1. Optimization of the cucumber concentration on the cucumber dye-binding assay. Error bars represent mean ±standard deviation (n = 3).

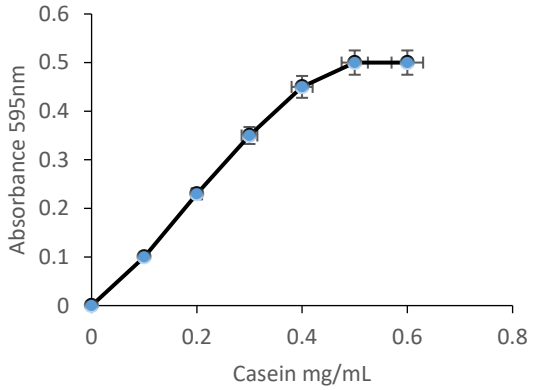

Fig. 2. Optimization of the casein concentration on the cucumber dye-binding assay. Error bars represent mean ± standard deviation (n = 3).

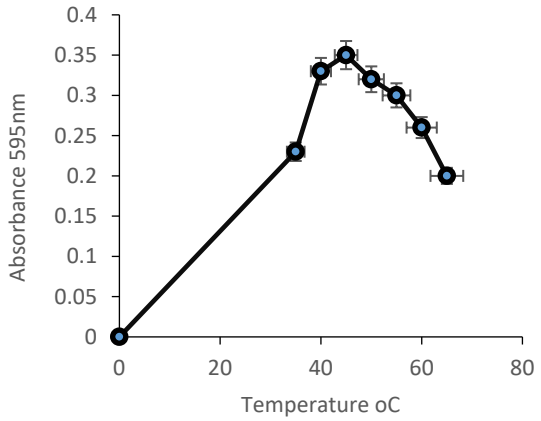

Fig. 3. Optimization of temperature for cucumber-dye binding assay. Data is Mean ± Standard Error of the Mean (n =3).

Optimization by Response Surface Methodology (RSM)

The central composite design (CCD) was employed to study the optimum concentration of the factors (**Table 1**). Twenty experiments were designed by the Design expert software 6.0 with six replicates of midpoints, which are useful to determine the experimental error (**Table 2**).

Table 1. Coded and actual values of significant factors used in central composite design (CCD).

Fig.	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded
A	Cucumber conc.	Mg/ml	Numeric	0.1	0.6	-1	+1
B	Casein Conc.	Mg/ml	numeric	1.0	3.0	-1	+1
C	Temperature	Degree	Numeric	35	60	-1	+1

Table 2. CCD experimental matrix generated by the Design expert software and the corresponding responses (actual and predicted).

Run	A	B	C	Actual absorbance 595nm	Predicted absorbance 595nm
1	0.35	1.75	47.50	0.39	0.41
2	0.10	1.50	35.00	0.30	0.33
3	0.35	3.05	47.50	0.80	0.73
4	0.35	4.00	35.00	0.52	0.54
5	0.35	2.75	47.50	0.55	0.54
6	0.10	1.50	60.00	0.20	0.15
7	0.35	1.75	47.50	0.75	0.89
8	0.35	1.75	47.50	0.45	0.38
9	0.60	1.50	60.00	0.42	0.40
10	0.60	1.50	35.00	0.50	0.49
11	0.60	3.75	47.50	0.81	0.83
12	0.10	1.00	35.00	0.78	0.81
13	0.35	1.75	60.00	0.30	0.33
14	0.10	3.75	47.50	0.080	0.10
15	0.35	1.75	47.50	0.88	0.78
16	0.10	1.50	35.00	0.40	0.44
17	0.60	2.50	35.00	0.100	0.074
18	0.60	1.50	35.00	0.100	0.13
19	0.60	3.00	60.00	0.25	0.22
20	0.35	1.00	47.50	0.14	0.16

The regression equation and the coefficient of determination (R^2) assessed the model's fitness. The model demonstrated high significance, as indicated by an F-value of 24.94, suggesting that the probability of this high F-value arising due to random noise is only 0.01%. Additionally, the model exhibited a very low p-value ($p < 0.0001$), confirming its statistical significance. The lack-of-fit test yielded an F-value of 1.9, indicating that the model adequately represents the experimental data.

An insignificant lack of fit suggests that observational errors are neither systematic nor directed, further supporting the model's reliability [30,31]. Additionally, the "Predicted R-squared" (0.9901**)** showed good agreement with the "Adjusted R-squared" (0.9891**),** further confirming the model's reliability.

Table 3. Analysis of variance (ANOVA) for Cucumber protease activity assay efficiency by CCD.

Source model	Sum Squares	of DF	Mean Square	F Value	Prob> F	
	1.76	20	0.088	24.94	<0.0001	Significant
A	0.16	1	0.16	46.30	<0.0001	
B	0.50	1	0.50	141.06	<0.0001	
C	1.07	1	1.07	0.30	0.0004	
A^2	0.046	1	0.46	13.05	0.0001	
B^2	0.027	1	0.027	7.51	0.0004	
C^2	0.038	1	0.038	10.76	0.0007	
AB	0.029	1	0.029	8.12	0.0080	
AC	0.20	1	0.20	57.53	<0.0001	
BC	0.082	1	0.082	23.36	<0.0001	
Residual	0.10	29	3.532E-003			
Lack of fit	0.088	22	3.987E-003	1.9	0.1959	not significant
Pure Error	0.015	7	2.104E-003			
Cor Total	1.86	19				

The investigation's low coefficient of variation (CV) of 4.3% underscores the reliability and consistency of the experimental results, signifying a high level of dependability. Regression analysis identified several significant model terms, including A, B, C, A^2, B^2, C^2, AB, AC, and BC, as detailed in **Table 3**. These findings highlight the substantial influence of all three experimental factors (cucumber concentration, casein concentration, and temperature) on the research outcomes, affirming their importance in shaping the efficiency of the assay. The 3D response plots (**Figs. 5 – 7**) show the response for each pair of components while holding the third variable constant. The curve of the contour lines in these maps offers information about factor interactions. Curved contour lines denote factor interactions; saddle-shaped plots suggest significant interactions, and circular contour plots imply non-significant interactions. Interaction efficiency of the assay may also be quantitatively analysed by looking at the numerical values in the coded equation. A big numerical value, either positive or negative, indicates a strong interaction between elements [32].

Effeciency =+0.43+0.21* A -0.21* B-0.037* C+0.062* A2-8.673E004* B2-0.032* C2+0.074* A*B+0.14* A * C+0.19* B * C

Final Equation in Terms of Actual Factors:

Effeciency=+2.70522-2.50656 * Conc. of Cucumber-1.02208* Casein conc.-0.028740*Temperature+0.98456* Conc. of Cucumber2-8.67325E-004 * Casein conc.2-2.05804E-004 * Temperature2+0.29800* Conc. of Cucumber * Casein conc.+0.043360 * Conc. of Cucumber * Temperature+0.015060* Casein conc. * Temperature

Perturbation plots, which are 2D representations of factor combinations, provide valuable insights into interactions between variables **Fig. 8**. Notable interactions were observed, particularly between cucumber and temperature, as well as between casein and cucumber concentrations. These interactions are evidenced by variations in the outcome of one factor depending on the levels of another, highlighting the interconnected influence of these factors on the response [32]. In this study, the interaction between cucumber concentration and casein concentration was found to be synergistic while between Temperature and cucumber concentration and casein concentration appears to be antagonistic. This is evident from the perturbation plots, where the lines representing these interactions do not cross and crossed at each other respectively [33].

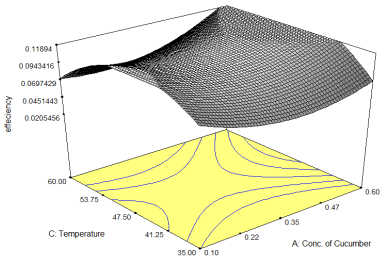

Fig. 5. 3D surface response view showing the response when temperature and concentration of cocumber were varied.

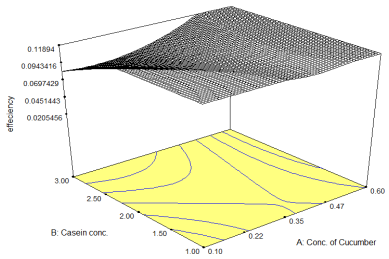

Fig. 6. 3D surface response view showing the response when concentration of cocumber and casein concentrations were varied.

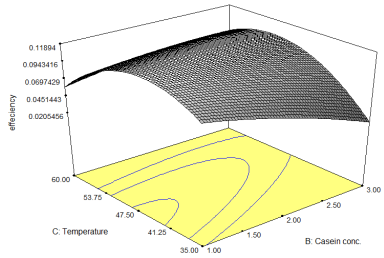

Fig. 7. 3D surface response view showing the response when temperature and casein concentrations were varied.

The suitability of the data for the selected model was evaluated using diagnostic model plots (**Figs. 9a–d**), which are essential for identifying discrepancies between experimental results and model predictions. These plots play a critical role in assessing and refining the model's adequacy [32]. The plot comparing actual versus predicted values (**Fig. 9a**) demonstrated a strong correlation, with data points closely aligning along the 45° line dividing the plot into equal halves, indicating a well-fitted model. Similarly, the plot of predicted values against studentized residuals (**Fig. 9b**) further confirmed the model's reliability.

Studentized residuals, which represent the differences between predicted and actual responses, exhibited no systematic patterns, reflecting the model's dependability. The normal probability plot (**Fig. 9c**) showed minimal to no abnormalities in the experimental data, verifying that the data followed a normal distribution. Moreover, the outlier plot (**Fig. 9d**) depicted the standard deviation of the actual responses, with all points falling within the range of -3.5 to 3.5. This absence of outliers further supports the model's accuracy and validity.

To compare the efficacy of RSM in optimizing the efficiency of the assay in the detection of Lead ion, the cucumber dye-binding assay is compared with OFAT, the results of the IC_{50} are shown in **Table 3**.

Table 3. Comparison of results between OFAT and RSM.

OFAT			RSM	
Metal	LOD (mg/L)	IC50 (mg/L)	LOD (mg/L)	IC50 (mg/L)
Pb2+	0.015 (0.013 to 0.017)	0.078 (0.057 to 0.27)	0.003 (0.002 to 0.004)	0.028 (0.022 to 0.037)

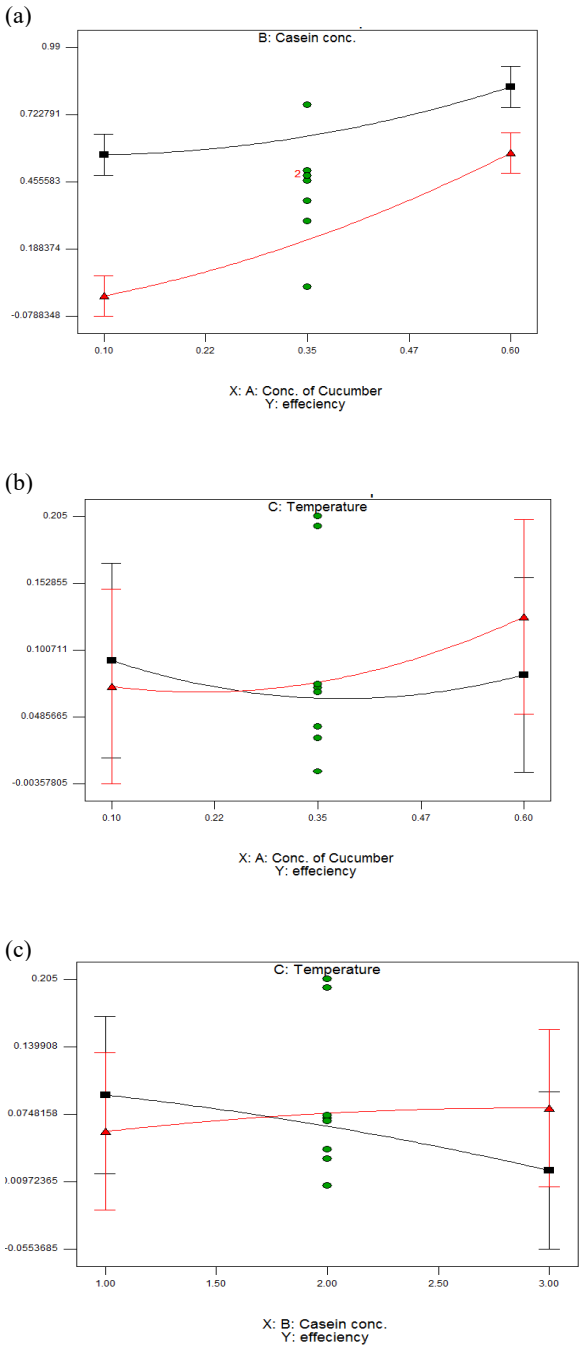

Fig. 8. Perturbation interaction curve (a) Casein vs Cocumber conc. (b) Temperature vs conc. of cucumber and (c) Temperature vs casein conc.

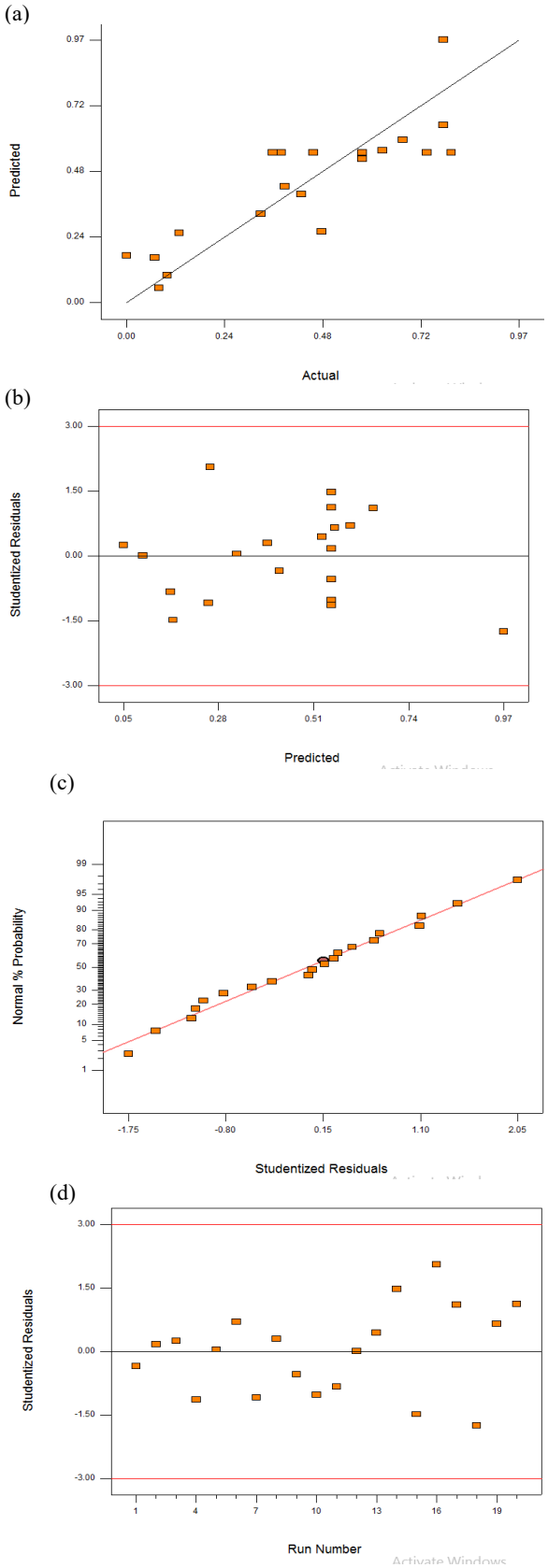

Fig. 9. Model diagnostic plots; (**a**) predicted vs actual, (**c**) studentized residue vs predicted, (**b**) normal plots of residue and (**d**) outlier T vs run.

A crucial indicator of a toxicant's potency in toxicology is its IC_{50} (Half Maximal Inhibitory Concentration), which is the concentration of a material required to 50% impede a biological or metabolic activity. It is frequently used in assays for enzyme inhibition to assess how different chemicals, such metals, inhibit enzymes like papain or ficin [22,34]. However, the sensitivity of analytical techniques used to detect traces of toxicants in environmental, dietary, or biological samples is determined by the Limit of Detection (LOD), which is the lowest concentration of a chemical that can be consistently detected [35]. In toxicant determination, both IC_{50} and LOD are essential. LOD ensures safety by identifying hazardous contaminants at levels below regulatory framework-permissible exposure limits, while IC_{50} is utilised to rank toxicants according to their inhibitory effects.

RSM has been extensively applied in analytical studies to improve efficiency and detection sensitivity and its effectiveness in enhancing the sensitivity of toxic metals in the cucumber assay further underscores its value. The one-phase exponential decay model employed in this study demonstrated an excellent fit to the experimental data, with coefficient of determination (R^2) values ranging from 0.9545 to 0.995. This assay is comparable to the previously established plant based assay methods **Table 4**.

Table 4. Comparison of lead detection between cucumber protease and other bioassays.

	IC_{50} mg/L							
Metal	Cucu-mber	Ficin	Papain	Bromelain	AChE from *Pangasius* sp.	48h *Daphnia magna*	15-min. Microtox™	Mo-Reducing Enzyme from *Serratia* sp. Strain DrY8
Hg^{2+}	nd	0.016-0.019	0.24-0.62	0.13-0.16	0.059-0.088	0.005-0.21	0.076–3.8	0.154-0.178
Pb^{2+}	0.022-0.037	nd	nd	nd	nd	nd	nil	nd
Ag^{+}	nd	0.022-0.037	0.33-0.49	nil	0.82-0.095	1.930	nil	0.018-0.046
Cu^{2+}	nd	0,022-0.027	0.004	0.163-0.305	0.065-0.096	0.020-0.093	0.029–0.050	0.295-0.435

Note: nd: not detected

CONCLUSION

The Bradford reagent has been applied successfully to the selective staining of proteins in protease based enzyme inhibition assays to detect heavy metals. One advantage of this assay is that it is shows the capability to detect the inability of polypeptides less than 2 kDa to interact with Coomassie dye, thereby offering a simple and effective means of detecting enzymatic inhibition by toxic metals. The sensitivity of the assay was found to be greatly enhanced by optimization using Response Surface Methodology (RSM) to the point that the IC50 value for lead detection was lower than that achieved using the One factor at a Time (OFAT) method. The model's reliability was statistically validated and a strong correlation was shown between the predicted and actual responses.

This assay is cost effective and practical in place of conventional analytical methods like AAS and ICP-MS which are expensive and require skilled personnel. It is anticipated that this method could be incorporated into portable biosensors for real time environmental monitoring in future applications. In summary, the optimized cucumber protease assay is presented as a significant advancement in heavy metal detection for environmental and food safety assessments.

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COMPETING INTERESTS

No competing interests as declare by the authors.

AI DECLARATION

We employed advanced AI-based tools during the initial phases of our research and manuscript preparation, specifically Mermaid, ChatGPT, ScholarAI, and Grammarly. These tools served as supplementary resources, assisting with data collection, analysis, and manuscript editing. However, they were not involved in data interpretation or formulating scientific conclusions. The final interpretations, conclusions, and scholarly work, including the structure and coherence of the arguments presented, are solely the responsibility of the authors.

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