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Improvement of Garlic Crude Protease-based Inhibitive Enzyme Assay for Mercury Using Response Surface Methodology

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ABSTRACT

Enzyme-based assays are an excellent preliminary screening tool with near real-time potential. An inhibitory enzyme assay using crude garlic protease, in conjunction with the casein and Coomassie dye-binding assay, is a sensitive method for detecting mercury. This study employed Response Surface Methodology (RSM) using a Central Composite Design (CCD) to optimize conditions for maximum mercury detection using crude garlic protease and casein as key variables. Twenty CCD experiments were generated with six center point replicates, and the resulting model was statistically significant ($F = 367.00$, $p < 0.0001$), showing strong predictive capability (Pred $R^2 = 0.9877$; Adj $R^2 = 0.9943$). Analysis of variance revealed that garlic protease (B), casein (C), their interaction (BC), and quadratic terms (A^2, B^2, C^2) significantly influenced the response. Diagnostic plots confirmed the model's robustness, showing minimal residual deviations and no significant outliers. The low coefficient of variation (CV = 5.46%) indicated experimental precision. 3D surface and contour plots highlighted a notable antagonistic interaction between garlic protease and casein concentrations, where their combined effects reduced individual contributions to the response. Model validation demonstrated an excellent correlation between predicted (A595 nm, 0.387) and experimental values (A595 nm, 0.381), confirming the accuracy of the optimization. Optimal conditions were identified at pH 6.78, with garlic protease at 0.534 mg/mL and casein at 0.1176 mg/mL, resulting in a verified response of A595nm = 0.381. Compared to traditional OFAT methods, RSM achieved a 26.27% improvement in the response. This optimized model provides an effective predictive framework for enhancing inhibitive crude garlic protease enzyme assay of mercury. These findings confirm the statistical soundness of RSM, validating its use for multifactorial optimization in an enzyme-based metal detection system.

INTRODUCTION

With a detection time of less than one hour, bioavailable mercury can be monitored in near real-time, enabling the temporal detection of harmful metals that are often overlooked by monitoring authorities [1–3]. For applications requiring near-real-time monitoring, instruments like Flow Injection Mercury Systems (FIMS) and Inductively Coupled Plasma (ICP) are too cumbersome and costly. For initial screening, rapid toxicological assays are great, and only positive results should be sent for instrumental validation. A number of luminescence-based toxicological assays are available, including MicrotoxTM and Xenoassay light™ [4,5] and protease-based systems [6–12]. Several sensitive assays for inhibiting toxic metals, based on proteases coupled with the casein-dye binding assay, have been developed, including those for papain [13], bromelain [14], and trypsin [15]. All of these assays demonstrate good performance in near real-time monitoring of toxic metal levels [5]. Previously, an inhibitive assay using Garlic crude protease was developed [20], showing one of the most sensitive responses to mercury with an IC_{50} (concentration causing 50% inhibition).

Analytical chemists typically optimize their processes by tweaking a single critical parameter at a time. Officially, this is known as one-factor-at-a-time (OFAT). The biggest problem with this method is that it doesn't take into account the interactive part of the factors. Consequently, this approach does not reveal the complete impact of the parameter on the outcome. One more thing that one-factor optimization does is make research more expensive and time-consuming by increasing the number of experiments needed to conduct the study. Additionally, OFAT utilizes more reagents and consumables [21]. The OFAT

limitations can be resolved using response surface methodology (RSM), a multivariate statistical technique. By fitting experimental data to polynomial equations, it uses mathematical and statistical techniques to forecast optimal conditions. When a large number of variables impact the responses of interest, RSM shines [22]. Optimal outcomes can be achieved by simultaneously optimizing the rates of these variables. Optimal detection of an analyte has been achieved in analytical chemistry on multiple occasions through the use of RSM [23–25]. The use of RSM in improving the sensitivity of toxic metal detection using a protease-based inhibitory assay has been successfully carried out with ficin to optimize detection [26]. With this in mind, the garlic protease metal assay is subjected to RSM-based optimization in this study to improve the response.

MATERIALS AND METHODS

Casein and Garlic crude protease solution preparation

Casein (Sigma) (2 g) was dissolved in 100 mL of deionized water at room temperature. To solubilize the mixture, the pH was adjusted to 8.0 using 5N NaOH and/or 5N HCl. To ensure maximum dissolution, the mixture was stirred overnight at 60 °C. The mixture was strained through multiple layers of cheesecloth to extract insoluble casein. Centrifugation at 10,000×g further clarified the slightly transparent filtrate (4 ºC). The Bradford assay, with crystalline BSA (Sigma) used as the standard, was used to measure the protein content of the clear supernatant. For future use, the solution is kept at 4 ºC. Alternatively, it can be frozen and stored at -20 °C. Continued with the previous step, a stock solution of 10.0 mg ml−1 of garlic crude protease was prepared at 4 °C in 20 mM sodium phosphate with a pH of 6.5 [26]. Working solutions of Garlic crude protease and casein were prepared from these stock solutions fresh daily.

Garlic crude protease optimization studies

The incubation duration was set at 30 minutes and the temperature at 30 degrees Celsius to mimic the field trial environment in Malaysia and to enable near real-time measurement [5,17,27]. The concentration of Garlic crude protease from the stock solution was adjusted to final concentrations ranging from 0.06 to 0.18 mg/mL in 20 mM phosphate buffer pH 6.5. A 30 µL casein solution was mixed completely with 50 µL of the garlic crude protease solution. The mixture was incubated at 30 ºC for 30 minutes after being topped up with 20 mM phosphate buffer pH 6.5, bringing the volume to 150 µL. A 10 µL portion was promptly removed and combined with 200 µL of Bradford dye-binding reagent once the incubation time had elapsed.

The absorbance at 595 nm for time zero was measured after incubation at room temperature for 5 minutes. After 30 minutes, a 200 µL mixture was prepared by mixing with Bradford dye-binding reagent. A second 10 µL aliquot was then taken, and the absorbance at 595 nm was measured after 5 minutes of incubation at room temperature. The absorbance measurement was done using a microplate reader from Bio-Rad Laboratories, Inc., located at 3110 Regatta Blvd, Richmond, CA 94804, United States. A final volume of 150 µL was used to optimize the substrate casein concentration, with the garlic crude protease fixed at 0.1 mg/mL and casein concentrations ranging from 0.2 to 0.8 mg/mL. We used a sodium phosphate buffer (20 mM) with a pH range of 5.8 to 7.8 (±1 pKa of phosphate), and we repeated the experiment as previously, with the sole modification being the change in pH [28].

Optimization Using RSM based on Central composite design (CCD)

We optimized three experimental factors—the incubation time of the enzyme-substrate, the concentrations of casein and garlic crude protease—using the central composite design (CCD). To achieve the best possible detection limit of 0.040 mg/L for mercury, a 23-factorial central composite experimental design was employed, resulting in a total of 20 experimental runs. The most desirable response is the one that shows the largest difference in absorbance, measured at 595 nm after 20 minutes of incubation at 30 °C in the Bradford dye-binding assay.

The pH range (from 5.8 to 7.8), casein concentration (from 0.2 to 0.8 mg/mL), and crude garlic protease concentration (from 0.06 to 0.18 mg/mL) were selected based on one-factor-at-a-time (OFAT) results from an earlier study [26]. Each range was chosen to span the region along the response curve where significant changes were observed, specifically covering the slope prior to plateauing to ensure effective optimization using response surface methodology (RSM).

Garlic crude protease mercury inhibition studies

To initiate the experiment, 50 µL of the crude protease from garlic, dissolved in 20 mM phosphate buffer with a pH of 6.5, as specified by the CCD experimental runs, was combined with 50 µL of mercury, resulting in a final concentration of 0.040 mg/L. The combination was left to incubate at 30 ºC for 10 minutes. The 20 mM phosphate buffer with a pH of 6.5 was used as a control in place of the mercury. Afterward, a 50 µL casein solution was prepared by adding and thoroughly mixing casein at the correct concentration. Without delay, a 20 µL portion was combined with 200 µL of the Bradford dye-binding reagent. A reading of 595 nm was taken as the time-zero absorbance after the mixture was incubated at room temperature for 5 minutes. Similar to the previous step, an additional 20 µL aliquot was taken and combined with the Bradford dye reagent after 30 minutes of incubation. The absorbance at 595 nm was measured after 5 minutes of incubation.

Data and Statistical Analysis

The per cent inhibition was calculated according to the following formula:

$$\%inhibition = 100x\frac{Test\ activity\ of\ sample - test\ activity\ of\ control}{Test\ activity\ of\ control}$$

In order to conduct the nonlinear regression, we used GraphPad Prism (Trial version 8.0.2) and the one-phase exponential decay model. Results were presented as means with standard deviations based on a minimum of three separate experiments. The RSM was conducted using Stat-Ease Inc.'s (USA) Design-Expert version 7.0 trial software.

RESULT AND DISCUSSION

Optimization using Response Surface Methodology (RSM)

A Central Composite Design (CCD) was utilized to determine the optimal values for the variables of interest in order to maximize the experiment's effectiveness. In a twenty-run experiment, the combined effects of pH (5.8-7.8), crude garlic protease concentration (0.20-0.80 mg/mL), and casein concentration (0.06-0.18 mg/mL) were evaluated (**Table 1**). The experimental responses were assessed using the absorbance at 595 nm (Abs595nm).

From a low of 0.032 (Run 17: pH 7.80, protease 0.20 mg/mL, casein 0.18 mg/mL) to a high of 0.401 (Run 6: pH 6.80, protease 0.50 mg/mL, casein 0.12 mg/mL), the response values varied widely. This indicates that the system is quite sensitive to changes in experimental conditions, as indicated by a large dynamic range in response (0.032-0.401 Abs595nm) (**Table 2**). The high responses (e.g., 0.397, 0.388, 0.381) were observed in replicates at the center point (pH 6.80, protease 0.50 mg/mL, casein 0.12 mg/mL), demonstrating that the experimental design is quite robust and relatively reproducible.

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

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded
A	pH	min	Numeric	5.8	7.8	-1	1
B	Garlic crude protease	mg/mL	Numeric	0.06	0.18	-1	1
C	Casein	mg/mL	Numeric	1	4	-1	1

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

	Factor 1	Factor 2 B:Crude Garlic protease	Factor 3 C:Casein	Response 1	Predicted
Run	A:pH	mg/mL	mg/mL	Abs595nm	Abs595nm
1	6.80	0.50	0.12	0.397	0.385
2	6.80	0.50	0.22	0.161	0.170
3	7.80	0.80	0.06	0.1	0.102
4	7.80	0.20	0.06	0.062	0.066
5	7.80	0.80	0.18	0.12	0.119
6	6.80	0.50	0.12	0.401	0.385
7	6.80	0.50	0.12	0.388	0.385
8	5.80	0.80	0.06	0.111	0.110
9	6.80	0.50	0.12	0.371	0.385
10	6.80	0.50	0.12	0.381	0.385
11	8.48	0.50	0.12	0.071	0.068
12	5.80	0.20	0.18	0.049	0.043
13	6.80	0.50	0.12	0.381	0.385
14	5.80	0.20	0.06	0.092	0.089
15	6.80	0.27	0.12	0.284	0.295
16	6.80	1.01	0.12	0.086	0.090
F17	7.80	0.20	0.18	0.032	0.028
18	5.80	0.80	0.18	0.127	0.119
19	5.12	0.50	0.12	0.078	0.087
20	6.80	0.50	0.02	0.198	0.195

The model's fitness was evaluated based on the regression equation and the determination coefficient (R^2) (Table 3). A significant model was indicated by an F-value of 367. A "model F-value" this high could only occur due to extremely rare events, with a probability of only 0.01%. The model's low probability value (<0.0001) and non-significant fit test F-value of 0.5903 indicated that it was a good fit. The importance of a non-significant, non-directed, and non-systematic lack of fit cannot be overstated [29–31]. The "Pred R-Squared" of 0.9877 is within reasonable agreement with the "Adj R-Squared" of 0.9943.

Table 3. Analysis of variance (ANOVA) for the Response Surface Quadratic Model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.3721	9	0.0413	367.00	< 0.0001	significant
A-pH	0.0004	1	0.0004	3.83	0.0787	
B-Crude Garlic protease	0.0077	1	0.0077	68.63	< 0.0001	
C-Casein	0.0007	1	0.0007	6.40	0.0299	
AB	0.0001	1	0.0001	0.9332	0.3568	
AC	0.0000	1	0.0000	0.3207	0.5837	
BC	0.0015	1	0.0015	13.18	0.0046	
A^2	0.1710	1	0.1710	1518.26	< 0.0001	
B^2	0.1239	1	0.1239	1099.74	< 0.0001	
C^2	0.0741	1	0.0741	657.70	< 0.0001	
Residual	0.0011	10	0.0001			
Lack of Fit	0.0005	5	0.0001	0.8067	0.5903	not significant
Pure Error	0.0006	5	0.0001			
Cor Total	0.3732	19				

Std. Dev.	0.0106	R^2	0.9970
Mean	0.1945	Adjusted R^2	0.9943
C.V. %	5.46	Predicted R^2	0.9877
		Adeq Precision	47.5078

In this study, the terms B, C, the interaction term BC, and the quadratic terms A^2, B^2, and C^2 were determined to be important to the model. On the other hand, p-values greater than 0.05 indicate that the terms are not statistically significant. When a model has numerous terms that are irrelevant (except for those necessary to maintain the model hierarchy), simplifying or reducing the model may improve its overall performance. Additionally, the Lack of Fit F-value of 0.81 indicates that the lack of fit is not substantial compared to the pure error, and there is a 59.03% chance that this result could be due to random noise. It is beneficial to have a lack of fit that is not significant, as it indicates that the model accurately captures the experimental data. The predicted R^2 value of 0.9877 is very close to the adjusted R^2 value of 0.9943, with a difference of less than 0.2. This means that the model is quite good at making predictions and not too much overfitting. The model's Adequate Precision, which examines the signal-to-noise ratio, was 47.508, significantly higher than the minimum acceptable level of 4. This means that the model has a good signal. Therefore, the model is considered reliable and can be used to navigate and understand the design area.

The experimental data are reliable, as evidenced by a low coefficient of variation (CV) of 5.46%, indicating that the measured responses were precise and consistent, meeting the requirements [32]. The analysis of variance (ANOVA) showed that the quadratic model was statistically significant ($F = 367.00$, $p < 0.0001$). There was a statistically significant difference ($p < 0.05$) between the model terms B (crude garlic protease), C (casein), BC, A^2, B^2, and C^2. There was no difference between A (pH), AB, and AC (**Table 3**). These results show that crude garlic protease, casein, and their interactions, especially in their quadratic forms, had a big impact on the response. This proves that all three independent variables in the model are important.

Diagnostic model plots (**Figures 1a-d**) were used to check how well the data fit into the chosen model. The graphs are especially helpful for determining how much the data differs from what the model predicted. This helps us figure out how good the model is and how to make it better. The graph of real vs. predicted values from the experiment (Fig. 1a) showed that the actual and predicted values were quite close to each other, as the data points were very close to the line that divides the graph into two halves (45º). We further verified the model's accuracy by visualizing the projected values and studentized residuals (Fig. 1b).

The difference between the anticipated value and the actual response from the model is referred to as the studentized residual. The plot of normal probability shows that the experimental data is not very anomalous or not at all (**Fig. 1c**). **Fig. 1d** displays an outlier plot, indicating how far the actual response's standard deviation deviates from the rest of the data. The plot didn't show any outliers because all the data points fell between -3.5 and 3.5.

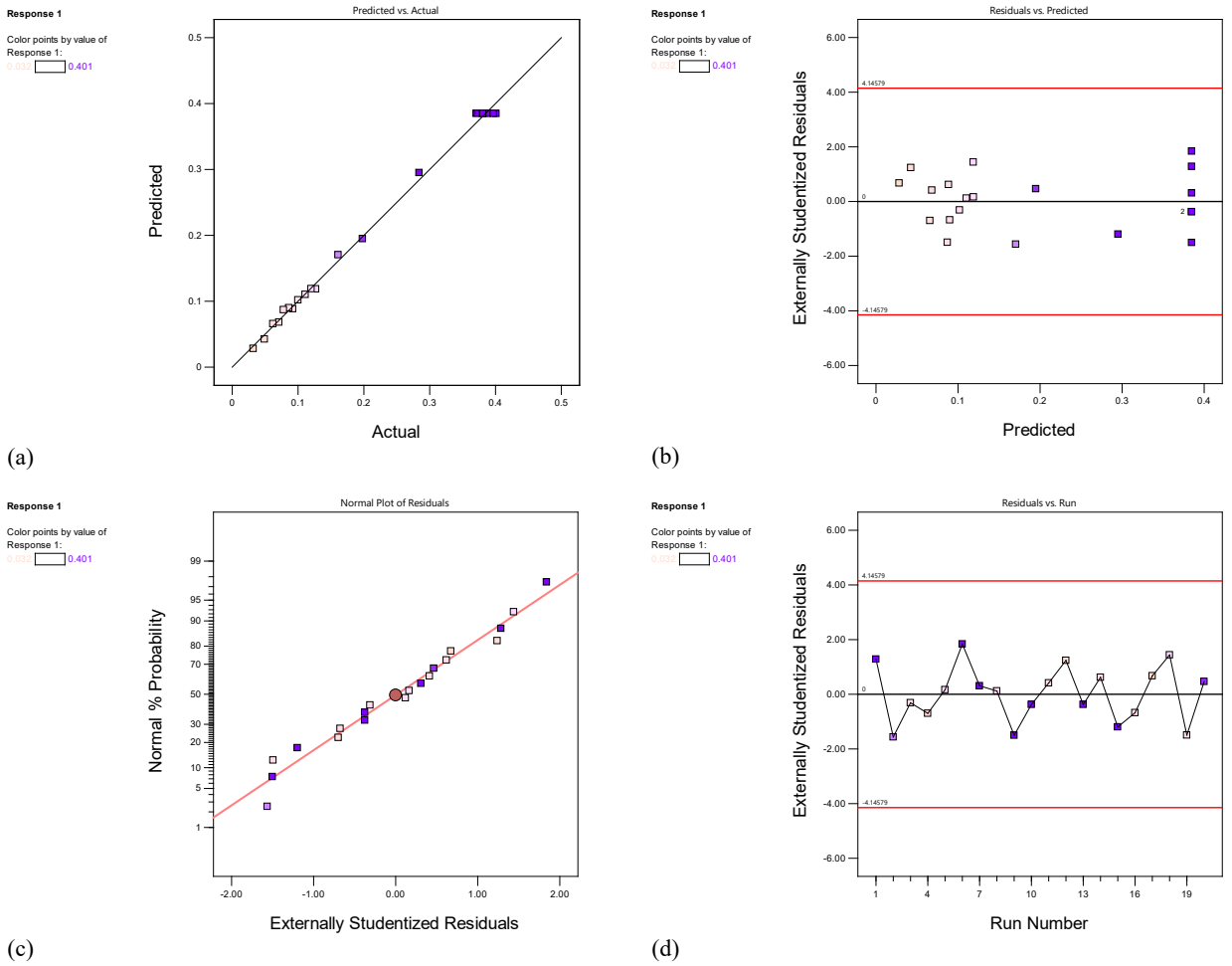

Fig. 1. Model diagnostic plots; (a) predicted versus actual, (b) studentized residue versus predicted, (c) normal plots of residue and (d) outlier T or externally studentized residuals versus run.

Figs. 2 to **4** show 3D plots and contour plots that display all the parameters necessary for optimal growth. Prior to conducting a real experiment, the plots are crucial for understanding the association at zero or intermediate levels of various combinations of independent factors. When one component remains constant, the 3D response plots reveal the maximal reaction for every set of factors. Curved contour lines indicate interaction, and there is a great deal of interaction shown by elliptical or saddle contour plots. There is no statistically significant interaction, as shown by circular contour plots. Examining the numerical value in the coded equation reveals significant interaction when it's either very high or very low, positive or negative [32].

Interactions between factors, particularly concentrations of garlic crude protease and casein, are shown in the perturbation plots and the 2D plot for the combination of factors (results not shown). For two factors to be considered to be interacting, the response to varying the outcome of one factor at different levels relative to the other must be different [33]. The fact that the perturbation lines for the concentrations of garlic crude protease and casein cross indicates that the two factors interact antagonistically, meaning that the influence of one factor reduces the effect of the other on the response [32].

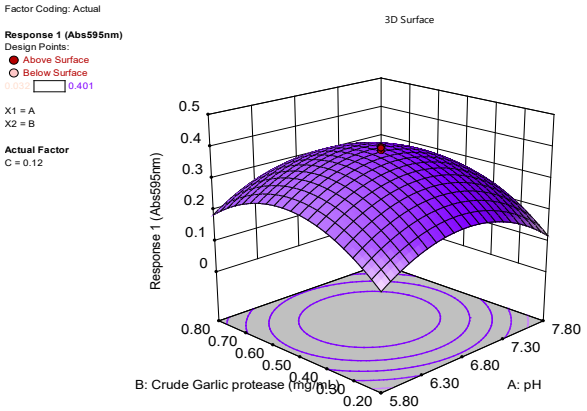

Fig. 2. A three-dimensional surface response view illustrating the effect of varying pH and concentration of garlic crude protease.

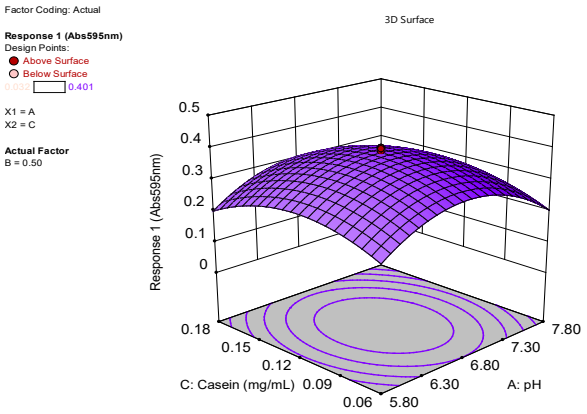

Fig. 3. View of the surface response in three dimensions illustrating the effect of changing the casein concentration and pH.

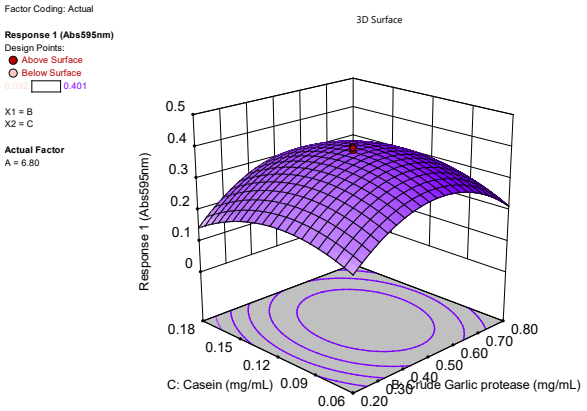

Fig. 4. Variations in the concentrations of garlic crude protease and casein are shown in this three-dimensional surface response view.

Verification of CCD experimental design of RSM

The CCD experimental design was found to be a successful tool for assessing the influence of pH, crude garlic protease, and casein concentrations on the maximum response for mercury detection. This study determined that crude garlic protease and casein concentrations are the primary factors that have a substantial impact on the response. The model's predictions on the ideal conditions for achieving the highest level of response were confirmed by experimental validation (**Table 4**). The strong correlation between the predicted and experimental results ($p > 0.05$) highlights the reliability of the model.

In order to attain the highest level of reponse, the ideal circumstances, as projected in **Table 5**, include a pH of 6.78, a crude Garlic protease concentration of 0.534 mg/mL, and a casein concentration of 0.1176 mg/mL with a predicted response of A595nm of 0.387, which was experimentally validated (**Table 5**). A comparison between the findings obtained via OFAT (reported in another source) and RSM demonstrated that the optimization using RSM resulted in a better response.

Table 4. The CCD design-based parameter suggestions and projected responses for each variable to achieve maximum response.

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	pH	6.78	5.80	7.80	0.0000	Actual
B	Crude Garlic protease	0.5344	0.2000	0.8000	0.0000	Actual
C	Casein	0.1176	0.0600	0.1800	0.0000	Actual

Table 5. Verification results between experiments and the predicted response.

RSM target solution	Desira-bility	Predicted % A595nm (95%, C.I.)	Experimental verification (95%, C.I.)	Statistical significant Difference between predicted and experimental
All studied factors are within range, Maximum response.	0.961	0.387 (0.377 to 0.396)	0.381 (0.341 to 0.421)	No significant Difference ($p>0.05$)

Comparison of optimisation parameters between OFAT and RSM

In comparison, results from OFAT [26] and RSM were gathered and compared to each other (**Table 6**). A higher response of 26.27% was achieved through RSM optimisation.

Table 6. Comparison of optimum conditions and results obtained between OFAT and RSM for optimum % degradation of glyphosate.

	OFAT		RSM	
Factors	Optimum value	Max A595nm	Optimum value	Max A595nm
pH	6.0	0.3017 (0.297 to 0.306)	6.78	0.381 (0.341 to 0.421)
Crude Garlic protease	1		0.5344	
Casein	0.2		0.1176	

CONCLUSION

The application of Response Surface Methodology (RSM) using a Central Composite Design (CCD) proved to be a statistically robust and efficient approach in optimizing conditions involving crude garlic protease and casein as a mercury detection system. The model demonstrated high predictive accuracy, with minimal lack of fit and a strong correlation between the predicted and experimental outcomes. It was discovered that the key variables—including protease and casein concentrations—significantly influenced the response, with diagnostic and interaction perturbation plots revealing an antagonistic relationship between the two factors. The validated optimal conditions (pH 6.78, 0.534 mg/mL protease, and 0.1176 mg/mL casein) resulted in a 26.27% improvement over the traditional OFAT methods. This confirms RSM as a powerful tool for enhancing the metal detection system.

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