

## DETERMINATION OF THE OPTIMUM CONDITIONS FOR THE REMOVAL OF HEAVY METALS FROM WASTEWATER BY UREASE ENZYME

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### ABSTRACT

The goal of the current investigation was to identify the ideal conditions for eliminating Pb(II) at a concentration of 100 mg/L from wastewater utilizing cotton-derived plant urease enzyme. According to the findings, the urease enzyme can remove Pb(II) up to 97% more effectively than the control at the same concentration after 3 hours of contact time and a 1:1 (v : v) ratio at pH 4.0. The AA test, “Fourier transform infrared spectroscopy” (FTIR), and “scanning electron microscopy” (SEM) were used to approve the results.

**Keyword:** urease enzyme, heavy metals, lead ions.

### INTRODUCTION

Environmental effects from toxic substances as well as other toxins produced as industrial waste can be considerable (11). Heavy metal removal from polluted sites can be costly and require large quantities of chemicals and energy when using conventional treatment methods. Due to the harmful consequences heavy metal removal has on the environment, plant development, and human health, it has received a lot of media attention (25, 27). Heavy metals are persistent, non-biodegradable contaminants that can create long-term issues, especially when they are present in concentrations that are greater than their baseline levels (5). Heavy metals removal from wastewater has traditionally been accomplished using a variety of conventional treatment techniques, including desorption, electrochemical treatment, electrodeposition, and chemical precipitation. Recent years have seen a lot of interest in biological remedies for heavy metal pollution, including biosorbent, biotransformation, and biomineralization (14, 5). Therefore, the removal of heavy metals and certain other waste pollutants with EICP may be an environmentally favorable solution. Chemical precipitation, one of the most used heavy metal removal techniques, could be added to the EICP to improve the removal of heavy metals (13). This process could change the soluble forms of heavy metals such hydroxides, phosphates, carbonates, and sulfides into their insoluble counterparts (15, 12). However, compared to other techniques, such electrocoagulation, chemical precipitation is a highly effective strategy for eliminating heavy metals in high concentrations. The rising price of disposing of the sludge created by chemical precipitation is another drawback of this process (8). The ability to control heavy metal separation in varied environments, such as soil and water, is another benefit of EICP. In order to immobilize and extract heavy metals and metalloids from polluted mining wastes, Panda *et al.*, (24) used the EICP technology. The effectiveness of the EICP method on the adsorption and desorption of soils mixed with various combinations of heavy metals was examined by Nam *et al.*, (23). This source's

contribution to the level of such metals in the environment is typically referred to as "Background Concentration" (16).

### MATERIAL AND METHODS

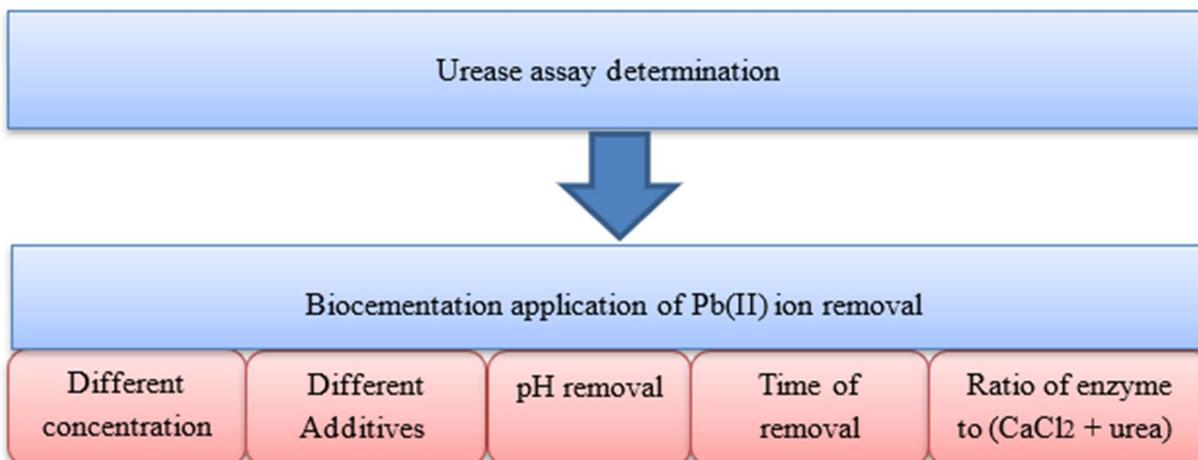
The chemicals and materials used in this study for enzyme extraction and removal of lead ions are listed in table 1 (17).

Table 1: lists the chemicals and materials employed in this study

Chemicals	Company	Origin
Phenol	Thomas Baker	India
Sodium nitroprusside Tris base Tris HCl (C <sub>4</sub> H <sub>11</sub> NO <sub>3</sub> ) Urea (CH <sub>4</sub> N <sub>2</sub> O)	Hi-media	India
Sodium hypochloride (NaClO)	Sehat	Iran
Ammonium chloride (NH <sub>4</sub> Cl)	Sigma	England
Hydroxide sodium(NaOH) Sodium acetate (CH <sub>3</sub> COONa), Coomassie Brilliant blue G-250 Lead (PbCl <sub>2</sub> )	BDH	England
Sodium Di-Hydrogen phosphate (NaH <sub>2</sub> PO <sub>4</sub> .12H <sub>2</sub> O), Dibasic sodium phosphate (Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O)	CDH	England

### Methodology

The approach of the experimental work used in this study is shown in Figure 1.



**Figure 1: Flow chart of the overall experimental work methodology..**

### **Urease assay determination**

Activity of urease enzyme has been determined using a modified Berthelot reaction (9) that relies on  $\text{NH}_4\text{Cl}$  standard curve across the ammonia released by enzyme. The sterilized glassware must wash in dilute hydrochloric acid and thoroughly cleaned by de-ionized and distilled water. The test reaction mixture was containing 0.8 ml of 100 mM phosphate buffer pH 6.8 with 1 ml of 500 mM urea prepared in same buffer and 1 ml of plant seeds extract, the mixture was incubated for one hour in a water bath at 37 °C. The reaction was halted by heating at 80 °C for 5 minutes to stop reaction. Urease activity was measured by measuring the absorbance rise at 625 nm after adding 10 ml of Berthelot's reagent for one hour in water bath at 37 °C to determine ammonia concentration. The quantity of enzyme released one mole from ammonia (1 min) under ideal conditions is known "an enzymatic activity unit" as follows, also Bradford's method was used to determine the concentration of protein (10).

$$\text{Urease Activity} = \frac{Ab}{\text{slope}} / (T \times C)$$

### **Where:**

$\frac{Ab}{\text{slope}}$  : is the concentration of ammonia,

T: is the time of reaction, 60 min.

C: is the constant, (14)

### **Ability of the urease enzyme to remove heavy metal at different metal concentration**

According to the best outcomes from the previous experiment, several doses of Pb(II) (5, 25, 50, 100, 250, 500, and 1000) mg/L were tested in each flask together with 15 ml of  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 15 ml of urea solution, and 15 ml of enzyme solution. The flasks were then agitated in a shaker for three hours at 150 rpm. The pH levels were evaluated three hours later. In order to measure the lead ion concentration, the mixture was filtered and examined using an "atomic absorption spectrometry" device (7).

### **Different Additives for the removal of heavy metals**

A significant amount of lead nitrate was dissolved in distilled water to create 100 (mg/L) of lead solution, which was used to test the enzyme's ability to remove lead ions from synthetic wastewater. Six flasks containing a 100 mg/L lead solution were created: the first flask contains only the lead solution and no other substances; the second flask contains the lead solution with a concentration of 110 mg/L and 10 ml of an enzyme solution; the third flask contains the lead solution with a concentration of 110 mg/L and 10 ml of CaCl<sub>2</sub>.6H<sub>2</sub>O solution; and the fourth flask contains the lead solution with a concentration of 110 mg/L. The final flask includes a 130 mg/L lead solution along with 15 ml of CaCl<sub>2</sub>.6H<sub>2</sub>O-urea solution and 10 ml of the enzyme. The fifth flask has a 120 mg/L lead solution along with 10 ml of CaCl<sub>2</sub>.6H<sub>2</sub>O-urea solution. After that, the flasks were placed in a shaker (Lsl-3016A, KOREA), where they were shaken for three hours at 150 rpm (7). The pH levels were determined following the experiment. In order to assess the lead concentration, the solution was filtered and examined using an atomic absorption spectrometry device ("atomic absorption spectrometry", Shimadzu AA-7000, Jaban).

#### **Fourier transform infrared spectroscopy (FTIR)**

The detection of the "surface functional groups" on reactive materials and spectrophotometric observation are made possible by the use of FTIR analysis. A Bruker Tensor 27 spectrophotometer and the KBr pellet technique were used to study the spectra in the 400–4000 cm<sup>-1</sup> region. It was completed at the College of Sciences at the University of Tehran.

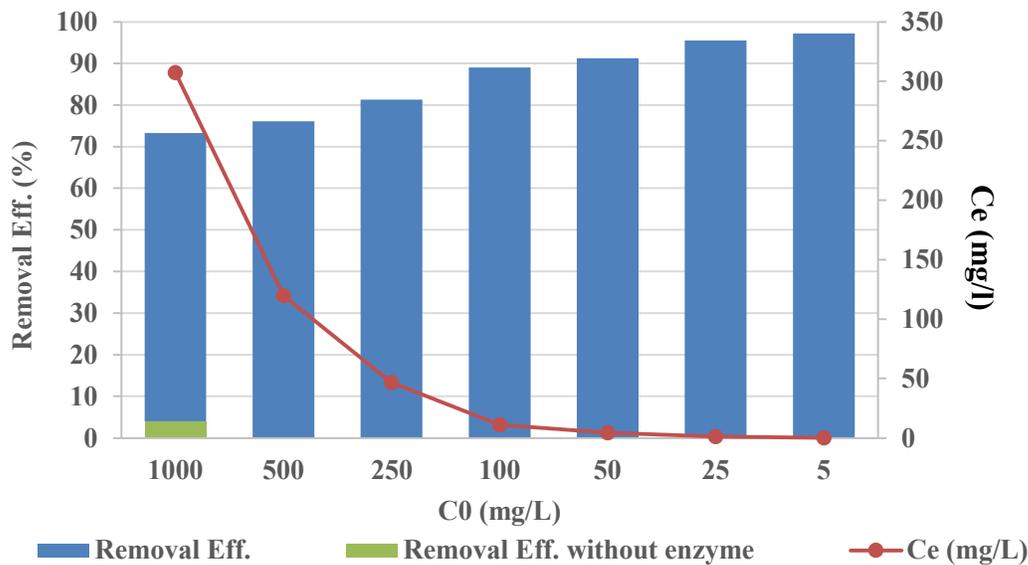
#### **Scanning electron microscopy (SEM)**

Under 10 KV voltage and a 6-mA flux, a SEM (NOVASEM, FEL450L) analysis was used to examine the surface morphology of calcite carbonate (CaCO<sub>3</sub>). In this study, scanning electron microscopy (SEM) technology was used for viewing and analysis of the surface of crystal shape and composition of the mineral carbonate deposition inside the lead ion specimens. It was done at the College of Science at the University of Tehran.

### **RESULTS AND DISCUSSION**

#### **Effect of different concentrations on the removal of heavy metal**

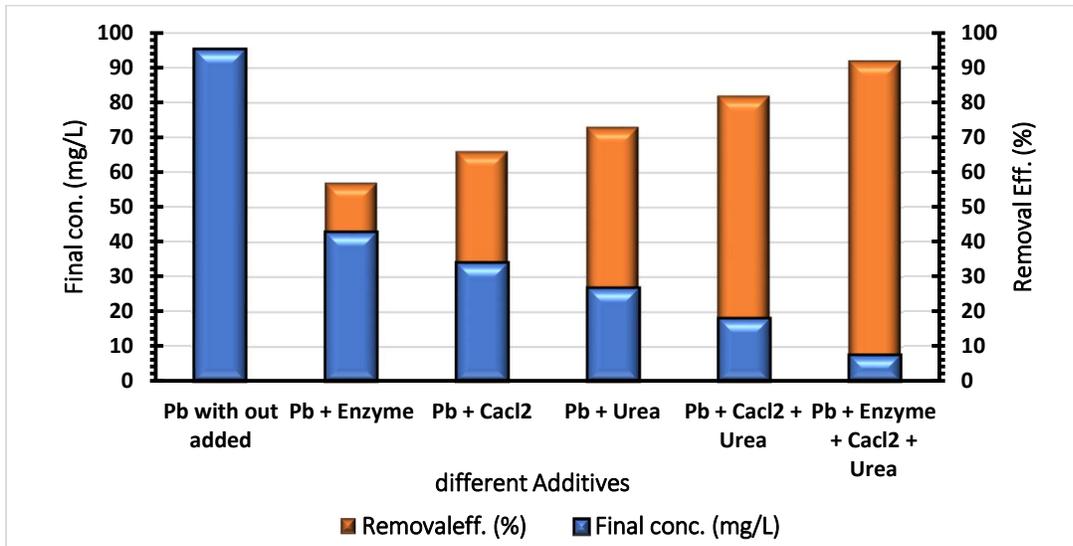
The results in Figure (2) demonstrated the removal rates of distinct lead ions by the urease enzyme at various initial Pb(II) concentrations (experimental conditions include: 10 ml of enzyme, and 10 ml of 1.25 M urea-CaCl<sub>2</sub>, at 3 hours of incubation). The enzyme-urea:CaCl<sub>2</sub> mixture demonstrates the same effectiveness in the bio-carbonation process when used to remove the lead ion from solutions. The outcome demonstrated that the effectiveness of the bio-removal process was only little impacted by the rise in Pb(II) content (2). This suggests that the concentration of lead ions has no effect on how well the enzyme: CaCl<sub>2</sub>-urea mixture removes heavy metals. Accordingly, when compared to bacterial strains that produce urease, the enzyme: CaCl<sub>2</sub>-urea combination is very effective at removing heavy metals (especially at high concentrations) (21, 19, 20). Furthermore, compared to the microbe-induced heavy metal removal time (24–72 h), the enzyme: CaCl<sub>2</sub>-urea mixture demonstrates a high removal rate in a shorter amount of time (8 h) (28).



**Figure 2. Removal efficiencies of Pb(II) by the enzyme at different concentrations.**

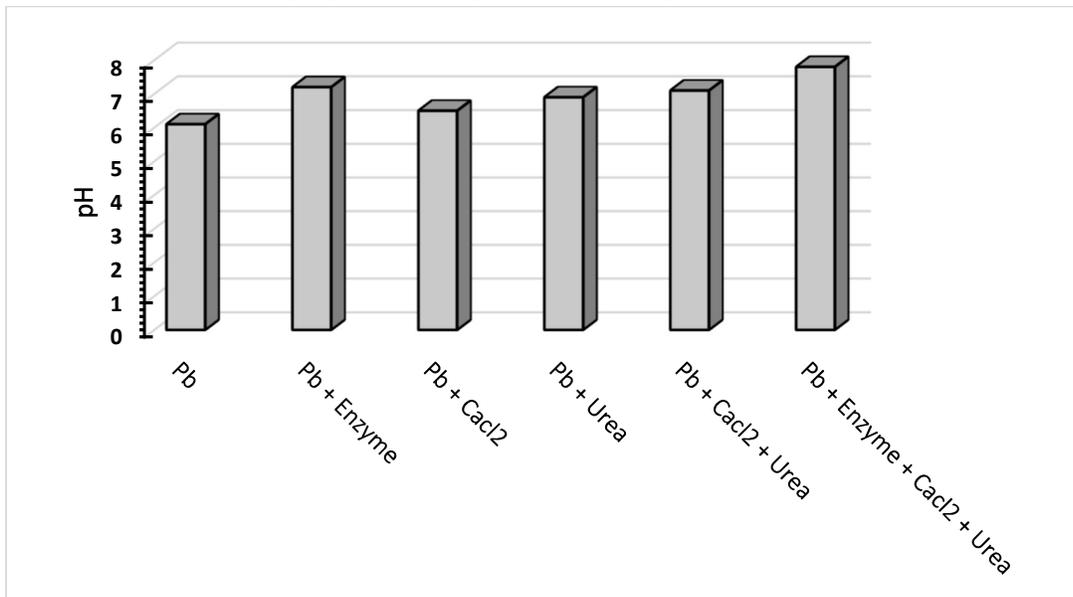
**Effect of different Additives on the removal of heavy metal**

After combining the normal heavy metal solutions with the plant-derived urease enzyme (PDUE)-urea/CaCl<sub>2</sub> solution, the bio-removal rates of precipitation started immediately and were finished after three hours. As part of the removal procedure, Pb(II) is taken up by a carbonate group (CO<sub>3</sub>: generated by PDUE's hydrolysis of urea), which causes carbonates of heavy metals that are insoluble to precipitate. Figure (3) displays the bio-removal rates of 100 mg/L Pb metal using enzyme, urea, CaCl<sub>2</sub>, and (enzyme+CaCl<sub>2</sub>+urea). The plant driven urease enzyme-urea mixture (urea+ CaCl<sub>2</sub>) was found to be extremely effective for the removal of lead from wastewater, according to the results. The range of the heavy metal removal efficiency was specifically 80% to 100%. The 20 ml and 30 ml of the enzyme were added to the Pb(II) solution, respectively, yielding clearance percentages of 80% and 100%. The urease enzyme was increased, which improved the rates at which heavy metals were removed (21).



**Figure 3. Removal efficiencies of Pb(II) by urease enzyme**

In contrast to the control solution's in Fig (4) the pH of 6.1, the pH values of the heavy metal control (without enzyme), Pb+enzyme, Pb+CaCl<sub>2</sub>, Pb+urea, Pb+CaCl<sub>2</sub>+urea, and Pb+enzyme+CaCl<sub>2</sub>+urea exhibit pH values of 6.1, 7.2, 6.5, 6.9, 7.1, and 7.8, respectively. The lead carbonates formed by the hydrolysis of urea's carbonates connected to the Pb(II) in the solution to form lead carbonates, which changed these soluble elements into insoluble form and decreased their toxicity by decreasing bioavailability (28).

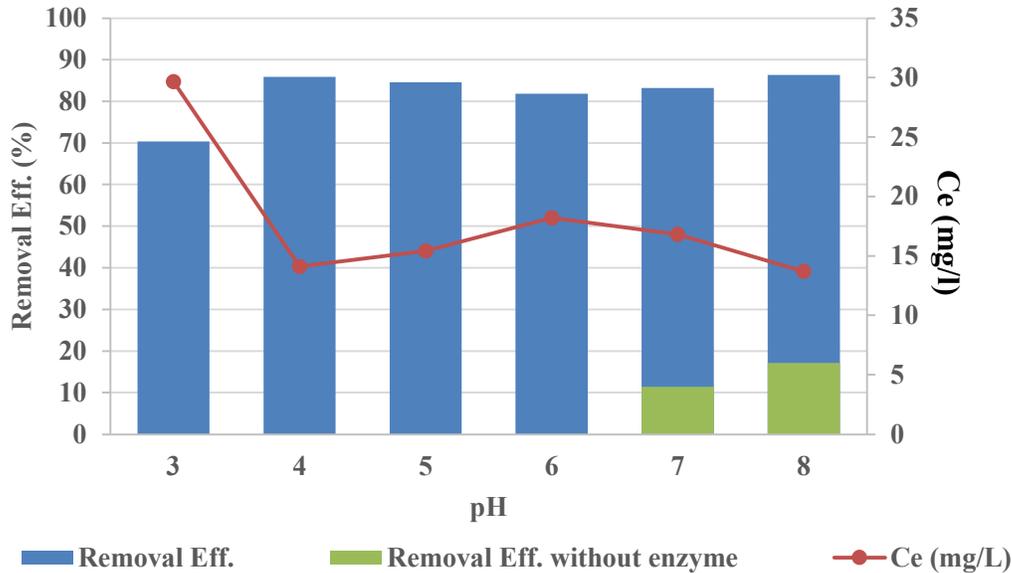


**Figure 4. Value of pH at the end of the experiment**

**Effect pH on the removal of heavy metal**

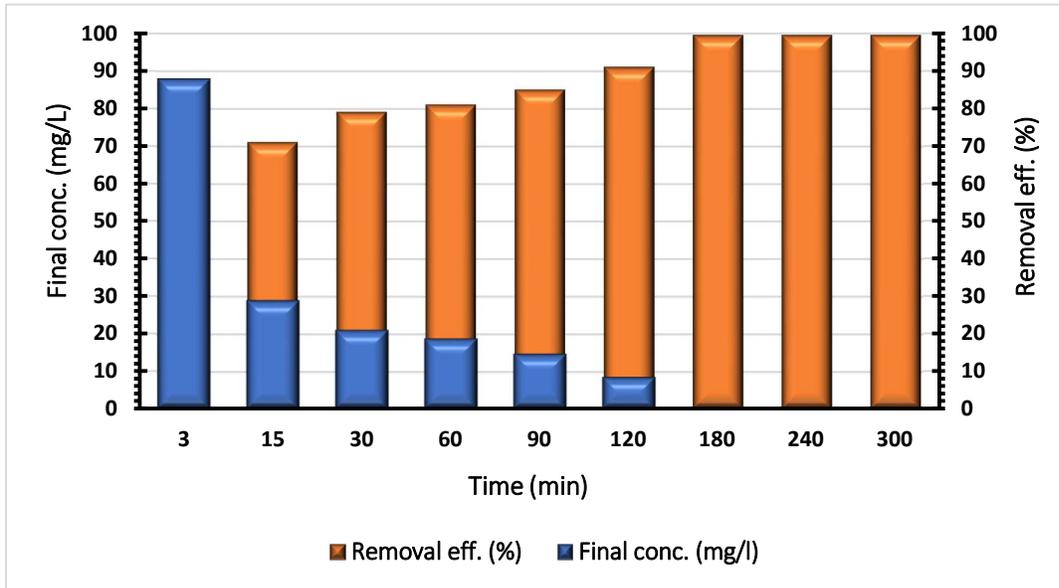
Figure (5) displays the pH readings for treatment samples that included Pb(II) at a concentration of 100 mg/L at the conclusion of incubation. After 180 minutes of incubation, the highest values were seen in all of the solutions. After this time, it was discovered that the highest removal efficiency at pH 4 was 85%, while the lowest removal efficiencies at pH 3, 5, 6, 7, and 8 were,

respectively, 70%, 84%, 82%, 83%, 85%, and (4%, 6% without enzyme). The enzymatic-urea hydrolysis-induced sequential production of ammonium ions is mostly responsible for the pH's small increase over time. When urea was hydrolyzed by urease-active bacteria (*Sporosarcina pasteurii*), the same outcomes were attained (1, 3, 4).



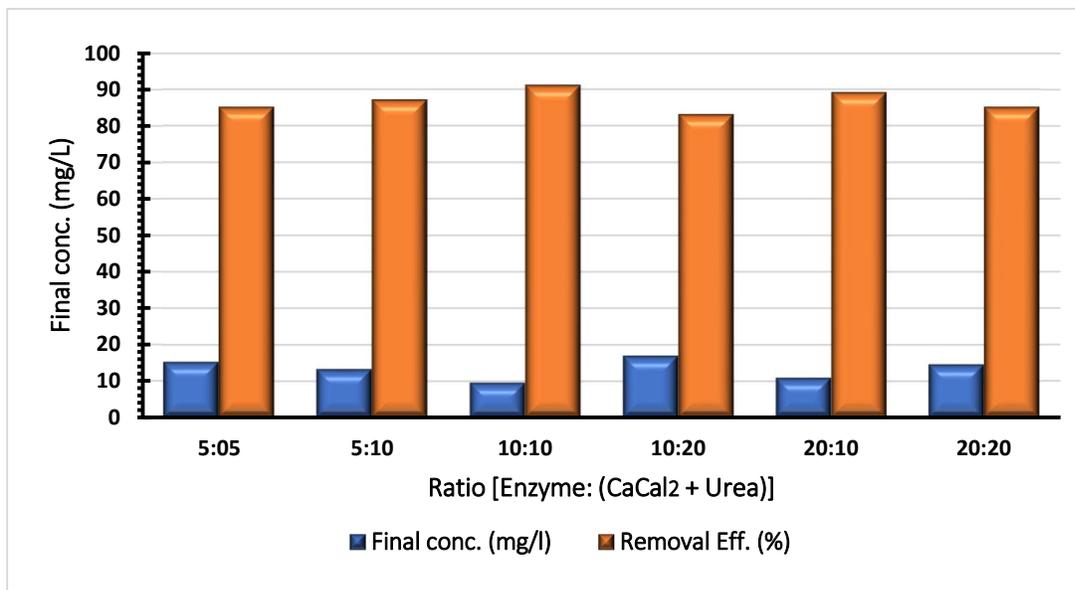
**Figure 5. Effect pH on the removal of Pb(II) by urease enzyme**  
**Effect of time period on the removal of heavy metal**

The elimination of Pb(II) by the urease enzyme and (CaCl<sub>2</sub> with urea) at a concentration of 100 mg/l is shown in Fig. (6). When the period was increased from 15 to 180 minutes, the removal efficiencies of Pb(II) improved from 71% to 99.5%, reaching equilibrium at that point. The best period for Pb(II) removal effectiveness was determined to be 180 minutes, which reached a 99.5% removal efficiency rate. Other intervals (15, 30, 60, 90, and 120 minutes) were found to be 71%, 79%, 81%, 85%, and 91% efficient, respectively. When the duration was extended from 30 to 180 minutes, Cr removal effectiveness rose from 60% to 90%, according to Saifuddin *et al.*, (26).



**Figure 6. Effect of time on removal Pb (II) by urease enzyme**  
**Effect the ratio on the removal of heavy metal**

The elimination of Pb (II) by the urease enzyme and (CaCl<sub>2</sub> with urea) at a concentration of 100 mg/L is shown in Fig. (7) in order to determine the ideal enzyme to (CaCl<sub>2</sub> with urea) ratio. The chosen ratios were 5:5, 5:10, 10:20, 20:10, and 20:20. (v : v). The 10:10 ratio was shown to have the highest removal effectiveness for Pb (II), reaching 91%, compared to the ratios with the lowest removal efficiency, which are 5:5, 5:10, 10:20, 20:10, and 20:20, which are, respectively, 85%, 87%, 83%, 89%, and 85%. Al-Dulimi, (7), discovered the ideal Cd (II) removal ratio of 1:1, achieving 99% removal.



**Figure 7. Effect the ratio on the removal of heavy metal by urease enzyme**

**Design expert modeling –RSM-CCD**

**Table 2. Design experiment program to obtain maximum removal efficiency**

	Factor 1	Factor 2	Factor 3	Response 1
Run	A:Con.	B:pH	C:Ratio	Removal eff.
unit	mg/L	--	%	%
1	83.65	7.97	34.84	94
2	83.65	4.03	34.84	92
3	52.50	6.00	25.00	89
4	52.50	6.00	40.00	85
5	21.35	7.97	34.84	77
6	21.35	4.03	15.16	89
7	52.50	6.00	25.00	83
8	52.50	3.00	25.00	82
9	21.35	7.97	15.16	91
10	21.35	4.03	34.84	87
11	83.65	4.03	15.16	95
12	52.50	6.00	25.00	89
13	100.00	6.00	25.00	88
14	52.50	6.00	25.00	85
15	52.50	6.00	10.00	84
16	5.00	6.00	25.00	93
17	83.65	7.97	15.16	93
18	52.50	9.00	25.00	95
19	52.50	6.00	25.00	84
20	52.50	6.00	25.00	83

**Optimize the enzyme and solution ratio to get maximum removal efficiency**

Using an ideal mixture design tool and the triplicate and average values as input data, the expert design program was utilized to examine 20 runs with various mixtures of enzyme, CaCl<sub>2</sub>, and urea. Table 3 displays the analysis of variance (ANOVA) results for the quadratic model, which clearly demonstrates the model's applicability with a highly significant model P-value of less than 0.050, a high F-value of 12.83, a small value for lack of fit, and a high value of the coefficient of determination R<sup>2</sup> equal to 0.8555. The corrected R<sup>2</sup> of 0.7888 and the expected R<sup>2</sup> of 0.639 are reasonably in agreement; the difference is less than 0.2. The ratio of signal to noise is measured

by Adeq Precision. A ratio of at least 4 is preferred. A strong signal is indicated by the ratio of 16.018. To move around the design space, utilize this model.

**Table 3. The target in this table was removal efficiency**

Source	Sum of Squares	df	Mean Square	F-value	p-value	
<b>Model</b>	740.71	6	123.45	12.83	< 0.0001	significant
A-Con.	133.86	1	133.86	13.91	0.0025	
B-pH	197.00	1	197.00	20.47	0.0006	
C-Ratio	133.86	1	133.86	13.91	0.0025	
AB	2.00	1	2.00	0.2078	0.6560	
AC	242.00	1	242.00	25.14	0.0002	
BC	32.00	1	32.00	3.32	0.0913	
<b>Residual</b>	125.12	13	9.62			
Lack of Fit	96.69	8	12.09	2.13	0.2110	not significant
Pure Error	28.43	5	5.69			
<b>Cor Total</b>	865.83	19				

**Final equation in terms of actual factors**

$$\text{Removal eff.} = 111.80972 - 0.295245 X_1 - 0.148983 X_2 - 1.89259 X_3 - 0.008156 X_1 X_2 + 0.017944 X_1 X_3 + 0.103314 X_2 X_3$$

Where

X1 : “is the concentration of Pb(II) (mg/L)”

X2 : “is the pH of solution”

X3 : “is the ratio [enzyme : (CaCl<sub>2</sub>-urea)”

It is possible to anticipate the reaction for specific levels of each factor using the equation expressed in terms of the real factors. Here, the levels for each factor should be stated in their original units.



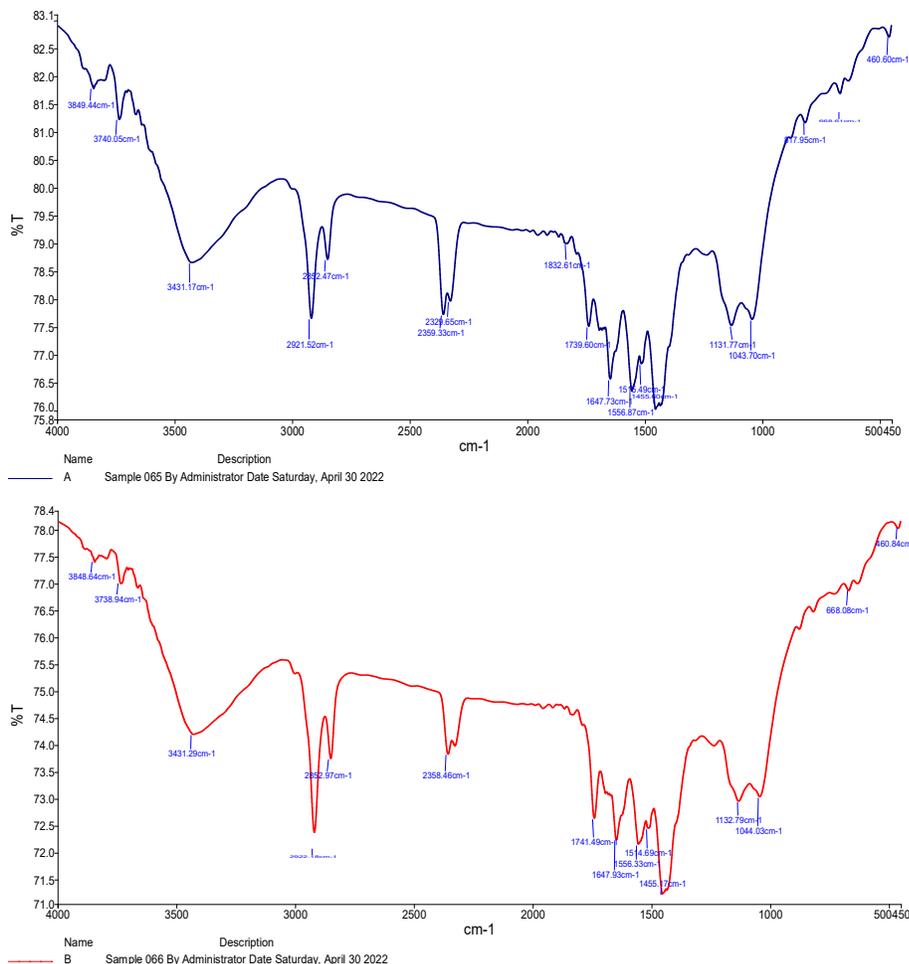
**Figure 9. A) The optimum and minimum removal efficiency of lead ions by urease enzyme the concentration is (75-84) mg/L at ratio (enzyme: CaCl<sub>2</sub>-urea) is (15-20) and is (21-35) mg/L at (27-37) respectively, B) The pH is best and worst is (6.98-7.97) at ratio (15-20) and is (4.03-5.02) at (30-35) respectively, and C) The pH is best and worst is (6.98-7.97) at concentration (75-84) mg/L and is (4.03-5.02) at (21-35) mg/L respectively.**

#### **FT-IR analysis of enzyme before and after biosorption of heavy metal**

Figure (10) depicts the study of the FT-IR spectra before and after the lead ion was adsorbent and precipitated by plant enzyme. The experimental setup included Co=100 mg/L Pb(II), 150 rpm, and a 3-hour contact duration. 100 ml of Pb(II) solution with a concentration of 100 mg/L received 10 ml of the urease enzyme. Table (4) lists the functional groups of the urease enzyme that were discovered from its FT-IR spectra before and after Pb loading (II). The active functional groups are represented by the FTIR curves before and after the experiment, and these groups were used to characterize the changes in vibration frequency of the functional groups in the biosorbents.

To determine whether functional groups are feasible for the biosorption process, the FT-IR spectra of the virgin and metal loaded urease were in the region of 450-4000 cm<sup>-1</sup>. The spectra show a variety of biosorption peaks, highlighting the complexity of the materials under investigation. According to reports in the literature, peaks in the FTIR spectrum based biosorbent are attributed to various groups and bonds according to their respective wavenumbers (cm<sup>-1</sup>) (22, 20, 16).

Indicative of the presence of hydroxyl (OH), the broad, intense absorption peaks that differences in waves (3439.17-3431.29) 7.88 cm<sup>-1</sup> are (2926.52-2922.18) Bounded alkyne groups (CC) are present if any of the following are present: 4.34 cm<sup>-1</sup>, (2858.47-2852.97) 5.5 cm<sup>-1</sup>, and (2359.33-2353.46) 5.87 cm<sup>-1</sup>. The difference between the wave peaks (1759.6-1741.49) was 18.11 cm<sup>-1</sup> (1647.73-1617.93) 29.8 cm<sup>-1</sup>, (1556.87-1514.69) (1556.87-1514.69) 42.18 cm<sup>-1</sup>, (1455.5-1455.17) 0.33 cm<sup>-1</sup>, (1139.77-1132.79) (1139.77-1132.79) 6.98 cm<sup>-1</sup>, and (1093.7-1044.03) The powerful band at (817.97-668.08) 149.89 cm<sup>-1</sup> can be attributed to the bending modes of aromatic compounds, whereas 49.67 cm<sup>-1</sup> are caused by the carbonyl (C=O) stretching vibration of the carboxyl groups. The majority of these peaks in the sample after biosorption exhibit a much lower absorbance than those in the raw sample as well as minor variations in the frequency bands. According to the findings, the alkyne and aromatic functional groups are crucial to the biosorption process. Because lead ions interact more strongly with the enzyme's alkyne (C≡C) and aromatic functional groups, lead ions exhibit greater enzyme absorption.



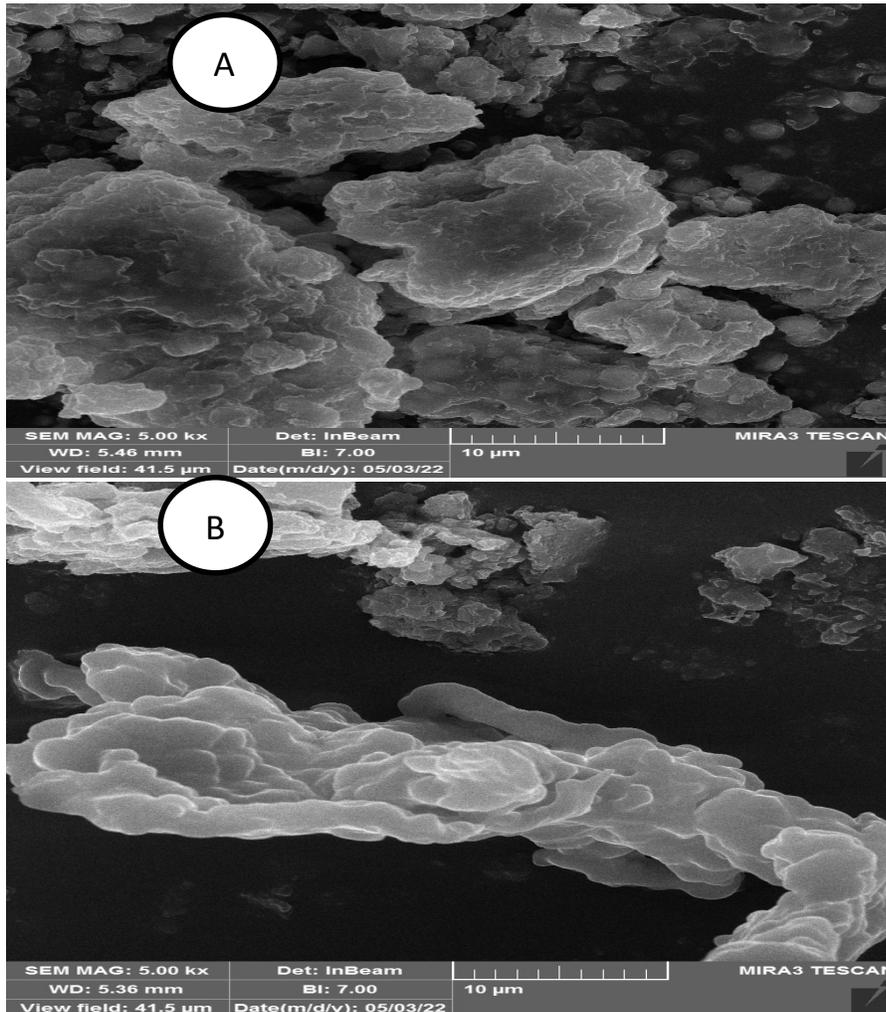
**Figure 10. FT-IR spectra for enzyme solution before and after loaded with 100 mg/L of Pb (II)**

**Table 4. Functional groups responsible for removal of heavy metals by enzyme**

FT-IR Peak	Assignment groups	Unloaded	Loaded	Difference in waves (cm-1)
1	Hydroxyl (OH)	3740.05	3738.4	1.65
2	Alkyne (C≡C)	2358.46	2329.65	28.81
3	Carbonyl (C=O)	1741.49	1739.6	1.89
		1556.33	1516.49	39.84
		1132.79	1131.71	1.08
4	Aromatic compound	817.95	668.08	149.87

### SEM analysis

SEM images of the precipitates collected from the precipitation of aqueous phase solution show densely aggregated rod shape and spherical calcite crystals as shown in Fig. (11). Mainly, there are two different types of calcite found, namely calcite spherical and calcite rod. Al-Thawadi *et al.*, (6) observed that the different shapes of calcium carbonate crystals morphology may be due to different phases of the sphere development and potential transformation into rod-shaped crystals.



**Figure 11. A) SEM images of calcite crystal before removal Pb(II), and B) after removal Pb(II) by urease enzyme.**

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