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Removal of mercury from its aqueous solution using charcoal-immobilized papain (*CIP*)

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ABSTRACT

In the present work mercury has been eradicated from its aqueous solution using papain, immobilized on activated charcoal by physical adsorption method. Operating parameters for adsorption of papain on activated charcoal like pH, amount of activated charcoal, initial concentration of papain in solution have been varied in a suitable manner for standardization of operating conditions for obtaining the best immobilized papain sample based on their specific enzymatic activity. The immobilized papain sample obtained at initial papain concentration 40.0 g/L, activated charcoal amount 0.5 g and pH 7 shows the best specific enzymatic activity. This sample has been designated as charcoal-immobilized papain (*CIP*) and used for further studies of mercury removal. Adsorption equilibrium data fit most satisfactorily with the Langmuir isotherm model for adsorption of papain on activated charcoal. Physicochemical characterization of *CIP* has been done. The removal of mercury from its simulated solution of mercuric chloride using *CIP* has been studied in a lab-scale batch contactor. The operating parameters viz., the initial concentration of mercury in solution, amount of *CIP* and pH have been varied in a prescribed manner. Maximum removal achieved in the batch study was about 99.4% at pH 7, when initial metal concentration and weight of *CIP* were 20.0 mg/L and 0.03 g respectively. Finally, the study of desorption of mercury has been performed at different pH values for assessment of recovery process of mercury. The results thus obtained have been found to be satisfactory.

1. Introduction

Mercury pollution is a global problem due to its wide distribution in nature and its toxicity to all forms of life ranging from bacteria to higher eukaryotes like plants and mammals. The total global input of mercury has been estimated to be 10^{10} g [1,2]. Chloro-alkali industries are the major sources for mercury pollution. Other industries discharging mercury-contaminated wastewaters include mining, smelting, tars and asphalt, coke ovens, textiles and those manufacturing cements, catalysts, paints, pesticides, pharmaceuticals and batteries. Thus, it is mandatory to abate mercury from industrial effluents before it is discharged to environment. Though bulk techniques like simple filtration or precipitation are suitable for removing a significant fraction of the metal, they are unable to decrease the concentration of contaminant from percentage to ppm level even in ppb level [3]. Thus, there is a burning need for a suitable finishing step which can remediate the metal in ppb level to meet the environmental agency regulations. Currently, the most common chemical modes of metal removal as a polishing step include ion exchangers or removal by chelation with synthetic crown ethers or other macrocyclic cage molecules [3]. The most significant drawback associated with typical ion exchangers is the lack of selectivity in metal binding and/or weak binding characteristics. While crown ethers are both selective and strong binders, they often exhibit slow release kinetics. This is a potential problem when metal reclamation is required. In addition, many crown ethers are also very toxic, so using them may simply add to the problem of contamination [3]. As a result of inherent problems with most of the current metal remediation strategies, researches are now turning toward natural systems. A number of studies have been performed in this line [4–10]. In addition to this, bioremediation with microorganisms can be carried out, but still that has the problem of disposal of the biofilms or filters. Thus, an environment-friendly, cost-effective, integrated, end-of-pipe remediation technology having suitable option of metal recovery are awaited for a long time.

In the present work a new technology, viz., immobilized enzyme technology has been employed for abatement of mercury from

Abbreviations: CIP, charcoal-immobilized papain; SEM, scanning electron microscope; *SEA*, specific enzymatic activity; TOC, total organic carbon; *f*, percentage removal of mercury.

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Nomenclature

C_0	Initial papain concentration in solution (g/L)
C_e	Papain concentration in solution at equilibrium condition (g/L)
C_{M0}	Initial concentration of mercury in solution (mg/L)
C_M	Final concentration of mercury in solution (mg/L)
f	Percentage removal of mercury ($f = [(C_{M0} - C_M)/C_{M0}] \times 100$)
k_2	Rate constant for enzymatic reaction [(g peptide formed)/(g papain^2 × h)]
K_F	Adsorption equilibrium constant used in Freundlich adsorption isotherm model (g papain/g activated charcoal)(g papain/L)^n.
K_L	Adsorption equilibrium constant used in Langmuir isotherm (L/g papain)
K_m	Michaelis–Menten constant (g casein/L)
n	Adsorption equilibrium constant used in Freundlich adsorption isotherm
q_e	Solid phase concentration of papain at equilibrium condition (g papain/g activated charcoal)
q^0	Solid phase concentration of papain for complete monolayer formation (g papain/g activated charcoal)
SEA	Specific enzymatic activity [(g peptide formed)/(g papain × h)]
T	Temperature (°C)
t	Time (min)
V_{max}	Max. forward velocity of the enzymatic reaction [(g peptide formed)/(g papain × h)]
W	Weight of activated charcoal (g)
W_{CIP}	Weight of charcoal-immobilized papain (g)

its aqueous solution. Papain, a proteolytic enzyme with a molecular weight of 23,000, is characterized as having a high degree of metal binding property due to the presence of four sulfhydryl (–SH) groups. Like all cysteine proteases, the catalytic activity of papain arises from the presence of catalytic cysteine and histidine residues at the active centre. Researches show that papain has the potential to bind heavy metals. Since the enzyme posseses an –SH group at the active site, its activity is altered due to binding of heavy metals at that site. The inhibitory effect of mercury ion to enzymatic action of papain proves the binding capability of mercury ion with papain. Moreover, with a high affinity towards organic compounds mercury has the possibility to bind with other amino acids [11–13]. Hence, papain, immobilized on suitable matrix, can be used in polishing treatment of industrial wastes for removal of mercury. Activated charcoal is unique, versatile and low-cost adsorbent having high surface area and high adsorption capacity [14]. Thus in the present study activated charcoal has been selected as the solid matrix for immobilization of papain. Papain has been immobilized on activated charcoal by physical adsorption method. An extensive experimental work has been carried out on immobilization of papain to establish the optimum process parameters in terms of initial papain concentration, amount of activated charcoal, pH, etc. The sample that shows the best enzymatic activity has been selected as the adsorbent for further studies of removal of mercury and has been designated as charcoal-immobilized papain or '*CIP*'. Physico-chemical characterization of *CIP* has then been done in terms of optimum pH, optimum temperature, pH stability and temperature stability, shelf life, etc. Topographical characterization has been performed by scanning electron microscope (SEM). To assess the enzymatic action of papain immobilized on activated charcoal, the kinetic study of protein hydrolysis by *CIP* using casein as substrate has been done. The values of 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) have been estimated using both Lineweaver–Burk method and Eadie–Hofstee method [15–20]. To study the mercury removal efficacy of *CIP*, an extensive investigation has been performed with an aim to develop a new cutting edge technology for mercury removal from its simulated solution of mercuric chloride by contacting the solution with *CIP* in a batch contactor. Mercuric chloride being a salt of weak base and strong acid should ionize in its aqueous solution as mercuric ion (Hg^{2+}) and chloride ion (Cl^-). When aqueous solution of mercuric chloride has been contacted with charcoal-immobilized papain (*CIP*), mercuric ion can bind with the thiol group of cysteine residue of immobilized papain molecule in addition to the physical adsorption on the surface of activated charcoal. Operating parameters like the initial concentration of mercury, the amount of *CIP* and pH have been varied in a prescribed manner. The result reveals that mercury can be removed maximum about 99.4% from its aqueous solution using *CIP* at pH 7, when initial metal concentration and weight of *CIP* were 20.0 mg/L and 0.03 g respectively. Any removal process of metal remains incomplete unless the recovery process has not been studied. In the present article to assess the efficiency of the process, the desorption of mercury from *CIP*–mercury complex has also been investigated at different pH values. It has been seen that low pH facilitates the desorption process which is in agreement with the observation made by Sluyterman and Wijdenes [12].

Therefore, it can be stated that papain, having the characteristics of metal binding due to presence of four sulfhydryl groups in its active sites, can be used to modify the free matter space of activated charcoal, a micro-porous solid matrix, and thereby increasing the number of active sites of charcoal for the removal of mercury from its aqueous solution.

2. Materials and methods

The procedure followed to carry out the experimental work in the present investigation along with the materials required for performing those experiments 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 have been presented. All chemicals, unless otherwise stated, were of AR grade.

2.1. Immobilization of papain on activated charcoal and selection of the best operating condition for preparation of charcoal-immobilized papain (CIP)

In the present work papain (SRL) had been immobilized over the activated charcoal (MERCK), selected as solid matrix for the immobilization of papain, by physical adsorption method. Different properties of activated charcoal (MERCK) were done by standard methods [21]. Pore size distribution of activated charcoal was tested using mercury intrusion method (Poremaster 60, Quantachrome) from 0 to 60,000 psi. The solution of papain of desired concentration was prepared by dissolving papain (SRL) in distilled water. For immobilization, the papain solution was stirred with specific amount of activated charcoal for 60 min at room temperature in a glass vessel. The solid sample obtained after separating it from solution by vacuum filtration method, was taken in a petridish and dried. Then the specific enzymatic activity of immobilized papain sample was determined spectrophotometrically extending the standard assay method for applying it to the solid sample containing immobilized enzyme [22]. As immobilized papain sample forms a heterogeneous mixture with casein

solution unlike free enzyme, there is a mass transfer resistance associated with the diffusion of substrate (casein) to the surface of solid sample for reaction with immobilized enzyme (papain). The mass transfer resistance can be eliminated by vigorous stirring of the solution during experimentation and this was done in each case for studying the enzymatic reaction rate with immobilized papain sample using cyclomixer (Remi Equipments Private Limited, Model No. CM 101). The protocol for estimation of specific enzymatic activity of immobilized papain sample was as follows: 0.03 g of immobilized papain sample was incubated with specific amount of substrate for 20 min at 35 °C. Freshly prepared casein solution (Hammarsten quality, SRL) (10.0 g/L) was used as substrate. EDTA–cysteine reagent was used as an activating agent and the pH of the solution was maintained with 0.1 M Tris–HCl buffer. During incubation period, the solution was vigorously stirred to eliminate the mass transfer resistance and to make the process reaction-rate-controlled. After incubation period the enzymatic action was terminated with 100.0 g/L trichloroacetic acid (TCA). Small peptides formed by enzymatic action of papain present in solid sample were separated from the unreacted protein by centrifugation and was measured spectrophotometrically (UV-VIS-NIR Spectrophotometer, U4100, HITACHI) at 280 nm. The same protocol was followed for 'zero hour' tubes. In 'zero hour' tubes the enzymatic action of sample was stopped by adding 100.0 g/L TCA before addition of substrate. The specific enzymatic activity (*SEA*) of immobilized papain sample has been expressed as [(g peptide formed)/(g papain × h)].

Amount of activated charcoal (0.3–1.0 g), initial concentration of papain in the solution (10.0–50.0 g/L) and pH (5–9) were varied individually during adsorption process in a prescribed manner. Effects of these various parameters on the activity of immobilized papain sample were examined. The sample that showed the best enzymatic activity was selected as the adsorbent for removal of mercury. The selected sample has been designated as charcoal-immobilized papain or '*CIP*' in the rest of this article.

2.2. Determination of loading of papain on activated charcoal using total organic carbon (TOC) analyzer and study of adsorption isotherm

In the present study loading of papain on activated charcoal was tested using differential method. When the solution of pure papain was tested using TOC analyzer, results demonstrate total organic carbon content of the solution [23,24]. After immobilization, the solution left in the container showed less amount of total organic carbon content. Thus, difference in the amount of TOC gives the amount of loading of papain on the activated charcoal. The protocol was as follows: The pure papain solution was tested for measurement of total organic carbon content using TOC analyzer (Online TOC – VCSH, SHIMADZU). Then papain solution of desired concentration was stirred with specific amount of activated charcoal for immobilization. The supernatant obtained after the centrifugation of the solution for separation of the activated charcoal loaded with papain, was tested for the measurement of total organic carbon content using the same instrument. This represents the measurement of concentration of papain which had not been adsorbed on the activated charcoal. The difference in values represents the amount of papain immobilized on activated charcoal in terms of total organic carbon content. Finally the TOC can be translated to the papain concentration from the value of total organic carbon content obtained for the pure papain solution of known concentration by TOC analysis.

In studying adsorption isotherm for adsorption of papain on activated charcoal, the papain solutions of varying concentrations (10.0–50.0 g/L) had been equilibrated with specific amount of activated charcoal (0.5 g) for a period of 2 h in an auto-temperature controlled shaker at 35 °C. As stated earlier, liquid phase concentration of papain remaining in the solution after adsorption was determined using TOC analyzer (Online TOC – VCSH, SHIMADZU) and from mass balance the solid phase concentration of papain was determined. Both the data were expressed in terms of papain concentration, translated from TOC concentration by the method as described earlier.

2.3. Physico-chemical characterization of CIP

Physico-chemical characterization of *CIP* was performed to find out optimum pH, optimum temperature, pH stability and temperature stability following standard methods. For assessing the shelf life period of *CIP*, stored at 30 °C, the enzymatic activity of *CIP* was determined using the standard method in every month up to six months. Casein (Hammarsten quality, SRL) was used as substrate in each case.

2.3.1. Determination of temperature optima and pH optima

0.03 g of *CIP* was used for determination of temperature optima. The specific enzymatic activity (*SEA*) of *CIP* was measured at different temperatures (35, 50 and 70 °C) at a constant pH following standard procedure (as described in Section 2.1). To determine the pH optima, *SEA* was determined at 35 °C using 0.03 g of *CIP* at different pH ranging from 5 to 9.

2.3.2. Determination of temperature stability and pH stability

To determine the temperature stability, 0.03 g of *CIP* was exposed to different temperatures separately like 4, 50 and 70 °C for 1 h. Then the enzyme assay was performed at 35 °C following the standard procedure (as described in Section 2.1). For pH stability, 0.03 g *CIP* was exposed to pH 5, 7 and 9 for 1 h. The *CIP* was washed and collected as the pellet after centrifugation. Enzyme assay was performed following the same procedure at 35 °C.

2.3.3. Scanning electron microscopy (SEM)

SEM studies of both activated charcoal and *CIP* were performed to obtain their topographical characterization. The samples were mounted on brass stubs using double-sided adhesive tape. SEM photographs were taken with scanning electron microscope (LEO – S440, UK) at the required magnification at room temperature. The working distance of 25 mm was maintained and acceleration voltage used was 15 kV, with the secondary electron image (SEI) as a detector.

2.3.4. Kinetic study of protein hydrolysis using CIP

The kinetics of the protein hydrolysis by *CIP* 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 get the proper values of these kinetic parameters, the experiment was carried out in a fashion so as to make the process reaction-rate controlled rather than mass-transfer controlled. As stated earlier, to make the system reaction-rate controlled, the solution was stirred vigorously during the experimentation using cyclomixer (Remi Equipments Pvt. Ltd., Model No. CM 201). The protocol for studying kinetics of protein hydrolysis was same as that described for determination of specific enzymatic activity in the Section 2.1. The only difference lies in the process is that here 0.03 g of *CIP* was incubated with the various amount of substrate. Remaining steps were same. The specific enzymatic activity vis-à-vis the velocity of enzymatic reaction using *CIP* was then evaluated for each case.

2.3.4.1. Data analysis of kinetic study of protein hydrolysis using CIP. Among the various approaches for graphical determination of

kinetic parameters (e.g., V_{max}, k_2, K_m), the most widely employed method is the Lineweaver–Burk method [19,20]. It is based on a double reciprocal plot of Michaelis–Menten equation and takes the form as follows:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{[S]}$$

where V is the initial velocity of enzymatic reaction and $[S]$ is the initial substrate concentration.

A plot of $1/V$ vs. $1/[S]$ gives the straight line. The intercepts on the X and Y axes give the values of $-1/K_m$ and $1/V_{max}$ respectively. Value of k_2 can be obtained from the following equation:

$$V_{max} = k_2[E_0]$$

where $[E_0]$ is the initial concentration of enzyme.

As the most accurate data are obtained at high values of substrate concentration, the Lineweaver–Burk plot is most suitable for determination of V_{max}, but it is less accurate for determination of K_m [19,20].

To overcome this difficulty, Eadie–Hofstee plot is used. It does not unduly emphasize points at low substrate concentrations [19,20]. The form of this is as follows:

$$V = V_{max} - K_m\frac{V}{[S]}$$

In this case, the velocity of enzyme action V has been plotted against $V/[S]$. Slope of the straight line gives the value of $-K_m$ whereas intercept on the Y axis gives the value of V_{max}. The value of k_2 can be determined by the same equation as mentioned above.

The Eadie–Hofstee plot provides more accurate kinetic parameters and it is frequently used to analyze the kinetic data, while the more straightforward and pragmatic Lineweaver–Burk plot continues to be most widely employed by enzymologists in general [19,20]. Therefore, in the present study kinetic parameters have been evaluated using very standard and practical methods like Lineweaver–Burk method and Eadie–Hofstee method which is in conformity with various research findings [15–20].

2.4. Removal of mercury

Batch mode contacting device was used to study the kinetics of removal of mercury from simulated solution of mercuric chloride (MERCK) using *CIP*. A systematic and thorough investigation was performed to see the effect of different parameters on the removal of mercury from its aqueous solution by varying the initial concentration of mercury in solution (0.1–50.0 mg/L), amount of immobilized papain (0.01–0.05 g) and pH (5–9) in a prescribed manner. In each case the solution was stirred vigorously with the cyclomixer to eliminate mass transfer resistance associated with the diffusion of mercury to the surface of *CIP*. Samples were collected at particular intervals. The solid was separated from the solution by filtration under vacuum and the concentration of mercury in the filtrate was analyzed using Atomic Absorption Spectroscopy (AA-240, Varian).

2.5. Recovery of mercury

Initially 0.03 g of *CIP* was incubated with mercuric chloride solution with mercury concentration of 1.0 mg/L for about 10 min. The solid containing *CIP*–mercury complex was separated from the solution by filtration under vacuum. The solid was washed with distilled water for several times and was kept for drying. Finally, to study desorption of mercury, the solid was incubated in buffer solutions having specific pH for 20 min. To see the effect of pH on the recovery rate, the pH of the buffer solution was varied from 4 to 9. After incubation period, the solid was separated from the solution by filtration method under vacuum and the concentration of mercury in the filtrate was analyzed using Atomic Absorption Spectroscopy (AA-240, Varian).

Table 1
Physical and textural properties of activated charcoal (MERCK).

Properties	Values
Bulk density (kg/m^3)	3100
Solid density (kg/m^3)	1083
Moisture content (%)	6.89
Ash content (%)	2.76
BET – surface area (m^2/g)	1188.4
Total pore volume (m^3/g)	0.4964×10^{-6}

3. Results and discussion

3.1. Immobilization of papain on activated charcoal and selection of the best operating condition for preparation of charcoal-immobilized papain (CIP)

Physical properties and textural characterization of activated charcoal (MERCK) have been presented in Table 1. It has been found that majority of the pores have the pore diameter between 3.475 μm and 6.21 μm. Specific enzymatic activities of papain samples immobilized on charcoal under different operating parameters viz., initial concentration of solution, weight of activated charcoal and pH respectively have been presented in Figs. 1–3. It is evident from Fig. 1 that specific enzymatic activity of immobilized papain sample obtained at initial papain concentration 40.0 g/L, adsorbent amount 0.5 g and pH 7 shows the maximum value. Figs. 2 and 3 confirm this result. Thus, the immobilized papain sample obtained at the present condition has been selected as the adsorbent for further studies of removal of mercury and has been designated as charcoal-immobilized papain or '*CIP*'.

In Fig. 1, it is seen that when initial papain concentration has been varied from 10.0 g/L to 50.0 g/L during adsorption of papain with specific amount of activated charcoal (0.5 g) at pH 7 and temperature 35 °C, initially there is a monotonous increase in *SEA* [(g peptide formed)/(g papain × h)] of the immobilized papain sample produced during the process. It has been observed up to 40.0 g/L initial concentration of papain solution. A subsequent decrease in values of *SEA* has been found for further increase in initial concentrations. This may be due to the fact that initially at low con-

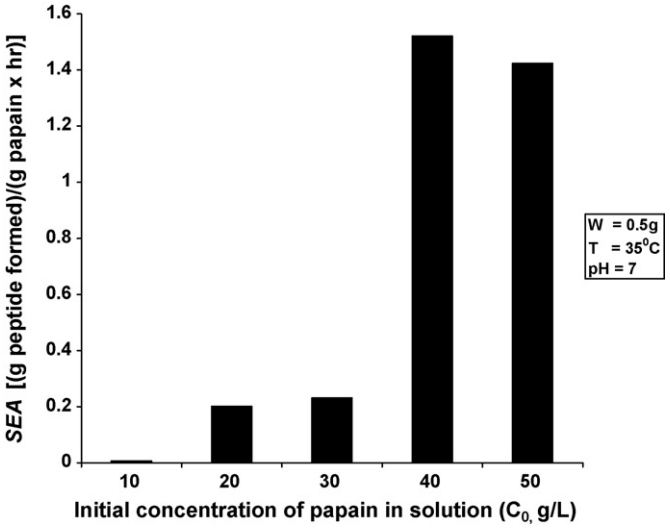

Fig. 1. Specific enzymatic activity (*SEA*) of immobilized papain sample obtained at different initial concentration of papain in solution.

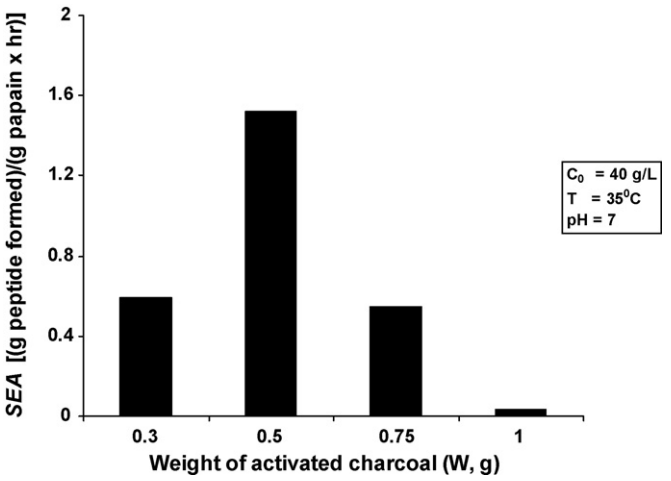

Fig. 2. Specific enzymatic activity (*SEA*) of immobilized papain sample obtained with different weight of activated charcoal.

centration, small amount of papain present in solution can interact with specific amount of activated charcoal and gets adsorbed on the activated charcoal easily. Thus, the enzymatic action of papain has been preserved. This phenomena occurs up to 40.0 g/L initial concentration of papain solution during adsorption process and the immobilized papain sample produced at this condition shows the best value of *SEA*. When initial concentration of papain in solution has been increased further, due to more interaction of papain molecule with activated charcoal, more amount of papain has been adsorbed on it but in the cost of enzymatic performance due to overburdening of papain on the surface of activated charcoal. Therefore, low values in *SEA* at higher initial concentration of papain have been found.

Similar kind of observation has been obtained when 40.0 g/L papain solution interacted with various amount of activated charcoal (0.3–1.0 g) during preparation of immobilized papain sample by physical adsorption method at pH 7 and temperature 35 °C (Fig. 2). With lower amount of activated charcoal, smaller amount of papain is immobilized with preservation of enzyme activity. Therefore, an increase in *SEA* is observed with increasing amount of activated charcoal. The trend is followed up to 0.5 g of activated charcoal. With higher amount of activated charcoal, more surface

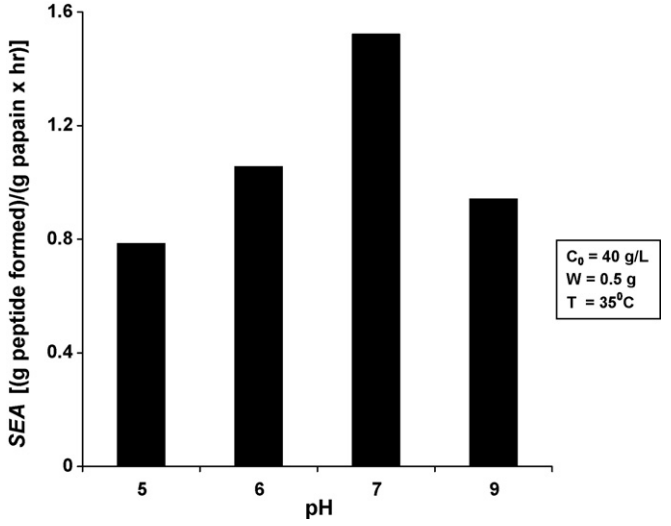

Fig. 3. Specific enzymatic activity (*SEA*) of immobilized papain sample obtained at different pH.

Table 2
Results of analysis of different adsorption isotherms.

Adsorption isotherm	Expression	Constants	R^2
Langmuir	$q_e = \frac{K_L q^0 C_e}{1+K_L C_e}$	$q^0 = 4.7037$, $K_L = 0.008897$	0.9844
Freundlich	$q_e = K_F C_e^{1/n}$	$K_F = 0.06796$, $n = 1.3466$	0.9298

area is available for papain to get spread over it. This may result in a change in conformation which in turn, decreases *SEA*.

The sample immobilized at pH 7 shows the highest activity (Fig. 3) when pH has been varied from 5 to 9 during immobilization procedure. This may be due to the change in conformation of papain at lower and higher pH. It gives rises to two possibilities. The first possibility is that altered conformation of papain prevents it from interacting with activated charcoal and thus leads to lower efficiency of immobilization. The second possibility is that extent of immobilization is unaltered but conformational change makes the active site less available to the substrates and results in lower *SEA*. However, the second possibility is less likely as immobilized papain sample has been found to retain its enzyme activity at pH 5 and 9 when tested later.

3.2. Determination of loading of papain on activated charcoal using TOC (total organic carbon) analyzer and study of adsorption isotherm

The equilibrium data of enzyme loading have been fitted to Langmuir adsorption isotherm model and Freundlich isotherm model. Values of the parameters obtained by non-linear method of analysis for these two models are shown in Table 2. It is evident from Table 2 that experimental data are fitted most satisfactorily to the Langmuir model. The values of the adsorption parameters according to Langmuir model viz., q^0 and K_L have been found to be 4.7037 g papain/g activated charcoal and 0.008897 L/g papain respectively as experiment reveals that 1.0 g TOC stands for 2.352 g papain.

3.3. Physico-chemical characterization of CIP

Study to characterize the *CIP* based on enzymatic activity reveals that pH 7 and 35 °C are the optimum conditions for its operation. Study of shelf life period of *CIP*, stored at 30 °C, shows that *CIP* retains its activity up to six months. Figs. 4 and 5 show the SEM photographs of activated charcoal and that of *CIP* respectively. SEM photographs of activated charcoal and *CIP* show their microporous

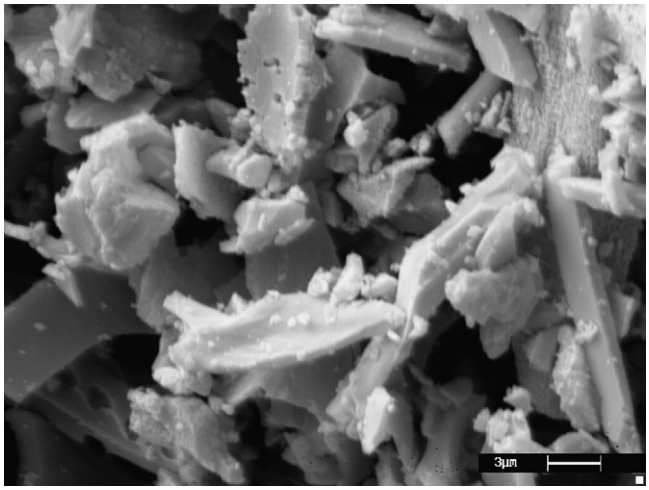

Fig. 4. SEM photograph of activated charcoal.

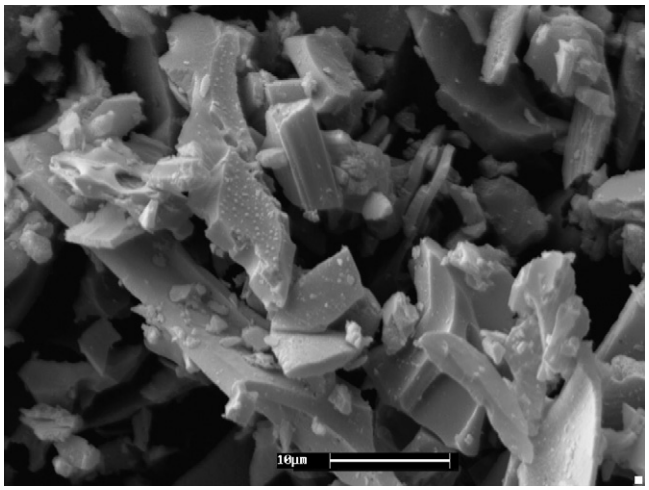

Fig. 5. SEM photograph of charcoal-immobilized papain (*CIP*).

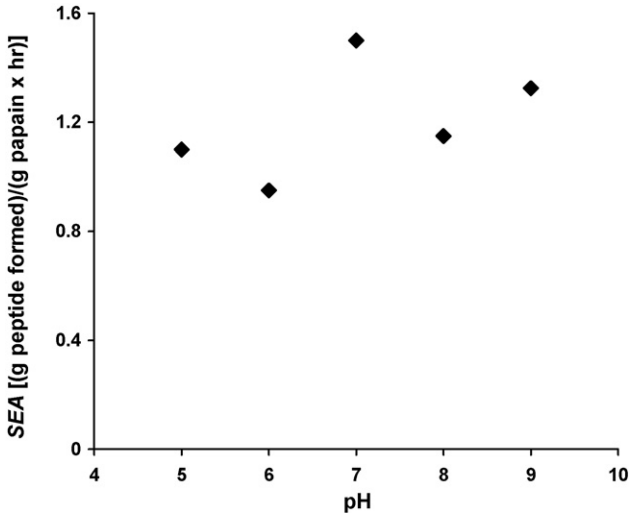

Fig. 6. Specific enzymatic activity (*SEA*) of *CIP* at different pH for determination of pH optima. In all cases temperature (T) = 35 °C.

structure. A polishing effect on the surface of *CIP* is observed due to adsorption of papain whereas, it is absent in case of native activated charcoal.

3.3.1. Determination of temperature optima and pH optima

The optimum temperature for *CIP* is found to be 35 °C. The *SEA* is found to be decreased approximately by 30.0% both at 50 °C and 70 °C (figure not shown). The optimum pH for *CIP* is 7 (Fig. 6). At pH 8 and 9, a slight decrease is observed in activity of *CIP*. The activity at pH 9 was found to be 87.1% of that obtained at pH 7. At pH 8, 76.6% of its optimum activity that has been obtained at pH 7 has been observed. At pH 5, *CIP* showed 72.3% of its optimum activity.

3.3.2. Determination of temperature stability and pH stability

CIP retains its activity when exposed to 4 °C for 1 h. However, very low activity is obtained when the enzyme has been exposed to 50 and 70 °C for 1 h. An exposure at high temperature for a long time gives *CIP* a chance to lose its folding and get denatured. The chance of getting denatured is higher when a small amount of papain, compared to its matrix i.e., activated charcoal is used for immobilization. In this situation, casein binds at a site other than the active site and acts as a stabilizer for papain binding, rather than a substrate. Exposure to high temperature for one hour provides enough time and energy for unfolding of papain in *CIP* and results in loss of activity.

Enzyme activity is retained when *CIP* is placed in a buffer of pH 7 for 1 h. Low activity is obtained with an exposure to other pH. Under the present experimental conditions, *CIP* is found to be stable neither at low pH nor at high pH. Being denaturing agents, acid and alkali, when in contact for a long time, help papain get unfold and form a monolayer on activated charcoal. This, in turn results in loss of activity.

3.3.3. Study of protein hydrolysis using CIP

The kinetic study of the protein hydrolysis by *CIP* has been done to determine the kinetic parameters like V_{max}, k_2 and K_m using varying amount of casein as substrate. The specific enzymatic activity vis-à-vis the velocity of enzymatic reaction using *CIP* is then evaluated for each case. The kinetic parameters have been evaluated by plotting the data using both the methods viz., Lineweaver–Burk method and Eadie–Hofstee method. The results are shown in Table 3. The values of kinetic parameters obtained with free enzyme are also shown in Table 3.

3.4. Removal of mercury

To assess the mercury removal efficacy of *CIP*, the simulated solution of mercuric chloride has been contacted with *CIP* in a batch contactor. Three parameters viz., the initial concentration of mercury in solution (0.1–50.0 mg/L), amount of *CIP* (0.01–0.05 g) and pH (5–9) have been varied in a prescribed manner. To establish the mechanism of mercury removal, raw activated charcoal has also been allowed to interact with simulated solution of mercuric chloride separately and the data is represented in Fig. 7. It is evident from the figure that with same amount of raw activated charcoal and *CIP* (0.014 g), the percentage removal of mercury by *CIP* (f) is much higher (72.0%) than that removed by raw activated charcoal (30.3%). For both the cases the initial concentration of mercury, pH and temperature were maintained at 15 mg/L, 7 and 35 °C respectively. It is clear that the surface area available for adsorption is almost same for raw activated charcoal and *CIP*. The higher removal of mercury by *CIP* indicates chemisorption nature of the process instead of physical adsorption which is the sole characteristics of raw activated charcoal. This confirms the chemical binding of mercury with papain immobilized on activated charcoal in case of *CIP*.

In Fig. 8, the percentage removal of mercury, obtained experimentally by varying the initial mercury concentration (C_{M0}) in the range of 0.1–50.0 mg/L, have been plotted against time keeping

Table 3
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	0.8903	0.429	35.6	0.943
Charcoal-immobilized papain (*CIP*)	0.8677	0.00201	53.661	0.9827	1.058	0.0027	64.85	0.7893

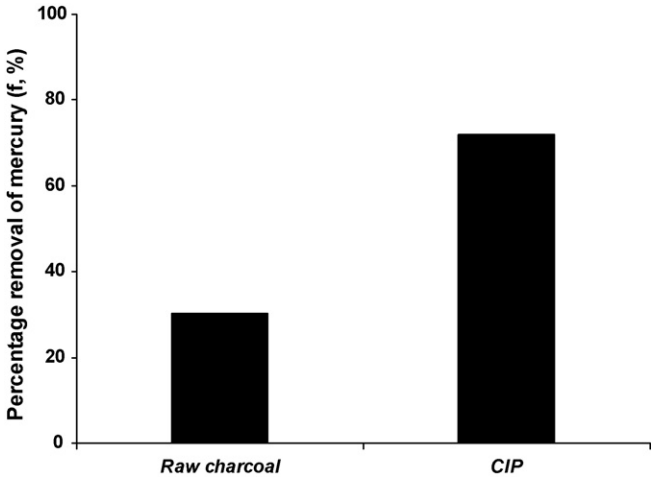

Fig. 7. Percentage removal of mercury using raw activated charcoal and *CIP*. In both the cases, initial concentration of mercury (C_{M0}) = 15 mg/L, pH = 7 and T = 35 °C.

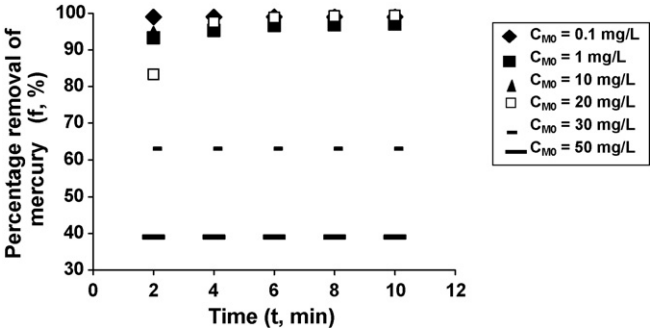

Fig. 8. Time histories of percentage removal of mercury using *CIP* with initial concentration of mercury (C_{M0}) as a parameter. In all cases W_{CIP} = 0.03 g, T = 35 °C and pH = 7.

other parameters like weight of *CIP*, pH and temperature constant at 0.03 g, 7 and 35 °C respectively. Fig. 9 represents the same percentage removal-time history obtained experimentally for a single initial concentration (C_{M0} = 1.0 mg/L). From the close observation of Figs. 8 and 9, it is evident that maximum removal for each case has been obtained within 2 min which is a clear indication of negligible mass transfer resistance present in the system. Moreover, attainment of the maximum removal within two minutes suggests that

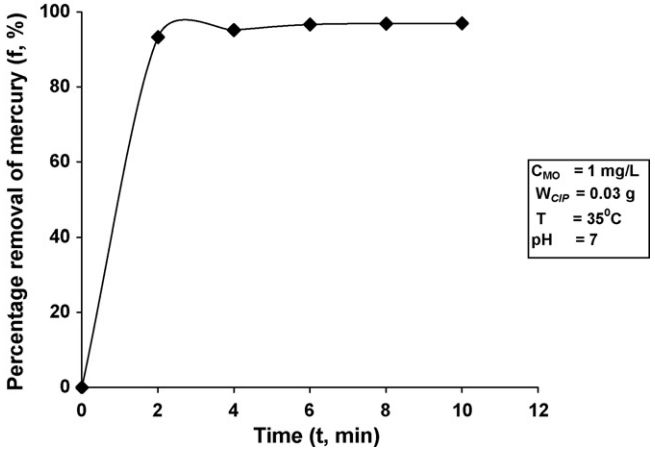

Fig. 9. Time history of percentage removal of mercury using *CIP*. C_{M0} = 1.0 mg/L, W_{CIP} = 0.03 g, T = 35 °C and pH = 7.

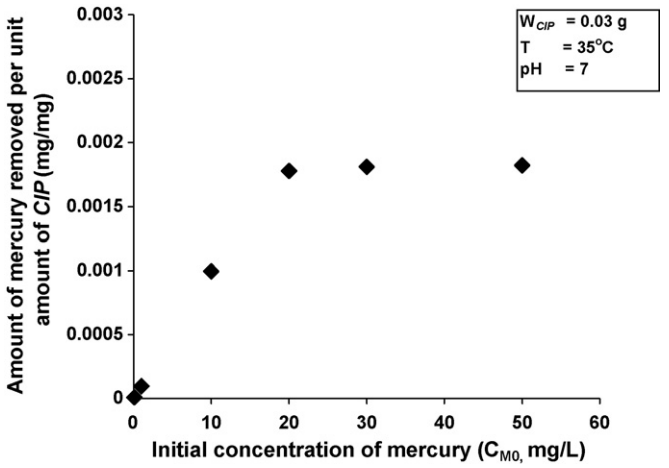

Fig. 10. Amount of mercury removed per unit amount of *CIP* at different initial concentration of mercury (C_{M0}).

the reaction between mercury and the immobilized enzyme to form mercury-enzyme complex is very fast. In Fig. 8, it is noted that with increase in initial concentration of mercury from 0.1 to 50.0 mg/L, the percentage removal decreases approximately from 99.0% to 39.0% and the decrease in percentage removal values are more prominent at higher concentrations like 30.0 mg/L and 50.0 mg/L. This may be due to the fact that at lower concentrations of mercury, binding capability of *CIP* with mercury has not reached to its saturation level. Here the term 'Binding Capability' is used to mean the amount of mercury adsorbed per unit gram of *CIP*. This can be explained in better way by Fig. 10 where the amount of mercury removed per unit weight of *CIP* has been plotted against initial concentration of mercury in solution. The figure reveals that when initial concentration of mercury is low (i.e., 0.1–10.0 mg/L), the binding capability of *CIP* has not reached to its saturation level, but at the initial concentration of 20.0 mg/L, it has reached to its saturation level. The figure shows that 1.0 mg *CIP* can bind utmost 0.002 mg mercury. This has been substantiated by the experimental findings obtained at still higher initial concentration of mercury like 30.0 mg/L and 50.0 mg/L.

To examine the effect of amount of *CIP* on the percentage removal of mercury, the later has been plotted against time in Fig. 11 by varying the amount of *CIP* (W_{CIP}) in the range of 0.01–0.05 g. For all the cases initial concentration of mercury, pH and temperature are kept constant at 1.0 mg/L, 7 and 35 °C respectively. The figure reveals that mercury has been removed from

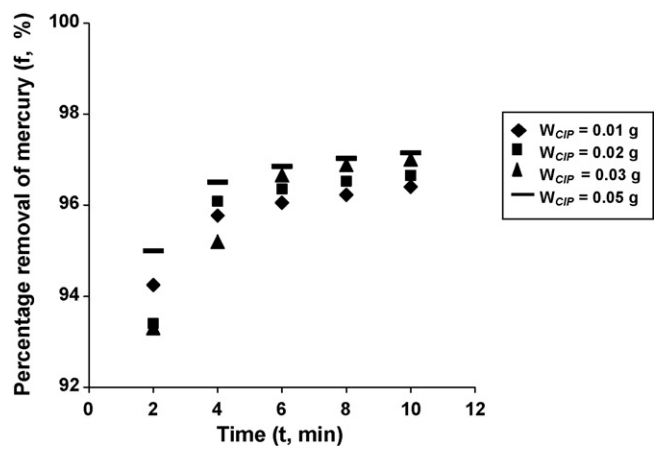

Fig. 11. Time histories of percentage removal of mercury using *CIP* with weight of *CIP* (W_{CIP}) as a parameter. In all cases C_{M0} = 1.0 mg/L, T = 35 °C and pH = 7.

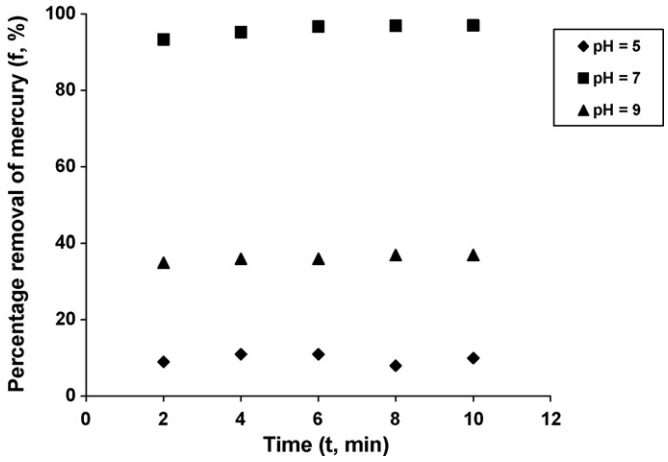

Fig. 12. Time histories of percentage removal of mercury using *CIP* with pH as a parameter. In all cases C_{M0} = 1.0 mg/L, W_{CIP} = 0.03 g and T = 35 °C.

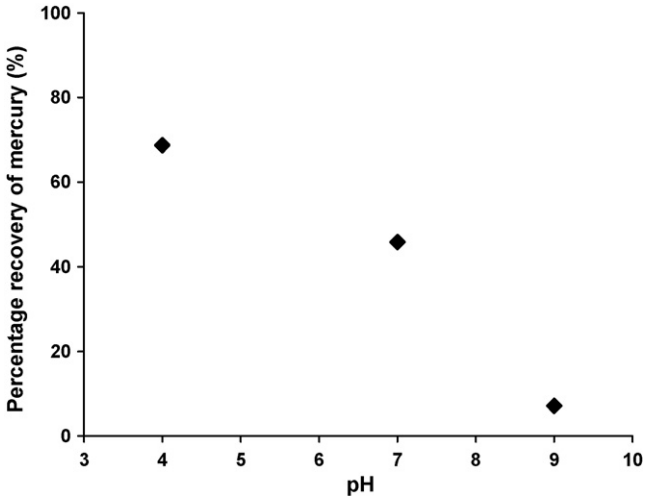

Fig. 13. Effect of pH on the percentage recovery of mercury. In all cases T = 35 °C.

96.4% to 97.2% with the amount of *CIP* from 0.01 g to 0.05 g. Similar to the previous cases, major portions of mercury are removed within two minutes which reconfirms the negligible mass transfer resistance and fast reaction between *CIP* and mercury. Since the initial concentration of mercury in solution is low (C_{M0} = 1.0 mg/L), the percentage removal of mercury remains unaffected with increase in amount of *CIP*.

Fig. 12 gives the result for mercury removal at different pH values. The neutral solution of pH 7 has been found to be the most effective one for removal of mercury followed by pH 9 and 5. Approximately 97.0% mercury has been removed at pH 7, whereas at pH 9 and 5 extent of mercury removal has been found to be 37.0% and 10.0% respectively when all other parameters remains constant (C_{M0} = 1.0 mg/L, W_{CIP} = 0.03 g, T = 35 °C). Being denaturing agents, acid and alkali both are likely to induce conformational change in papain which reduces its metal binding capacity. Moreover, dissociation of mercury from papain is favored at low pH. From the molecular model of papain it is clear that binding of metal ions to papain involves cysteine 25 and the imidazole group of histidine 159 and the process is accompanied by a proton release [12]. Therefore at low pH, the process is kinetically driven to the dissociation of papain–metal complex. Masoom and Townshend [25] reported significantly less binding of metal ions on a poly-cysteine (PLCys) matrix immobilized on controlled pore glass in acidic pH. They postulated that at lower pH, cysteine groups of PLCys remain in the protonated form leading to a decreased capacity of metal binding.

3.5. Recovery of mercury

Experiment was carried out at three different pH (4, 7, 9) and the result is shown in Fig. 13. From the figure it is seen that 68.72% mercury was recovered at pH 4, while at pH 7 recovery was about 45.8% and at pH 9 percentage recovery was negligible. At acidic pH, papain reduces its metal binding capacity due to conformational change. Thus, metal recovery is maximum at lower pH. At pH 7, the recovered metal includes mercury adsorbed at the surface due to physical adsorption process which was observed in case of free activated charcoal (refer Fig. 7). At high pH, metal release is unfavorable due to proton uptake.

Similar observation was made by Masoom and Townshend [25] who reported quantitative recovery of metal ion from a poly-cysteine (PLCys) matrix immobilized on controlled pore glass using 0.1 M HNO_3. According to their findings, the metal-binding column becomes fully regenerated and reusable after acid treatment in spite of strong binding of metals.

4. Conclusion

The present study deals with the removal of mercury from its aqueous solutions using Charcoal-Immobilized Papain (*CIP*). The work is primarily based on the principle of modification of the free matter space in porous materials by introducing chemicals to increase the number of active sites for the removal of mercury ions from its aqueous solution. So far, most research work on this topic has been carried out using silica and zeolite-based molecular sieve mesoporous materials. The use of carbonaceous adsorbents with the same purpose has been much less frequent. The present study shows that papain, immobilized on activated charcoal can be used to remove mercury from industrial waste water. Papain has been immobilized on activated charcoal through physical adsorption process and the data obtained during equilibrium studies have been best fitted to Langmuir model. The immobilized papain sample obtained at initial papain concentration 40.0 g/L, activated charcoal amount 0.5 g and pH 7 shows the best specific enzymatic activity and it has been designated as charcoal-immobilized papain (*CIP*) and used for further studies of mercury removal. Study to characterize the *CIP* based on enzymatic activity reveals that pH 7 and 35 °C are the optimum conditions for its operation. To assess the mercury removal efficacy of *CIP*, the simulated solution of mercuric chloride has been contacted with *CIP* in batch mode of operation. The operating parameters viz., the initial concentration of mercury in solution, amount of *CIP* and pH have been varied in a prescribed manner during batch study. Maximum removal for each case has been obtained within 2 min. This suggests that the reaction between mercury and the immobilized enzyme to form mercury–enzyme complex is very fast. Highest conversion achieved in the batch study is about 99.4% at initial mercury concentration 20.0 mg/L, weight of *CIP* 0.03 g and pH 7. Finally, the study of desorption of mercury has been performed at different pH values for assessment of recovery process of mercury. The results thus obtained have been found to be satisfactory. Thus, it can be concluded that the removal of mercury using papain immobilized on activated charcoal having the suitable option of metal recovery can be a better technology for industrial waste water treatment.

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