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Removal of mercury (II) from aqueous solution using papain immobilized on alginate bead: Optimization of immobilization condition and modeling of removal study

Aparupa Bhattacharyya^a, Susmita Dutta^a, Parameswar De^b, Parthasarathi Ray^b, Srabanti Basu^{c,*}

^a Department of Chemical Engineering, National Institute of Technology, Durgapur 713209, India

^b Department of Chemical Engineering, University of Calcutta, Kolkata 700009, India

^c Department of Biotechnology, Heritage Institute of Technology, Kolkata 700107, India

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ABSTRACT

Papain having the characteristics of metal binding ability is immobilized on alginate bead. Design Expert Software (Version 7.1.6) uses Response Surface Methodology (RSM) for statistical designing of operating condition for immobilization of papain on alginate bead considering concentration of papain, concentration of sodium alginate, concentration of calcium chloride and pH as numeric factors and Specific Enzymatic Activity (SEA) of immobilized papain sample as response. Immobilization using 25.96 g/L papain, 20 g/L sodium alginate and 20 g/L calcium chloride at pH 7 gives the desired product as indicated by ANOVA (Analysis of Variance). Three parameters viz., initial concentration of mercury (II), amount of AIP and pH are varied in a systematic manner. Maximum 98.88% removal of mercury (II) has been achieved within 8 min when simulated aqueous solution of mercury (II) with initial concentration of 10 mg/L has been contacted with 5 g of AIP at pH 9 and at 35 °C in a batch reactor. A mathematical model has been developed and the value of equilibrium constant for binding of mercury (II) with AIP has been found to be 126797.3.

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1. Introduction

Classified as a heavy metal, mercury and its compounds are toxic to living systems and difficult to be eliminated by the body. This leads to bioaccumulation i.e., even small amount can act as a cumulative poison and over a period of time they reach the toxic level (Abuein et al., 2008; Starvin and Rao, 2004). Since the beginning of the industrial era anthropogenic activities like increased mining, high rate of fossil-fuel burning, widespread use of raw materials containing mercury in the industries are important contributors of mercury to the environment. Of different chemical species of mercury, organomercurials are the most toxic form followed by mercuric [Hg (II)] ions. Organomercurials including methylmercury and phenylmercury are avidly accumulated by fish and marine animals and finally enter human body leading to neurological disorders and other health problems (Abuein et al., 2008; Krishnan and Anirudhan, 2002; Starvin and Rao, 2004). Industrial waste water coming from different industries like chloro-alkali, paper and pulp, oil refinery, paint, pharmaceutical and batteries add to mercury pollution severely if they are discharged to the environment without any proper treatment (Anirudhan et al., 2008; Donia

et al., 2008; Krishnan and Anirudhan, 2002; Starvin and Rao, 2004). Therefore abatement of mercury from industrial waste water to attain the stipulated limit as prescribed by EPA is mandatory. In recent years considerable attention has been given to remove mercury by adsorption process on various adsorbent (Dutta et al., 2009; Ghodbane and Hamdaoui, 2008; Krishnan and Anirudhan, 2002; Lohania et al., 2008; Natale et al., 2006; Rmali et al., 2008; Velicu et al., 2007). It has been seen that papain, a proteolytic enzyme, has the characteristic of metal binding capability due to presence of sulfhydryl group in its active site and on immobilization over activated charcoal can be used for removal of mercury from its simulated aqueous solution (Dutta et al., 2009). At the same time it has been observed that at pH 7, 45.8% recovery of mercury is possible from *Charcoal Immobilized Papain* (CIP)–mercury complex, which means that there is always a possibility of leakage of mercury ions during an actual continuous process or during any neutral wash. The reason may be due to the fact that papain gets immobilized on the surface of activated charcoal by physical adsorption method and therefore there is a scope for mercury to leach out from papain after binding. Thus CIP may not be able to give effective removal of mercury (II) from its aqueous solution at pH 7. This shortcoming leads the present investigators to search for another suitable matrix over which papain may be immobilized and can be used for removal of mercury (II) from its

* Corresponding author. Tel.: +91 33 2443 0456; fax: +91 33 2443 0455.
E-mail address: srabanti_b@yahoo.co.uk (S. Basu).

Nomenclature

<i>A</i>	concentration of papain, g/L, as mentioned in Eq. (10)	K_m	Michaelis–Menten constant, g casein/L
<i>B</i>	concentration of sodium alginate, g/L, as mentioned in Eq. (10)	$[M_0^{+2}]$	initial concentration of metal in solution, mg/L
<i>C</i>	pH, as mentioned in Eq. (10)	$[M^{+2}]$	final equilibrium concentration of metal in solution, mg/L
<i>D</i>	concentration of calcium chloride, g/L, as mentioned in Eq. (10)	$[M_t^{+2}]$	experimental concentration of metal in solution at any time <i>t</i> , mg/L
$[E_0]$	loading of papain in the solution, g mole/L	$[M_{theo}^{+2}]$	theoretically calculated final equilibrium concentration of metal, mg/L
$[E^-]$	concentration of deprotonated papain, g mole/L	<i>R1</i>	Specific Enzyme Activity, (g peptide)/(g papain × hr)
$[EH]$	concentration of undissociated papain, g mole/L	<i>R²</i>	correlation coefficient as mentioned in Table 2
$[EM^*]$	concentration of metal which binds with papain, g	<i>V</i>	volume of solution, ml
<i>f</i>	percentage removal of mercury $f = \frac{[M_0^{+2}] - [M_t^{+2}]}{[M_0^{+2}]} \times 100$	<i>V_{max}</i>	maximum forward velocity of the enzymatic reaction, [(g peptide formed)/(g papain × hr)]
$[H^+]$	concentration of hydrogen ion, g mole/L	<i>W(AIP)</i>	weight of AIP taken in solution, g
<i>K₁</i>	equilibrium constant for first reaction as represented by Eq. (1)	α	distance of the axial point from the center
<i>K₂</i>	equilibrium constant for second reaction as represented by Eq. (2)	β	loading of papain in alginate bead, g mole/g
<i>k₂</i>	rate constant for enzymatic reaction, [(g peptide formed)/(g papain ² × hr)]		

simulated aqueous solution effectively without any leakage at its operating condition i.e., at pH 7. An organic matrix viz., alginate bead has been selected for the present study. Though a number of studies have been carried out to immobilize papain on alginate bead with various objectives (Roy et al., 2004; Sankalia et al., 2005; Willner et al., 1991), no investigation was made for removal of mercury from waste water using papain immobilized on alginate bead. In the present study, the possibility of using papain immobilized on alginate bead for eradication of mercury (II) from simulated waste water has been explored.

Alginate is commercially available as sodium salt of alginic acid at low cost. As a skeletal component of marine algae it has the property of being strong and at the same time flexible. Papain immobilized on alginate beads at the optimum condition, referred as AIP, was used for removal of mercury (II) from simulated solution in a batch contactor under laboratory condition. Optimum conditions for removal of mercury (II) were determined by altering the operating parameters. Recovery of mercury (II) from spent alginate beads was studied to check the possibility of reuse of the alginate bead and mercury (II).

The aims of the present work are to develop a new environment-friendly and less expensive adsorbent suitable for removal of mercury from industrial waste water and to check whether the adsorbed metal can be recovered from the spent adsorbent for reuse.

2. Methods

The techniques followed to perform experiments in the present study along with the materials used have been described below. All the experiments were carried out at least three times to ensure the reproducibility and accuracy of the data, checked by calculating the standard deviation. Arithmetic mean of the data has been presented. All chemicals, unless otherwise stated, were of AR grade.

2.1. Immobilization of papain on alginate beads and optimization of immobilization condition for preparation of Alginate Immobilized Papain (AIP)

Papain (SRL Pvt. Ltd.) was immobilized on calcium alginate beads by ionotropic gelation method. The general method is as follows: sodium alginate (Loba Chemie Pvt. Ltd.) solution was pre-

pared by dissolving requisite amount of sodium alginate in distilled water and specific amount of papain was added to it. This mixture was then allowed to fall drop wise into an ice cold calcium chloride (Loba Chemie Pvt. Ltd.) solution having suitable concentration and was kept in refrigerator. After 30 min, the beads were taken out from the cold calcium chloride solution and stored in distilled water.

Specific Enzymatic Activity (SEA) was determined spectrophotometrically extending the standard assay method for applying it to the solid immobilized sample (Arson, 1970; Dutta et al., 2009). The protocol for estimation of SEA of immobilized papain sample was same as that described by Dutta et al. (2009). The only difference lies in the process is that here 0.5 g of AIP was incubated with the freshly prepared casein solution (Hammerstein quality, SRL). Remaining steps were same. To eliminate the external mass transfer resistance for bulk diffusion the solution was stirred using magnetic stirrer (Remi Equipments Pvt. Ltd., Model No. 1MLH) during the process. Concentration of the small peptides formed due to enzymatic action of papain immobilized on alginate beads was measured spectrophotometrically (UV 2300, TECHCOM) at 280 nm. SEA of immobilized papain sample has been expressed as [(g peptide formed)/(g papain × h)]. Micro-Kjeldahl method was used to determine the loading of papain in alginate bead (Miller and Houghton, 1945).

Design Expert Software (Version 7.1.6) was used to optimize the immobilization condition. Design Expert Software works on the principle of Response Surface Methodology (RSM) for determination of optimal response. A number of experiments have been performed to optimize experimental process-parameters using RSM (Hameed et al., 2008; Kalil et al., 2000; Ravikumar et al., 2006; Ravikumar et al., 2007; Sahu et al., 2009). Papain immobilized in optimized condition has been designated as Alginate Immobilized Papain (AIP) and used for further studies on mercury removal.

Concentrations of sodium alginate, papain and calcium chloride and pH were considered as operating (independent) parameters for immobilization of papain and their +1 and –1 levels were fixed separately at 20 and 50 g/L, 20 and 50 g/L, 20 and 50 g/L and 4 and 10, respectively. SEA of immobilized papain sample was regarded as the response or output variable. Response Surface Methodology (RSM) explores the relationships between independent variables and dependent response variables. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. The most popular RSM is the Central Composite

Design (CCD) which is an experimental design methodology for building a second order (quadratic) model for the response variables. Normally CCD consists of a 2^n factorial runs with $2n$ axial runs and n_c centre runs where n is the number of input parameters or numeric factors. To assess the effects of these four input independent parameters on the activity of immobilized papain sample, CCD was employed to design experiments statistically which consisted of 16 factorial points, eight axial points and six replicates at the centre points. This resulted in a total of 30 experiments which would have to be performed. Factorial points consist of all possible combination of +1 and -1 levels of the factors. The axial points were located at $(\pm\alpha, 0, 0, 0)$; $(0, \pm\alpha, 0, 0)$; $(0, 0, \pm\alpha, 0)$ and $(0, 0, 0, \pm\alpha)$, where α was the distance of the axial point from the centre. The centre points $(0, 0, 0, 0)$ were repeated six times to determine the experimental error and reproducibility of the data. The condition having concentration of papain 35 g/L, concentration of alginate 35 g/L, pH 7 and concentration of calcium chloride 35 g/L was considered as the centre point. On completion of 30 experiments as designed by CCD and after determination of SEA of each immobilized sample, ANOVA was used to analyze the data for assessing the best immobilized condition. The immobilized sample obtained at best operating condition was designated as Alginate Immobilized Papain (AIP) and used for further studies.

2.2. Physicochemical characterization of AIP

Physicochemical characterization was done in terms of temperature and pH optima, temperature and pH stability and kinetic parameters for protein hydrolysis. Scanning electron microscopy was done to obtain the surface topography of AIP. Energy Dispersive X-ray Spectrometry (EDS) study was performed with AIP sample treated with mercury to check the existence of mercury.

2.2.1. Determination of temperature optima, pH optima, temperature stability and pH stability

Temperature optima and pH optima of AIP were determined by measuring SEA at different temperatures and pH following the protocol as described by Dutta et al. (2009). Temperature stability of AIP was assessed by exposing AIP to different temperatures (30–70 °C) for 1 h and enzyme assay was performed at 35 °C following the same protocol. For determination of pH stability, AIP was incubated in different pH (4–9) at room temperature for 1 h. It was then washed thoroughly with distilled water. SEA of washed AIP was determined at 35 °C at a constant pH (Dutta et al., 2009). The stability of AIP was determined on the basis of SEA.

2.2.2. Scanning electron microscopy and energy dispersive X-ray spectrometry

Scanning electron microscopy (SEM) was performed for calcium alginate, AIP and AIP with bound mercury separately to obtain the surface topography of these samples. The samples were coated with platinum for 60 s and were mounted on brass stubs using double-sided adhesive tape. SEM photographs were taken with scanning electron microscope (JSM 6700F, JEOL, Japan) at the required magnification at room temperature. The working distance of 25 mm was maintained and acceleration voltage used was 20 kV, with the secondary electron image (SEI) as a detector. Energy dispersive X-ray spectrometry (EDS) study was done for AIP bound with mercury using the same instrument.

2.2.3. Kinetic study of protein hydrolysis using AIP

The kinetics of the protein hydrolysis by AIP using casein as substrate were studied to determine the kinetic parameters like maximum forward velocity of the enzymatic reaction (V_{max}), rate constant for enzymatic reaction (k_2) and Michaelis–Menten constant (K_m). To eliminate the external mass transfer resistance for

bulk diffusion, the solution was stirred during reaction using magnetic stirrer (Remi Equipments Pvt. Ltd., Model No. 1MLH). The study was performed following the protocol as described by Dutta et al. (2009) using 0.5 g of AIP in solution.

2.3. Removal of mercury

The mechanism of mercury removal using papain immobilized on solid matrix has already been proved (Dutta et al., 2009). Thus, in the present study, emphasis was given on the kinetics and extent of removal of mercury using AIP. Batch mode contacting device was used to study the kinetics of depletion of mercury (II) from simulated solution of mercuric chloride (MERCK) using AIP. The effect of three parameters viz., initial concentration of mercury (II), amount of AIP and pH on the removal of mercury from its aqueous solution was examined by varying them in the range of 1–30 mg/L, 3–8 g and 5–9, respectively in a systematic manner. In each case the solution was stirred with magnetic stirrer (Remi Equipments Pvt. Ltd., Model No. 1MLH) to eliminate external mass transfer resistance associated with the bulk diffusion of mercury (II) to the surface of AIP. Samples were collected at particular intervals. The solid was separated from the solution by filtration under vacuum and the concentration of mercury (II) in the filtrate was analyzed using Atomic Absorption Spectroscopy (Varian AA240, Graphite Tube Atomizer GTA 120, Flame mode AA240).

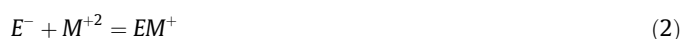
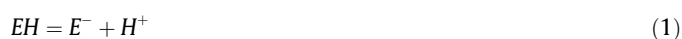
2.4. Recovery of mercury

Initially 5 g of AIP was incubated with mercuric chloride solution with the initial mercury (II) concentration of 10 mg/L for 10 min. The alginate beads containing AIP–mercury complex were then separated from the solution by filtration under vacuum. The beads were washed several times and kept for drying. The treated beads were incubated in buffer solution having various pH in the range of 4–9 for 10 min to examine the effect of pH on desorption. After incubation, the beads were separated from the solution and concentration of mercury (II) in the filtrate was analyzed using Atomic Absorption Spectroscopy.

3. Mathematical modeling

In the present study papain has been immobilized on alginate bead that has been used for metal removal. For development of mathematical model it is assumed that immobilization on alginate bead does not affect the ionization process of papain and the binding of metal with it. Thus, it can be presumed that papain bound with alginate behaves like free papain in solution. Experimental results also indicate that adsorption equilibrium is attained fairly rapidly within 8–10 min in general irrespective of the parameters (viz., initial concentration of mercury, pH of solution and weight of AIP). Therefore, a general equilibrium model has been proposed to express the reaction mechanism and finally, the value of equilibrium constant for binding of mercury with enzyme has been determined by nonlinear regression method.

The reactions involved in removal of mercury using papain may be written as follows:



Eq. (1) represents deprotonation of the thiol group present in papain and Eq. (2) represents binding of metal ion with the deprotonated thiol group of enzyme. The pK_a value for deprotonation of the thiol group has been reported as 7.55 (Wong, 1989) and corresponding value of equilibrium constant (K_1) is 2.818×10^{-8} . From

reaction (2), it is evident that after formation of EM^+ complex, bound mercury will no longer be available in solution. Value of the equilibrium constant (K_2) for the second reaction has to be determined.

The value of K_2 physically signifies the strength/extent of metal–enzyme binding taking place during adsorption. Dissociation of the enzyme (E), in this case papain, into E^- form (due to dissociation of the $-SH$ group of the cysteine residue in this case) is an established phenomenon. The reaction is usually fast and the equilibrium is reached quickly. Binding of metal ion with the dissociated state E^- is ionic in nature and therefore expected to be fast. According to Sluyterman and Wijdenes (1976), the reaction is reversible. These are the considerations for assuming the equilibrium reaction (2), which represents binding of metal ion to E^- and their dissociation, as part of the proposed mechanism. K_2 represents the equilibrium constant of the aforementioned reaction.

From Eqs. (1) and (2) it can be written,

$$K_1 = \frac{[E^-][H^+]}{[EH]} \quad (3)$$

$$K_2 = \frac{[EM^+]}{[E^-][M^{+2}]} \quad (4)$$

The overall mass balance for enzyme can be written as follows:

$$[E_0] = [E^-] + [EH] + [EM^+] \quad (5)$$

where $[EH]$ = concentration of undissociated papain, $[E^-]$ = concentration of deprotonated papain, $[H^+]$ = concentration of hydrogen ion, $[EM^+]$ = concentration of metal which binds with papain, $[M^{+2}]$ = final equilibrium concentration of metal in solution after contacting with papain and $[E_0]$ = loading of papain in the solution.

The value of $[E_0]$ can be calculated as follows:

$$[E_0] = \frac{W(AIP)\beta}{V}$$

where $W(AIP)$ = weight of AIP taken in solution, β = loading of papain in alginate bead and V = volume of solution. The β value has been found to be 2.17×10^{-6} g mol papain/g AIP and volume of solution in each case has been kept at 30 ml.

Eliminating the unknown concentrations viz., $[E^-]$ and $[EH]$ from Eqs. (3)–(5) and on rearrangement, one can get the following equation:

$$K_1[EM^+] + [EM^+][H^+] + K_1K_2[M^{+2}][EM^+] - K_1K_2[E_0][M^{+2}] = 0 \quad (6)$$

$$\text{Again one can write } [EM^+] = [M_0^{+2}] - [M^{+2}] \quad (7)$$

where $[M_0^{+2}]$ is the initial concentration of metal in solution.

Substituting Eq. (7) in Eq. (6) and solving for K_2 one can write,

$$K_2 = \frac{\{[M_0^{+2}] - [M^{+2}]\}\{K_1 + [H^+]\}}{K_1[M^{+2}]\{[E_0] - [M_0^{+2}] + [M^{+2}]\}} \quad (8)$$

Again, on rearrangement Eq. (8) can be expressed as quadratic equation of $[M^{+2}]$ as follows:

$$\begin{aligned} K_1K_2[M^{+2}]^2 + [M^{+2}]\{K_1 + [H^+] + K_1K_2[E_0] - K_1K_2[M_0^{+2}]\} \\ - \{K_1[M_0^{+2}] + [M_0^{+2}][H^+]\} \\ = 0 \end{aligned} \quad (9)$$

4. Results and discussion

4.1. Immobilization of papain on alginate beads and optimization of immobilization condition for preparation of Alginate Immobilized Papain (AIP)

Optimum conditions for immobilization were determined using Design Expert Software (Version 7.1.6). The list of experiments as designed by RSM and the values of response for each sample obtained at corresponding experimental conditions are shown in Ta-

Table 1
Experimental design and results.

Std	Run	Factor 1 A:A (g/L)	Factor 2 B:B (g/L)	Factor 3 C:C	Factor 4 D:D (g/L)	Response1 R1
8	1	50.00	50.00	10.00	20.00	448.744×10^{-5}
3	2	20.00	50.00	4.00	20.00	264.483×10^{-5}
1	3	20.00	20.00	4.00	20.00	426.182×10^{-5}
17	4	5.00	35.00	7.00	35.00	34.011×10^{-5}
25	5	35.00	35.00	7.00	35.00	770.887×10^{-5}
9	6	20.00	20.00	4.00	50.00	666.849×10^{-5}
4	7	50.00	50.00	4.00	20.00	409.18×10^{-5}
19	8	35.00	5.00	7.00	35.00	956.402×10^{-5}
14	9	50.00	20.00	10.00	50.00	1076.74×10^{-5}
10	10	50.00	20.00	4.00	50.00	722.002×10^{-5}
26	11	35.00	35.00	7.00	35.00	770.887×10^{-5}
5	12	20.00	20.00	10.00	20.00	72.419×10^{-5}
28	13	35.00	35.00	7.00	35.00	770.887×10^{-5}
12	14	50.00	50.00	4.00	50.00	601.668×10^{-5}
27	15	35.00	35.00	7.00	35.00	770.887×10^{-5}
13	16	20.00	20.00	10.00	50.00	366.015×10^{-5}
16	17	50.00	50.00	10.00	50.00	1079.24×10^{-5}
2	18	50.00	20.00	4.00	20.00	880.21×10^{-5}
20	19	35.00	65.00	7.00	35.00	668.103×10^{-5}
22	20	35.00	35.00	13.00	35.00	13.3×10^{-5}
24	21	35.00	35.00	7.00	65.00	1501.66×10^{-5}
18	22	65.00	35.00	7.00	35.00	842.336×10^{-5}
15	23	20.00	50.00	10.00	50.00	156.684×10^{-5}
21	24	35.00	35.00	1.00	35.00	21.2×10^{-5}
30	25	35.00	35.00	7.00	35.00	770.887×10^{-5}
11	26	20.00	50.00	4.00	50.00	195.542×10^{-5}
6	27	50.00	20.00	10.00	20.00	833.561×10^{-5}
23	28	35.00	35.00	7.00	5.00	1366.29×10^{-5}
29	29	35.00	35.00	7.00	35.00	770.887×10^{-5}
7	30	20.00	50.00	10.00	20.00	169.219×10^{-5}

ble 1. The regression analysis was performed to fit the response. As suggested by the software, square root transformation was chosen and quadratic process order was selected to analyze data. The final regression function for SEA in terms of coded factors is given below:

$$\begin{aligned} \sqrt{R1} = & +0.088 + 0.018 \times A - 6.315E - 003 \times B - 1.454E \\ & - 003 \times C + 4.443E - 003 \times D - 2.163E - 004 \times A \times B \\ & + 7.505E - 003 \times A \times C + 8.069E - 004 \times A \times D + 3.604E \\ & - 003 \times B \times C - 7.404E - 004 \times B \times D + 4.217E \\ & - 003 \times C \times D - 8.314E - 003 \times A^2 + 3.503E - 004 \times B^2 \\ & - 0.019 \times C^2 + 7.837E - 003 \times D^2 \end{aligned} \quad (10)$$

where R1 represents SEA (g peptide formed)/(g papain × hr) A = concentration of papain (g/L), B = concentration of sodium alginate (g/L), C = pH, D = concentration of calcium chloride (g/L).

Based on the analysis done by ANOVA, it has been observed that three input parameters viz., concentration of papain, concentration of sodium alginate and concentration of calcium chloride have significant individual effect on response i.e., SEA of immobilized papain sample. It has also been observed that the quadratic effects of concentration of papain, pH, concentration of calcium chloride and the interaction effect between concentration of papain and pH on response factor are quite significant. The conjugate effects of pH and concentration of papain are shown in Fig. 1. It is found that with increase in concentration of papain, SEA increases monotonously, whereas in case of pH, SEA shows a different pattern. Initially SEA increases up to the value of pH 7 and then decreases with further increase in pH. An increase in papain concentration in solution increases the probability of immobilization of papain on alginate bead which leads to higher SEA values. On the other hand, there is an optimum value for pH, in this case 7, which gives the best immobilization of papain on alginate and hence the highest SEA. The findings result in a convex surface. In both the cases, concentration of sodium alginate and concentration of calcium chloride are fixed at zero level i.e., both the concentrations have the values of 35 g/L. The maximum value of SEA is obtained when concentration of papain is 50 g/L and at pH 7.

The major diagnostic method is residual analysis. The plot of actual vs. predicted response (Fig. 2) displays the real response data against the predicted response. Points above and below the diagonal line are the areas of over and under prediction. Actual values

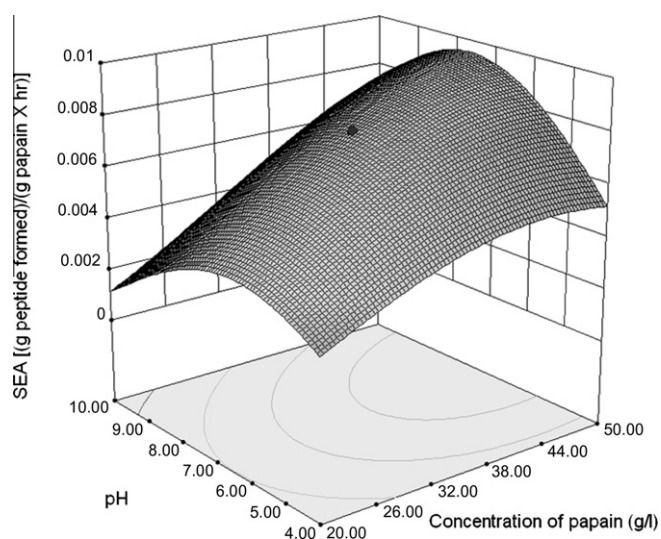


Fig. 1. Conjugate effect of pH and concentration of papain on SEA of immobilized papain sample.

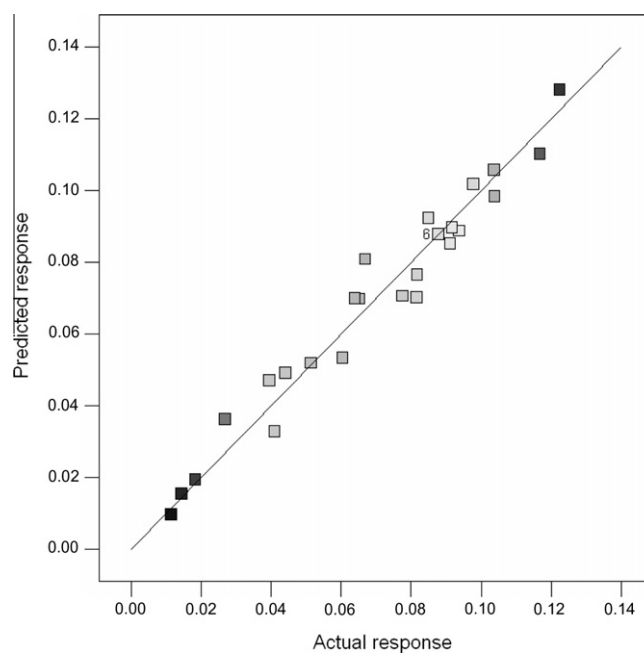


Fig. 2. Actual vs. predicted response plot.

are the measured response data for a particular run, and the predicted values are evaluated from the model and are generated by using the approximating functions. According to the Design Expert Software, the optimized condition for immobilization of papain on alginate bead is obtained when concentration of papain, concentration of sodium alginate, concentration of calcium chloride and pH have the values of 25.96 g/L, 20 g/L, 20 g/L and 7, respectively. The immobilized sample obtained at this condition has been termed as Alginate Immobilized Papain (AIP) and used for further studies on mercury removal.

4.2. Physicochemical characterization of AIP

4.2.1. Determination of temperature optima, pH optima, temperature stability and pH stability

Studies on physicochemical characterization of AIP reveal that temperature optima and pH optima of AIP are 70 °C and 5, respectively. Temperature has a direct effect on the value of diffusivity. Diffusivity increases with an increase in temperature. Thus at higher temperature, diffusion of the substrate (casein) to inner core of AIP happens to be more and this has been substantiated by the higher value of SEA. Though the optimum temperature for enzyme action has been found to be 70 °C further experiments with AIP have been carried out at 35 °C to reduce the cost of heating. The pH is maintained at 7 for further experiments instead of the optimum pH 5 to make the process simple. AIP is found to be stable between a wide range of temperature (4–70 °C) and pH (2–12).

4.2.2. Scanning electron microscopy and energy dispersive X-ray spectrometry

SEM study was performed to check topographical changes, if there is any, in porous nature of alginate bead due to immobilization of papain and binding of mercury (II) to the immobilized papain. SEM pictures of a calcium alginate bead with immobilized papain (AIP) shows a smoother surface than that of calcium alginate bead (figures not shown). The smoothing effect was even more in the mercury-bound AIP sample (figures not shown) which can be considered as an indication of binding of mercury with papain. EDS study shows a peak of mercury in the mercury-bound AIP

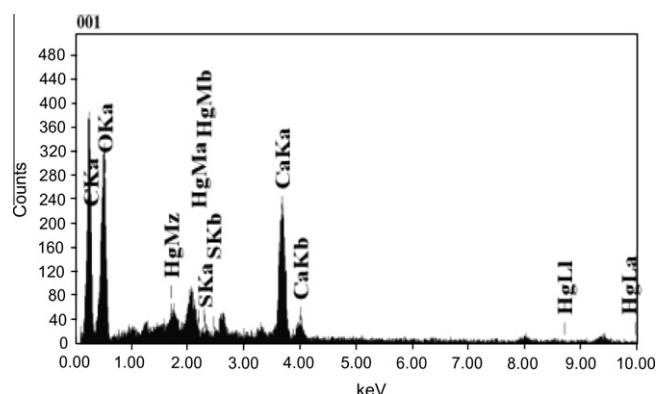


Fig. 3. EDS picture of AIP bound with mercury.

sample which gives the evidence for binding of mercury with AIP (Fig. 3)

4.2.3. Kinetic study of protein hydrolysis using AIP

The kinetic study of protein hydrolysis by AIP has been performed to determine the kinetic parameters like V_{max} , k_2 and K_m . The kinetic parameters are calculated by Lineweaver–Burk method and Eadie–Hofstee method. Kinetic parameters of free enzyme and immobilized enzyme are shown in Table 2.

The kinetic parameter V_{max} indicates the maximum velocity of product formation. The other parameter K_m gives the amount of substrate required to reach half the maximum velocity signifying affinity of the enzyme towards its substrate. After immobilization of papain the V_{max} value is decreased 10 times leading to a lower rate of product formation. K_m is increased 10 times due to immobilization which indicates that affinity of the enzyme towards its substrate is reduced after immobilization. The finding is in agreement with Li et al. (2001) who reported lower K_m value after immobilization of papain on nitrilon fiber. The rate constant k_2 represents rate of breakdown of enzyme–substrate complex to product(s). The value has been found to be decreased after immobilization.

4.3. Removal of mercury

To investigate the efficacy of AIP as a means of mercury (II) eradication from waste water and to substantiate the theory stated in Section 3, a systematic and thorough experimentation has been done by contacting the simulated solution of mercuric chloride with AIP in a batch contactor. Three parameters viz., the initial concentration of mercury (II) in solution (1–30 mg/L), amount of AIP (3–8 g) and pH (5–9) have been varied during the experimentation in a prescribed manner.

In Fig. 4, the percentage removal of mercury (II) obtained experimentally by varying the initial concentration of solution in the range of 1–30 mg/L have been plotted against time keeping all other parameters like weight of AIP, pH and temperature constant at 5 g, 7 and 35 °C, respectively. Figure shows that in all the cases, maximum removal has been achieved approximately within 8 min which indicates that systems have reached their saturation/equi-

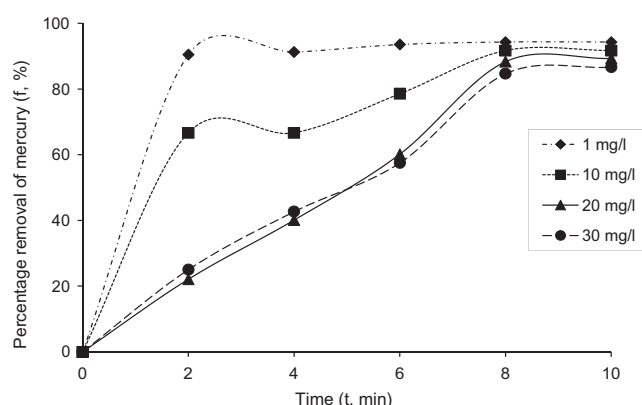


Fig. 4. Time histories of percentage removal of mercury using AIP with initial concentration of mercury as parameter. In all cases weight of AIP = 5 g, pH 7 and temperature = 35 °C.

librium values within that specific time. It is also seen from the Fig. 4 that for lower initial concentration of mercury (II) like, 1 mg/L, the system has attained its saturation value within 2 min whereas, for higher concentration it takes longer time. Therefore, in general it can be stated that for lower initial concentration (1 mg/L) of mercury (II), attainment of the saturation value is much faster than that for the higher ones. This may be due to the fact that for lower concentration mercury (II) can bind with E^- easily whereas, for the higher concentrations free mercury (II) faces EM^+ complex more readily than it faces E^- . 94.31% mercury (II) has been removed when solution having 1 mg/L initial mercury (II) concentration is contacted with 5 g AIP at pH 7 and 35 °C.

Fig. 5 represents the time histories of percentage removal of mercury (II) using varying amount of AIP ranging from 3 to 8 g when all other parameters like initial concentration of mercury

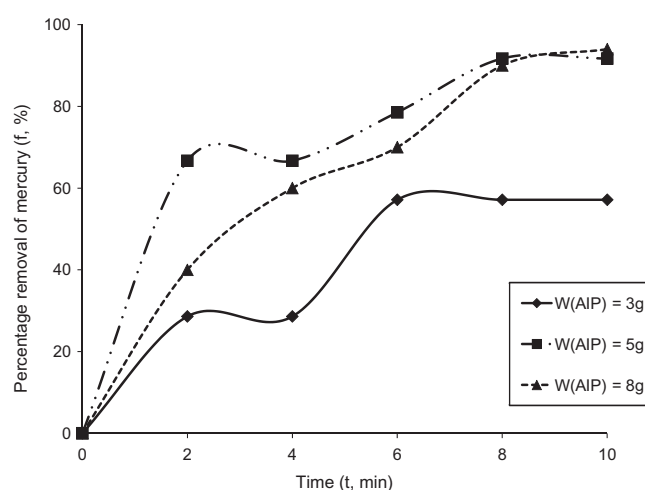


Fig. 5. Time histories of percentage removal of mercury using AIP with weight of AIP as parameter. In all cases initial concentration of mercury = 10 mg/L, pH 7 and temperature = 35 °C.

Table 2
Values of kinetic parameters.

	Lineweaver–Burk equation				Eadie–Hofstee equation			
	V_{max}	K_m	k_2	R^2	V_{max}	K_m	k_2	R^2
Free papain	0.897	0.439	35.88	0.991	0.890	0.429	35.6	0.943
AIP	0.018	5.609	0.712	0.999	0.017	5.537	0.68	0.997

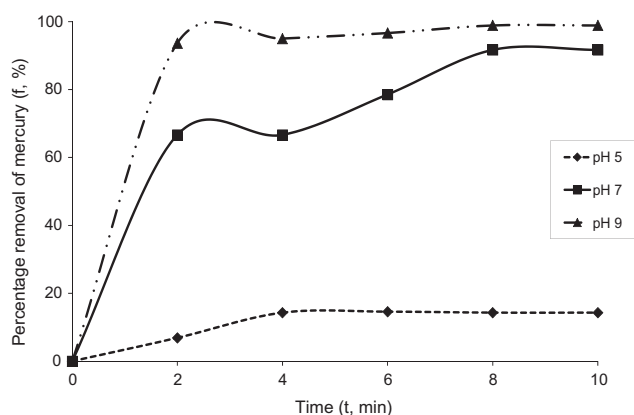


Fig. 6. Time histories of percentage removal of mercury using AIP with pH as parameter. In all cases initial concentration of mercury = 10 mg/L, weight of AIP = 5 g, and temperature = 35 °C.

(II), pH and temperature are kept constant at 10 mg/L, 7 and 35 °C. It is evident from the figure that with increase in the amount of AIP from 3 to 8 g, the percentage removal of mercury (II) from solution has been increased from 50% to 80%. The same pattern was observed with CIP [Dutta et al., 2009]. This may be due to the availability of more amount of enzyme (papain) in the system which acts as the reactant of the reaction between enzyme and metal as depicted by Eq. (2). Here too the maximum possible removal has been achieved within 8 min.

Fig. 6 embodies the effect of pH on the removal of mercury (II) from simulated solution. The percentage removal of mercury (II) is plotted against time considering pH as a parameter by varying it in the range of 5–9 keeping all other parameters viz., initial concentration of solution, weight of AIP and temperature at 10 mg/L, 5 g and 35 °C, respectively. From the figure it is seen that pH has a direct effect on percentage removal of mercury (II). With an increase of pH of the solution from 5 to 9, percentage removal of mercury (II) has been increased from 14.28% to 98.88%. As represented by Eq. (1), higher pH favors the removal of mercury (II) due to formation of more deprotonated enzyme molecule (E^-), which is capable of binding metal. On the other hand, lower pH favours the backward reaction leading to formation of more un-ionized enzyme molecules which are incapable to bind metal. These findings are in agreement with observations made by Dutta et al. (2009). The maximum adsorption capacity of AIP based on experimental findings has been found to be 0.156 mg Hg/g AIP with 0.05 g papain loading.

4.4. Recovery of mercury

Recovery of mercury (II) from the spent AIP has been carried out to check the reusability of AIP. This is important because otherwise AIP would gradually accumulate mercury and finally get saturated with the bound metal ions. This would result in loss of metals binding capacity of AIP and create a disposal problem.

Table 3
Determination of K_2 .

Sl no.	pH	$[H^+]$	$[M_0^{+2}]$	W(AIP)	$[E_0]$	$[M^{+2}]$	$[M_{theo}^{+2}]$	Error	$(\sum error^2)$	K_2
1	7	1×10^{-7}	1	5	3.623188E-04	0.05693	9.11E-02	3.42E-02	7.28E-01	126797.3
2	7	1×10^{-7}	13.5	5	3.623188E-04	1.12	1.43E+00	3.13E-01		
3	7	1E-07	18.7	5	3.623188E-04	2	2.13E+00	1.26E-01		
4	7	1E-07	30	5	3.623188E-04	4	4.01E+00	6.57E-03		
5	7	1E-07	10.5	3	2.173913E-04	2.5	1.79E+00	-7.05E-01		
6	7	1×10^{-7}	7.5	8	5.797101E-04	0.45	4.63E-01	1.35E-02		
7	5	1×10^{-5}	10.5	5	3.623188E-04	9	9.32E+00	3.17E-01		
8	9	1E-09	9	5	3.623188E-04	0.1	2.25E-01	1.25E-01		

The experiments to study recovery of mercury have been carried out at three different pH, viz., 4, 7 and 9. Maximum amount of mercury was recovered at pH 4 (96%) whereas, at relatively higher pH like 7 and 9, recovery of mercury was negligibly small (0.5%). This is in accordance with the fact that at lower pH, papain reduces its metal binding property as depicted by Eqs. (1) and (2) whereas at higher pH, due to formation of more amount of deprotonated enzyme molecule, probability of formation of metal-enzyme complex is more and thereby, chances of recovery of metal ion is less. These findings are in good agreement with the observations made by Dutta et al. (2009).

Papain immobilized on alginate beads may be a better choice than CIP for removal of mercury. On activated charcoal, papain gets immobilized on the surface by physical adsorption method, whereas, in calcium alginate papain gets entrapped inside the calcium beads during immobilization. Since papain is immobilized on the surface of activated charcoal therefore, there is a scope for mercury to leach out from CIP after binding. A recovery of 45.8% adsorbed mercury at pH 7 indicates that there is a possibility of leakage of mercury ions during an actual continuous process or during any neutral wash. On the other hand, papain is entrapped within calcium alginate beads and hence the chance of leaching of mercury is low once mercury gets bound with immobilized papain. This results in higher effective removal of mercury by papain immobilized on alginate while using for industrial purposes.

4.5. Determination of K_2

The errors and minimization principle are as follows:

$$error = [M_{theo}^{+2}] - [M^{+2}] \quad (11)$$

$$\sum error^2 = \sum \{ [M_{theo}^{+2}] - [M^{+2}] \}^2 \quad (12)$$

$$\frac{\partial \sum error^2}{\partial K_2} = 0 \quad (13)$$

The values of theoretically calculated final equilibrium concentration of metal i.e., $[M_{theo}^{+2}]$ have been obtained using Eq. (9).

Initial estimates of K_2 have been obtained from Eq. (8) using known values of K_1 , $[M^{+2}]$, $[M_0^{+2}]$, $[E_0]$ and $[H^+]$ for different experimental runs. Final value of K_2 has been determined by minimizing 'Sum of Error Square' i.e., $(\sum error^2)$ with respect to K_2 by solving Eq. (13) numerically. Table 3 represents those data. The high value of K_2 obtained indicates that forward reaction of Eq. (2) is favored, which in turn, indicates a strong binding between mercury (II) and AIP.

5. Conclusion

In the present study, papain has been immobilized on calcium alginate bead. The immobilization condition was optimized by Design Expert Software (Version 7.1.6). The immobilized sample obtained at optimum condition is designated as AIP and used for

removal of mercury (II) from simulated solution. Best removal (98.88%) was obtained when 30 ml of mercury (II) solution of concentration of 10 mg/L was kept with 5 g AIP pH 7 and 35 °C for 8 min. The adsorbed mercury (II) can be recovered from the spent alginate beads at low pH. The work leads to the development of a new adsorbent for removal of mercury using a low-cost enzyme.

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