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# Response surface design for the optimization of enzymatic detection of mercury in aqueous solution using immobilized urease from vegetable waste

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ABSTRACT

Soluble and alginate immobilized urease was utilized for detection and quantitation of mercury in aqueous samples. Urease from the seeds of pumpkin, being a vegetable waste, was extracted and purified to apparent homogeneity (sp. activity 353 U/mg protein; $A_{280}/A_{260}$ = 1.12) by heat treatment at 48 ± 0.1 °C and gel filtration through Sephadex G-200. Homogeneous enzyme preparation was immobilized in 3.5% alginate leading to 86% immobilization, no leaching of enzyme was found over a period of 15 days at 4 °C. Urease catalyzed urea hydrolysis by soluble and immobilized enzyme revealed a clear dependence on the concentration of $Hg^{2+}$. Inhibition caused by $Hg^{2+}$ was non-competitive ($K_i = 1.2 \times 10^{-1}$ μM for soluble and $1.46 \times 10^{-1}$ μM for alginate immobilized urease.). Time-dependent inhibition both in presence and in absence of $Hg^{2+}$ ion revealed a biphasic inhibition in activity. For optimization of this process response surface methodology (RSM) was utilized where two-level-two-full factorial ($2^2$) central composite design (CCD) has been employed. The regression equation and analysis of variance (ANOVA) were obtained using MINITAB® 15 software. Predicted values thus obtained were closed to experimental value indicating suitability of the model. 3D response surface plot, iso-response contour plot and process optimization curve were helpful to predict the results by performing only limited set of experiments.



## 1. Introduction

The toxic effects of mercury have been well known since the 18th century. In 1889, Charcot's *Clinical Lectures on Diseases of the Nervous System* attributed some rapid oscillatory tremors to mercury exposure. Toxicity from mercury exposure occurs with both organic and inorganic forms. Minamata disease is an example of organic toxicity. Toxicity from inorganic mercury can result from direct contact through the skin or gastrointestinal tract or from inhalation of mercury vapors. Extensive research has been done to investigate the possibilities offered by enzymes in biotechnological and environmental applications [1,2]. Enzymatic reactions have proved to be very promising tool to identify major pollutants such as heavy metals [3,4]. However, use of free enzymes show some major drawbacks such as thermal instability, susceptibility to attack by proteases, activity inhibition, high sensitivity to several denaturing agents, the impossibility of separating and reusing free catalyst at the end of the reaction, etc. The use of immobilized enzymes has proved to be more advantageous than the free enzymes [5]. However, high cost and limited availability of immobilized enzyme preparations are two important limitations in the wider applications of enzymes for routine detection of heavy metal ions [6,7]. Among the techniques used for immobilization, entrapment in natural biopolymers is favored for various reasons; e.g., non-toxicity of the matrix, possibility of the variation in the bead size and high percentage of immobilization and cost effectiveness [8,9]. Considering these, calcium alginate mediated entrapment has attracted much attention. Apart from the matrix, enzyme's availability and behaviour towards the pollutant are also important issues of consideration.

Enzymes are often specific to inhibitor and in many cases the inhibitory effect of investigated pollutant is related to its biological toxicity [10]. Owing to its pronounced sensitivity, urease (urea amidohydrolase, EC 3.5.1.5) has been considered as a model enzyme for application as a probe for heavy metal ions. Interestingly, different metals exhibit quite different behaviour in their ability to act as urease inhibitor [11–13]. Most of the studies have utilized urease obtained from jack bean which being expensive. Therefore, there still exist a need to have urease from a non-conventional, unutilized and cheaper source for versatile applications. Employing very simple steps, urease was purified to apparent homogeneity from an agricultural waste, i.e., the dehusked seeds of pumpkin (*Cucumis melo*) and entrapped into alginate beads. In the present communication, we describe the interaction of thus

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purified soluble as well as calcium alginate immobilized urease with mercury ion. In addition to this, the effect of the variables, i.e., $Hg^{2+}$ ion concentration and time of interaction which are affecting the activity of immobilized urease enzyme were evaluated by using response surface methodology (RSM); a statistical and graphical technique. Different factorial designs are available in RSM techniques [14,15]. Here two-level–two-factor full factorial central composite design (CCD) model was used [16]. The predicted result by the response surface CCD model was then compared with the experimental results.

### 1.1. Design of experiments (DOEs)

A two-level–two-factor ($2^2$) full factorial experiment was designed to observe the event of the parameters influencing activity of immobilized urease enzyme. The two factors considered are (i) concentration of $Hg^{2+}$ (low 0.001 mM and high 0.1 mM) (ii) interaction time of $Hg^{2+}$ with enzyme (5–30 min).

## 2. Materials and methods

### 2.1. Materials

Pumpkin seeds were procured from the local market and dehusked just before soaking. Tris was obtained from Boehringer Mannheim Gmbh, Germany. Bovine serum albumin was obtained from Sigma Chemical Co., USA. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Urea (enzyme grade), Mercuric acetate, Nessler's and Folin–Ciocalteau reagents were from Qualigens Fine Chemicals, Mumbai. All other reagents were analytical grade chemicals either from BDH or E. Merck, India.

### 2.2. Isolation of urease

Dehusked pumpkin seeds (6 g) were soaked in 25 mM Tris–acetate buffer (pH 7.5) for 8 h at 4–6 °C, homogenized in a kitchen blender for 2 min, filtered through muslin cloth and centrifuged for 15 min at 0–4 °C at 21,500 × *g*. The supernatant was filtered through a thick layer of prewashed glass wool to remove excess fat layer.

### 2.3. Purification of urease

The following steps were taken to purify the crude enzyme preparation.

#### 2.3.1. Heat treatment

Crude enzyme preparation (3.0 ml) was heated at 48 ± 0.1 °C in a water bath for 10 min and was immediately chilled in crushed ice. This was centrifuged at 21,000 × *g* for 15 min at 0–4 °C. The supernatant was collected.

#### 2.3.2. Gel filtration

Heat treated enzyme (1.5 ml, 2.4–2.6 mg protein) was loaded on a Sephadex G-200 column (1.5 cm × 40 cm). The elution was carried out at 4–6 °C at a flow rate of 20 ± 2 ml/h with degassed extraction buffer. Various 2.0 ml fractions containing urease activity were pooled and concentrated against solid sucrose. The enzyme preparation was kept frozen (at −20 °C) in small aliquots until further use.

The enzyme preparation (sp. activity 353 ± 12 U/mg protein), which showed a single enzyme and protein band on native 7.5% PAGE (at pH 8.3), was employed for the study.

### 2.4. Calcium alginate beads preparation

A 3.5% solution of sodium alginate was prepared in 25 mM Tris–acetate buffer (pH 7.5) by stirring for 2 h at room temperature and was stored at 4 °C. Suitably diluted enzyme solution (0.7 mg protein/ml) was mixed in chilled alginate solution and dropped in 100 ml of chilled and continuous stirring 400 mM calcium chloride solution with the help of a micropipette. The beads were allowed to stir for 90 min at 4 °C for complete calcium alginate formation. Beads were thoroughly washed with the buffer and stored at 4 °C.

### 2.5. Urease activity assay

Enzyme activity was assayed in 50 mM Tris–acetate buffer (pH 8.0). An aliquot (0.8 ml) of buffer and 1.0 ml of 250 mM urea in the same buffer were brought to 30 °C. The reaction was started by adding 0.2 ml of suitably diluted enzyme. After 10 min, 1.0 ml of 10% trichloroacetic acid was added to stop the reaction. The total reaction mixture was transferred to a measuring flask (50 ml) and the volume was made to 50 ml with distilled water after adding 1.0 ml of Nessler's reagent. The amount of ammonia liberated was measured at 405 nm in a Spectronic 21UVD spectrophotometer.

For assay of immobilized enzyme, the beads were incubated at 30 °C for 10 min in standard assay medium comprised of 50 mM Tris–acetate buffer (pH 8.0) containing 250 mM urea. Following incubation, an aliquot of 1.0 ml was withdrawn from the reaction mixture and assayed as described above. The beads were recovered from the reaction mixture, washed thoroughly with the buffer and stored at 4 °C.

A unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of ammonia in 1 min under the test conditions defined above (30 °C, 50 mM Tris–acetate buffer, pH 8.0, 250 mM urea). Protein was estimated by the method of Lowry et al. [17] with Folin–Ciocalteau reagent calibrated with crystalline bovine serum albumin.

### 2.6. Effect of mercuric ions on the activity

A stock solution of mercuric acetate was made in 50 mM Tris–acetate buffer (pH 8.0) and diluted with the same buffer as required. The activity of suitably diluted enzyme was determined in the presence of varying concentrations of $Hg^{2+}$ added in the standard assay mixture. For the direct effect of $Hg^{2+}$, enzyme alone was incubated with the desired concentration of inhibitor for 10 min at 30 °C and the treated enzyme was assayed for the activity. The inhibition pattern and inhibition constant ($K_i$) was calculated from a Lineweaver–Burk plot.

The results reported are the mean of five to eight replicate experiments carried out with a fresh batch of purified enzyme.

### 2.7. Design of experiment

RSM is an empirical statistical technique employed for multiple regression analysis by using quantitative data. It solves multivariable data which is obtained from properly designed experiments to solve multivariable equation simultaneously. The graphical representation of their functions is called response surface, which was used to describe the individual and cumulative effect of the test variables and their subsequent effect on the response. Easy way to estimate response surface, factorial designs is the most useful schemes for the optimization of variables with a limited number of experiments. A variety of factorial designs are available to accomplish this task. The most successful and best among them is the CCD which is accomplished by adding two experimental points along each coordinate axis at opposite sides of the origin and at a distance equal to the semi-diagonal of the hyper cube of the factorial

design and new extreme values (low and high) for each factor added in this design. If the factorial is a full factorial then,

$$\alpha = [2^k]^{1/4} \quad (1)$$

In this study, $k = 2$ factor (concentration of $Hg^{2+}$ and interaction time), so $\alpha = 1.414$.

The total number of experimental point in a CCD will be $2^k + 2k + x_0$ where $k$ is the number of central points. In order to understand the variation of the response with the change in independent variables and their possible interaction mathematical analysis of the data became necessary. Thus, this methodology allows the modeling of a second order equation that describes the process.

Inside the first region considered the response might be expressed as a function of independent variable of the form:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_{ij} \quad (2)$$

where, $Y$ = predicted response, $X_i^2$, $X_i$, $X_j$ = independent variables in coded values, $\beta_0$ = offset term, $\beta_i$ = linear effect, $\beta_{ii}$ = squared effect, $\beta_{ij}$ = interaction effect.

This equation may be a linear one, but it may be unsuitable to explain fully the wide range included in the factor space or to describe completely influence of all the variables and their interactions. Hence it is necessary to go for polynomial equation for analyzing and understanding the geometric nature of the surface, the maxima and minima of the response and the significance of the coefficients of the canonical equation.

A second order polynomial Eq. (1) was used to fit the data. The analysis of results was performed with statistical and graphical analysis software MINITAB® Release 15 developed by Minitab Inc., USA. This software was used for regression analysis of the data obtained and to estimate the coefficient of regression equation. The goodness of fit of the regression model obtained was given by the multiple correlation coefficients and by the coefficient of determination.

This response surface model was also used to predict the result by iso-response contour plots and three-dimensional surface plots. Contour plot is the projection of the response surface as a two-dimensional plane where as 3D surface plots is the projection of the response surface in a three-dimensional plane. For optimization of enzyme activity by addition of $Hg^{2+}$ ion and the interaction time, a $2^2$ factorial CCD with 4 axial ($\alpha = 1.414$) points, 4 cube points and 6 central points resulting in a total of 13 or 14 experimental points will be used in a single block. This design helps to get a good idea about the interaction of variables and this interaction can be inspected with the help of contour plots and 3D surface plots.

## 3. Results and discussions

### 3.1. Extraction of enzyme

The procedure for extraction of urease from dehusked seeds of pumpkin was optimized by varying the pH and molarity of the extraction buffer. The seeds were soaked overnight at 4–6 °C in 25 mM Tris–acetate buffer of pH range varied from 6.0 to 8.5. The soaked seeds along with the buffer were crushed in a kitchen blender giving high strokes for 2 min. Longer duration of crushing caused an increased extraction of extraneous proteins, whereas shorter crushing periods or crushing at lower strokes resulted in an incomplete extraction of enzyme. The supernatant obtained after centrifugation (21,500 × *g* for 15 min at 0–4 °C) revealed an increase in the activity with increasing pH of the extraction buffer and attained its maximum at pH 8.5 (results not shown). The specific activity, on the other hand, increased with increase in the pH up to 7.5. Further increase in pH notably reduced the specific activity. Therefore, the optimum pH for extraction of urease in Tris–acetate buffer appeared to be 7.5.

The suitable concentration of the buffer for substantial extraction of urease at pH 7.5 was explored by varying the molarity of the buffer from 5 to 100 mM, and proceeding as above. It was observed that the maximum enzyme activity as well as specific activity could be obtained at 25 mM of concentration. Use of concentrations lower than 25 mM resulted in less enzyme activity (as also its specific activity). At higher concentration, more protein and less enzyme units were extracted. Therefore, Tris–acetate buffer of 25 mM concentration at pH 7.5 was selected as the extraction buffer.

### 3.2. Purification of enzyme

#### 3.2.1. Heat fractionation

Heat fractionation of crude extract (48 ± 0.1 °C, for 10 min) purified the enzyme by about 1.4-fold with 76% recovery.

#### 3.2.2. Gel filtration

Heat fractionated enzyme was further purified by gel filtration through Sephadex G-200 column. The enzyme was eluted in a single peak. This step further purified urease by about 5.2-fold with 48% recovery.

Native 7.5% PAGE revealed single protein band under coomassie brilliant blue staining as well as under silver and urease specific staining. The purified enzyme showed a typical protein spectrum with maximum absorption at 278 nm and $A_{280}/A_{260}$ of 1.12 suggesting the enzyme preparation is free of nucleotides.

#### 3.2.3. Characterization of alginate immobilized urease

The conditions for optimal immobilization of pumpkin urease in calcium alginate beads were explored. In order to search the suitable concentration of sodium alginate, its concentration was varied from 1 to 6%. With 1–2% of alginate, there was only 30% immobilization. The enzyme activity was also lost after a single assay, probably due to the larger pore size in the beads, resulting into leaching of the enzyme during assay. With 5–6% of alginate, 50% immobilization was achieved but the shape and size of the beads were not uniform probably due to very high viscosity of the alginate solution. Sodium alginate in the concentration range of 3–4% resulted into stable beads of more or less uniform shape and size. The beads obtained with 3.5% solution of alginate were homogeneous and exhibited 86% immobilization with uniform distribution of enzyme per bead. Therefore, 3.5% sodium alginate was employed for further study.

Each bead contained 3.5–4.0 μg protein and the specific activity of the immobilized urease was found to be 327 ± 21 U/mg of protein. There was no leaching of the enzyme (less than 1%) over a period of 15 days at 4 °C. When the same beads were repeatedly used for 7 days (with an interval of 24 h between each use), they still retained 40–45% activity. The pH optimum of soluble and alginate entrapped urease in 50 mM Tris–acetate buffer was 8.0 and 7.0, respectively. There was a shift of 1.0 unit towards an acidic pH value upon immobilization. Urease immobilized in alginate beads revealed an apparent increase in $K_m$ and $V_{max}$ values. $K_m$ was approximately 1.17-fold higher than the $K_m$ of the soluble enzyme (16.66 ± 0.2 mM for soluble and 19.5 ± 0.3 mM for immobilized urease; results not shown).

### 3.3. Effect of $Hg^{2+}$ ions on soluble and alginate immobilized enzyme

The effect of $Hg^{2+}$ on the activity of soluble and alginate entrapped urease was studied in the concentration range that had

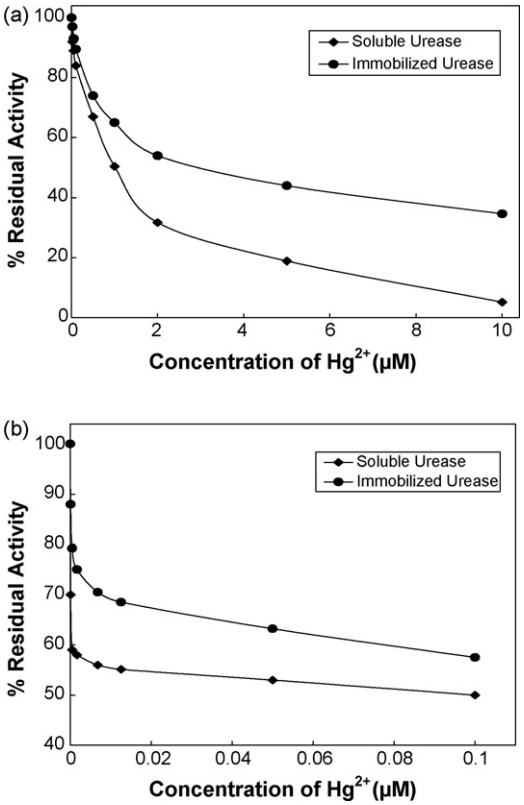


**Fig. 1.** (a) Effect of $Hg^{2+}$ on the activity of soluble and alginate entrapped urease. Suitably diluted enzyme (14.0–16.1 U/ml, 6.7–8.9 μg protein/ml) and two beads (3.5 μg protein/bead) was incubated in presence of varying concentration of $Hg^{2+}$ (0.02–10.0 μM) added into the standard assay mixture and assayed the activity after incubating for 10 min. (b) Inactivation of soluble and alginate entrapped urease by $Hg^{2+}$. Suitably diluted enzyme (14.8–16.0 U/ml, 7–9 μg protein/ml) and two beads (3.6 μg protein/bead) was incubated with varying concentrations (0.0001–0.1 μM) of $Hg^{2+}$ in absence of urea for 10 min at 30 °C in 50 mM Tris–acetate buffer (pH 8.0) and then assayed for the activity.

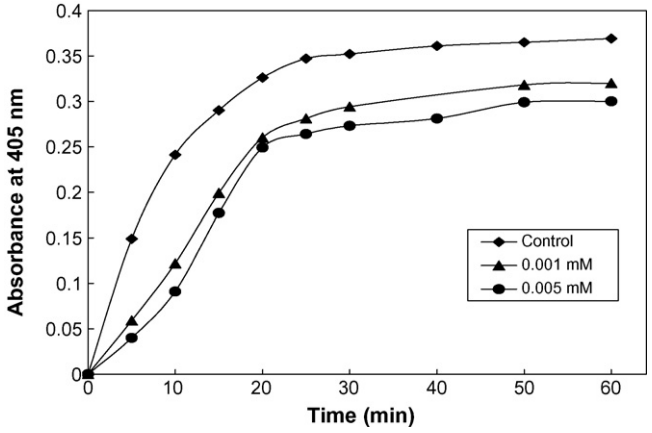


**Fig. 2.** Time-dependent hydrolysis of urea by alginate immobilized pumpkin urease. Suitably diluted enzyme (12.8–14.7 U/ml, 8.0–8.5 μg protein/ml) was immobilized and one bead (3.0 μg protein/bead) was incubated in the presence of 0.001 and 0.005 mM of $Hg^{2+}$ added in the standard assay mixture. The samples withdrawn at different time intervals were assayed for the ammonia formed using Nessler's reagent.

measurable inhibition. The desired concentration (0.02–10.0 μM) was added into the standard assay mixture and assayed the activity after incubating for 10 min. The results revealed a concentration-dependent inhibition in the activity. The soluble enzyme was more sensitive towards the inhibitor than the immobilized preparation. The soluble enzyme exhibited 50% inhibition at 1.0 μM of $Hg^{2+}$, while 5 μM of inhibitor was required by immobilized enzyme for same degree of suppression, Fig. 1(a). Similarly, at 10 μM of $Hg^{2+}$, only 5.2% and 35% activity was observed for soluble and immobilized preparations, respectively.

In order to study the direct interaction of $Hg^{2+}$ with urease, the soluble as well as alginate immobilized enzyme were directly exposed to $Hg^{2+}$ (0.1–1.0 μM, in absence of urea for 10 min at 30 °C) and then assayed for the leftover activity. There was an instant loss in the activity. Therefore, reasonably low concentration (0.0001–0.1 μM) had to be employed to obtain a measurable rate of inhibition. There was a rapid inactivation. Thus, at 0.0005 μM $Hg^{2+}$ the soluble enzyme was inhibited by about 40% while immobilized enzyme by about 20%, Fig. 1(b). Further increase in the concentration, a much slower suppression was noticed. The observations suggest that even 0.0005 μM of $Hg^{2+}$ is sufficient to produce measurable inactivation.

In order to explore $K_i$, the urea concentration in the assay mixture was varied from 2 to 125 mM. The results, when expressed by a Lineweaver–Burk double reciprocal plot of substrate concentration versus velocity (absorbance at 405 nm), revealed a non-competitive inhibition. The $K_i$ was found to be $1.26 \times 10^{-1}$ μM for soluble and $1.46 \times 10^{-1}$ μM for alginate immobilized urease.

Time-dependent interaction of soluble urease was studied by incubating the enzyme alone with 0.01 μM $Hg^{2+}$ at 30 °C in the absence of urea. Aliquots were withdrawn at specific time intervals and assayed for the activity. The results exhibited a biphasic pattern of inhibition. The data revealed that about half of the initial activity (50% in 10 min) was lost rapidly and remaining in a slow and sustained decay (63% in 50 min, results not shown). In fact, the rate of urea hydrolysis in presence of metal ion progressed at a reduced rate throughout the course of the study. Moreover, the interaction of the metal ion with the enzyme protein was so strong that the inhibition could not be reversed by dialysis (25 mM Tris–acetate buffer, pH 7.5, 0 °C, 24 h). Similar biphasic inactivation by heavy metal ions has also been reported for watermelon urease [12].

Time-dependent interaction of immobilized urease in the standard assay medium, on the other hand, exhibited quite differently. The beads were fairly active even at higher level of inhibitor (i.e., 0.005 mM of $Hg^{2+}$ could inhibit the activity by about 20% in 60 min) (Fig. 2). This less inhibition at a higher concentration of inhibitor requiring longer time duration might be due to the protection provided to the enzyme by alginate entrapment. Also, the hindered accessibility of the inhibitor to the enzyme in the immobilized form cannot be ruled out.

The enzyme requiring the presence of free –SH groups are generally inhibited by heavy metallic ions and the metal ions that form the most insoluble sulfides are the strongest inhibitors [18]. Rather extensive experimental data are available supporting the contention that urease contains one or more sulfhydryl groups as integral part of its catalytically active site [19–21]. The time-dependent inhibition of urease with metal ion in absence of urea appears to be due to the interaction of metallic ions with the –SH groups of the enzyme protein.

Further, titration of pigeonpea urease with 5,5′-dithiobis-(2-nitrobenzoate) revealed the presence of two categories of free sulfhydryl groups in the enzyme protein; one group being readily accessible while the other was titrable only after protein denaturation [21]. The observations reported above support the view that not all the –SH groups of the enzyme react with $Hg^{2+}$ at the same time. The readily available –SH groups react faster than the "masked" groups. However, inactivation of watermelon

**Table 1(a)**
Experimental data for the residual activity of the enzyme at different concentration and interaction time

| Concentration (mM) | Time (min) | % Residual activity |
|---|---|---|
| 0.100 | 20 | 23.48 |
| 0.010 | 20 | 43.50 |
| 0.005 | 20 | 72.00 |
| 0.001 | 20 | 79.00 |
| 0.005 | 5 | 6.05 |
| 0.005 | 10 | 31.50 |
| 0.005 | 15 | 49.60 |
| 0.005 | 20 | 68.47 |
| 0.005 | 30 | 75.92 |
| 0.001 | 5 | 11.80 |
| 0.001 | 15 | 56.36 |
| 0.001 | 20 | 76.56 |
| 0.001 | 30 | 80.37 |

urease by specific –SH blocking reagents like iodoacetic acid, *N*-ethylmaleimide and *p*-hydroxy-mercuricbenzoate has revealed a biphasic inactivation kinetics where half of the initial activity was lost in fast phase and remaining half in a slow phase [22]. This characteristic behaviour is suggestive of 'half-site reactivity' in the enzyme, where all the sites contribute to the activity but differ with respect to their rate of inactivation with these reagents. This phenomenon of molecular asymmetry in urease is a consequence of its unique quaternary structure and is not induced by any specific ligand.

### 3.4. Response surface factorial design for the optimization of the process

The variables that predominantly affecting the enzymatic detection of $Hg^{2+}$ by immobilized urease were (i) concentration of $Hg^{2+}$ solution and (ii) interaction time, and each parameter was having one lower value and one higher value. Thus for the two-level–two-full factorial CCD, 14 experimental values were required because in the programme itself there are four cube points, three center points in cube, four axial points and three central points in axial (Table 1(a)). Experiments were performed according to the experimental plan and the response thus obtained for each combination of the variables are given in Table 1(b) where the concentration of $Hg^{2+}$ ion was changed from 0.001 to 0.10 mM maintaining the interaction time 5–30 min, the response varied from 11.8 to 80.37%. Significant changes in enzyme activity were observed for all the combinations, implying that both these variables significantly affecting the enzyme activity.

**Table 2(a)**
Estimated regression coefficients for % residual activity

| Term | Coefficient | S.E. coefficient | *T* | *p* |
|---|---|---|---|---|
| Constant | −13.27 | 17.136 | −0.775 | 0.464 |
| Concentration (mM) | −22.09 | 12.749 | −1.733 | 0.127 |
| Time (min) | 14.33 | 59.791 | 0.240 | 0.817 |
| Concentration (mM) × concentration (mM) | 61.10 | 16.058 | 3.805 | 0.007 |
| Time (min) × time (min) | −20.68 | 3.670 | −5.635 | 0.001 |
| Concentration (mM) × time (min) | −21.69 | 62.522 | −0.347 | 0.739 |

$S = 5.52932$, PRESS = 13065350. $R^2 = 97.46\%$; $R^2(\text{pred}) = 0.00\%$; $R^2(\text{adj}) = 95.65\%$.

**Table 2(b)**
Analysis of variance for % residual activity

| Source | DF | Seq SS | Adj SS | Adj MS | *F* | *p* |
|---|---|---|---|---|---|---|
| Regression | 5 | 8224.42 | 8224.42 | 1644.884 | 53.80 | 0.000 |
| Linear | 2 | 6972.57 | 1157.61 | 578.804 | 18.93 | 0.002 |
| Square | 2 | 1248.17 | 1246.07 | 623.035 | 20.38 | 0.001 |
| Interaction | 1 | 3.68 | 3.68 | 3.678 | 0.12 | 0.739 |
| Residual error | 7 | 214.01 | 214.01 | 30.573 | | |
| Lack-of-fit | 5 | 204.81 | 204.81 | 40.961 | 8.90 | 0.104 |
| Pure error | 2 | 9.21 | 9.21 | 4.604 | | |
| Total | 12 | 8438.43 | | | | |

| Observation | StdOrder | % Residual activity | Fit | S.E. fit | Residual | St resid |
|---|---|---|---|---|---|---|
| Unusual observations for % residual activity | | | | | | |
| 1 | 1 | 23.480 | 23.443 | 5.529 | 0.037 | 2.10 RX |
| 2 | 2 | 43.500 | 51.289 | 4.099 | −7.789 | −2.10 R |

R denotes an observation with a large standardized residual. X denotes an observation whose X value gives it large leverage.

The response surface regression results give the coefficient for all the terms in the model (Table 2(a)) and each effect is estimated independently. Therefore, the coefficient for the linear terms will be the same where it fit just to the linear model. The $R^2$ value is 97.46% for residual enzyme activity suggested that 97.4% results of the total variations can be explained by this model. In addition to this estimated regression coefficient small *p* values for $Hg^{2+}$ concentration (0.127), time (0.817), concentration square (0.007) and time square (0.001) suggesting these effects may be important in the model.

Analysis of variance (ANOVA) was utilized for statistical testing of the model in the form of linear terms, squared terms and the interaction (Table 2(b)). The *p*-value (0.002) for linear and 0.001 for square terms confirm the applicability of the model.

**Table 1(b)**
Data processed by MINITAB® software

| StdOrder | RunOrder | PtType | Blocks | Concentration (mM) | Time (min) | % Residual activity | Predicted value |
|---|---|---|---|---|---|---|---|
| 3 | 1 | 1 | 1 | 0.100 | 20 | 23.48 | 23.4426 |
| 7 | 2 | −1 | 1 | 0.010 | 20 | 43.50 | 51.2892 |
| 2 | 3 | 1 | 1 | 0.005 | 20 | 72.00 | 64.6811 |
| 9 | 4 | 0 | 1 | 0.001 | 20 | 79.00 | 76.2923 |
| 12 | 5 | 0 | 1 | 0.005 | 5 | 6.05 | 3.7078 |
| 10 | 6 | 0 | 1 | 0.005 | 10 | 31.50 | 30.6496 |
| 6 | 7 | −1 | 1 | 0.005 | 15 | 49.60 | 50.9740 |
| 13 | 8 | 0 | 1 | 0.005 | 20 | 68.47 | 64.6811 |
| 11 | 9 | 0 | 1 | 0.005 | 30 | 75.92 | 72.2432 |
| 8 | 10 | −1 | 1 | 0.001 | 5 | 11.80 | 13.2162 |
| 5 | 11 | −1 | 1 | 0.001 | 15 | 56.36 | 61.8843 |
| 1 | 12 | 1 | 1 | 0.001 | 20 | 76.56 | 76.2923 |
| 4 | 13 | 1 | 1 | 0.001 | 30 | 80.37 | 85.2563 |

Central composite design: factors, 2; base runs, 13; base blocks, 1; replicates, 1; total runs, 13; total blocks, 1. Two-level factorial: full factorial: cube points, 4; center points in cube, 5; axial points, 4; center points in axial, 0. Alpha, 1.41421. Response surface regression: % residual activity versus concentration (mM), time (min). The analysis was done using coded units.

**Table 2(c)**
Estimated regression coefficients for % residual activity using data in uncoded units

| Term | Coefficient |
|---|---|
| Constant | −18.7174 |
| Concentration (mM) | −2351.47 |
| Time (min) | 7.54880 |
| Concentration (mM) × concentration (mM) | 24936.5 |
| Time (min) × time (min) | −0.132347 |
| Concentration (mM) × time (min) | −35.0476 |

Multiregression analysis was performed as the data to obtain a quadratic response surface model (Table 2(c)) and second order equation obtained was

$$y = -18.7174 - 2351.47 \times \text{concentration of } Hg^{2+} + 7.54880 \times \text{interaction time} + 24936.5 \times (\text{concentration of } Hg^{2+})^2 - 0.132347 \times (\text{interaction time})^2 - 35.047 \times \text{concentration of } Hg^{2+} \times \text{interaction time.} \quad (3)$$

The predicted values of residual activity of enzyme obtained using Eq. (3) are closed to the experimental values proving that the model is fully applicable.

### 3.4.1. Interpretation of residual graph

The normal probability plot, Fig. 3(a), shows that the distribution of residual value which is defined as the difference between the predicted (model) and observed (experimental) are forming a straight line and residual value are normality distributed on both the side of the line indicating that experimental point are reasonably aligned with predicted value.

A histogram, Fig. 3(b), of the residuals shows the distribution of the residuals for all the observations and one long tail in the plot indicating skewness in the data whereas one bar is far from the others, these points were outlined. The plot between individual residual values and in the fitted value shows that all the residuals are scattered randomly about the zero and one or two points are outliners (Fig. 3(c)).

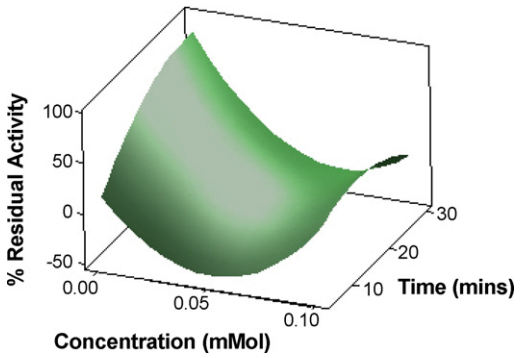


**Fig. 4.** 3D surface plot of the combined effect of concentration of $Hg^{2+}$ and interaction time on residual activity of the enzyme.

This last plot of Fig. 3(d) is the residual value and the order of the corresponding observations. The plot is useful when the order of the observations may influence the results which can occurs when data are collected in a line sequence. This plot can be helpful to a designed experiment in which the runs are not randomized. For residual activity data, the residuals appear to be randomly scattered about zero. No evidence exists that the regression terms are correlated with one another.

### 3.4.2. Interpretation of response 3D surface plots

The surface plot, Fig. 4, which is a three-dimensional graph where residual activity of enzyme was represented by varying simultaneously $Hg^{2+}$ concentration from 0.001 to 0.1 mM and time from 5 to 30 min. From this response surface plot this is also clear that residual enzyme activity to 50% concentration of $Hg^{2+}$ should be 0.005 mM and exposure time should be nearly 15 min.

The surface plot also describing individual and cumulative effect of these two test variable and test their subsequent effect on the response.

### 3.4.3. Contour plot

The iso-response contour plots between the variables such as $Hg^{2+}$ concentration and interaction time is given in Fig. 5. The lines of contour plots (center of the circle) predicting the values

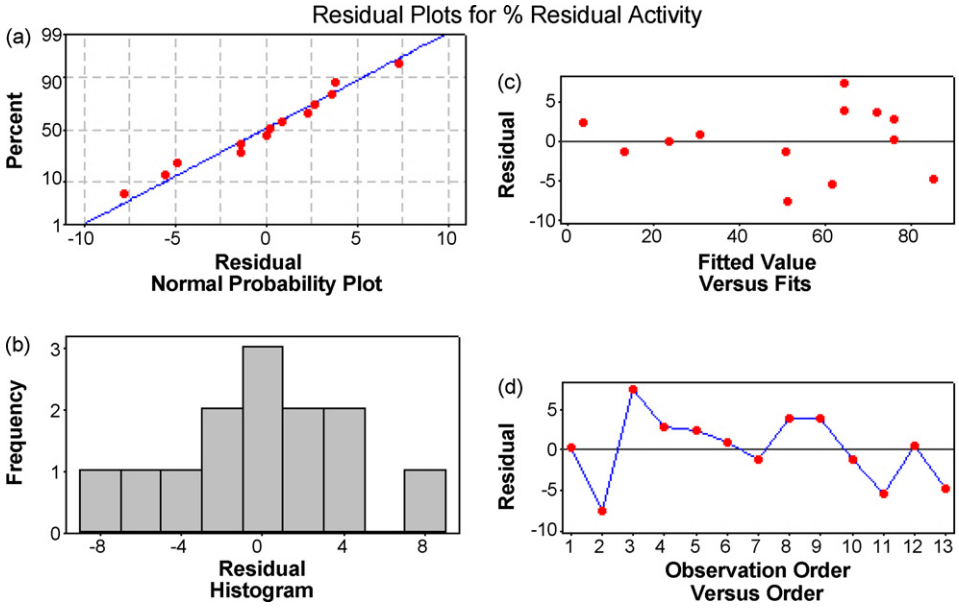


**Fig. 3.** (a) Normal probability plot of the residuals. (b) Histogram of the residuals. (c) Residual versus the fitted value. (d) Residual versus the order of the data.

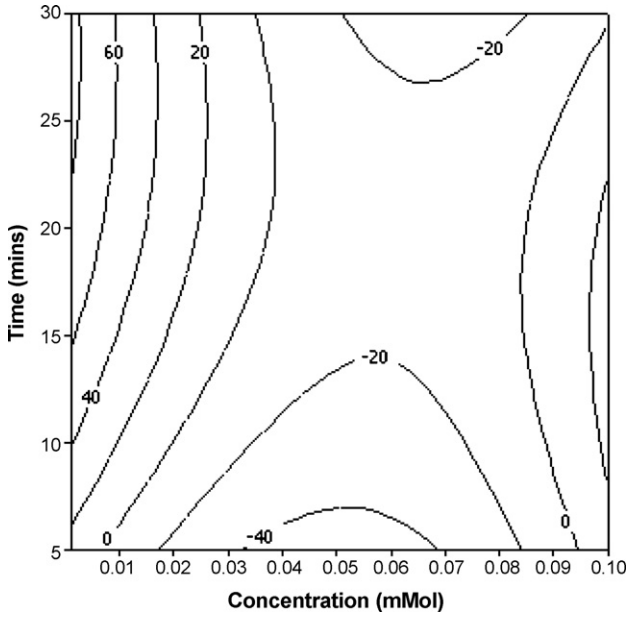


**Fig. 5.** Contour plot of the combined effect of concentration of $Hg^{2+}$ and interaction time on residual activity of the enzyme.

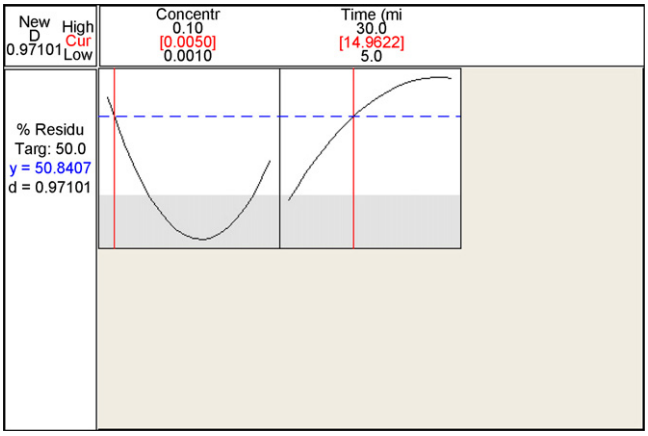


**Fig. 6.** Optimization plot to confirm the experimental results that 86.91% enzyme activity when 0.05 mM of $Hg^{2+}$ was added and interaction time was 50 min.

of residual enzyme activity for different $Hg^{2+}$ ions concentration at different interaction time. These values are more or less same to the experimental values.

### *3.4.4. Process optimization curve*

In order to confirm the experimental results that 50% enzyme activity when 0.005 mM of $Hg^{2+}$ was added and interaction time was 15 min. A response surface optimization curve was plotted by MINITAB® 15 Software Programme where minimum to maximum values of residual activity which was fixed (11.80–80.37%), concentration (0.001–0.1 mM), time (5–30 min) and target value of 50% residual activity was fixed. The result thus obtained was shown in Fig. 6 which is clearly indicating that 50.89% (*y* value given in Fig. 6) residual activity was predicted if the $Hg^{2+}$ concentration was 0.005 mM (curve value) and time of interaction was for 15 min (curve values).

## 4. Conclusion

The present study was aimed to work out an inexpensive and simple procedure for immobilization of urease obtained from a rather cheap and non-conventional source, which could be utilized for detection and quantization of $Hg^{2+}$ present in polluted wastewater/industrial effluents. The study conducted and results reported above suggest the versatile application of urease obtained from an agricultural waste, i.e., the discarded seeds of pumpkin and revealed its suitability for the detection of $Hg^{2+}$ in water, industrial effluents, soil, etc. Furthermore, with the help of experimental data a model was predicted by statistical and graphical technique response surface methodology (RSM) where two-level–two factor ($2^2$) was used. The predicted value thus obtained using MINITAB software has been found close to the experimental value indicating the applicability of the model.

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