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Role of Green Macro-Alga *Enteromorpha Intestinalis* in its Free and Immobilized State to Remove Copper Ions from Aqueous Medium

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Abstract - Heavy metals presence in industrial effluents may be a significant source of pollution that leads to considerable environmental issues. In the present work, the potential adsorptive behavior of algal cells *Enteromorpha intestinalis* for copper ions removal from aqueous medium has been studied in the states of alginate-immobilized algal cells and free algal cells. The impact of initial copper concentrations (5, 10 and 15) mg/l, contact time ranging from 0– 8 days has been studied at temperatures 25 °C, pH 7± 2 and agitation speed of 120 rpm.

The algae achieved the highest efficiency at an initial concentration of 5 mg/l, reaching 74.49% on the fourth day of the experiment when treated with alginate-immobilized algae. In contrast, the free algae recorded a removal rate of 66.51% on the second day of the experiment. The alginate beads, on the other hand, achieved the lowest removal rate at the same concentration, reaching 38.75% on the second of the experiment. The analysis of the biosorption process using scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) before and after process showed that copper ions were primarily biosorped onto the algae cells surface.

دور الطحالب الكبيرة الخضراء *Enteromorpha intestinalis* في حالتها الحرة والمثبتة لإزالة أيونات النحاس من الوسط المائي

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المستخلص - وجود المعادن الثقيلة في مياه الصرف الصناعي قد يشكل مصدرًا كبيرًا للتلوث الذي يؤدي إلى مشكلات بيئية كبيرة. في العمل الحالي، تم دراسة السلوك الامتصاصي لخلايا الطحالب *Enteromorpha intestinalis* لإزالة أيونات النحاس من الوسط المائي في حالتي الخلايا الطحلبية المثبتة باستخدام الالجنيت والخلايا الطحلبية الحرة. تم دراسة تأثير تركيزات النحاس الأولية (5 و 10 و 15) ملغم/لتر، ومدة التلامس التي تتراوح من 0 إلى 8 أيام، عند درجة حرارة 25 درجة مئوية، ودرجة حموضة2 ± pH 7 ، وسرعة تحريك 120 دورة في الدقيقة. حققت الطحالب أعلى كفاءة عند تركيز ابتدائي قدره 5 ملغم/لتر، حيث وصلت إلى 74.49% في اليوم الرابع من التجربة عندما تم معالجتها باستخدام الطحالب المثبتة بالالجنيت. بالمقابل سجلت الطحالب الحرة معدل إزالة قدره 66.51% في اليوم الثاني من التجربة. أما حبيبات الالجنيت، فقد حققت أقل معدل إزالة عند نفس التركيز، حيث بلغت 38.75% في اليوم الثاني من التجربة. أظهرت تحليل عملية الامتصاص البيولوجي باستخدام المجهر الإلكتروني الماسح (SEM) وتقنية التحليل الطيفي بالأشعة السينية المشتتة للطاقة (EDX) قبل وبعد العملية أن أيونات النحاس تم امتصاصها بشكل أساسي على سطح خلايا الطحالب.

الكلمات المفتاحية: الجنيت ، الامتصاص الحيوي، *Enteromorpha* ، النحاس ، التثبيت

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Introduction

Heavy metal pollution released into the environment by industrial effluent is regarded as one of the worst environmental issues due to their toxicity to both humans and other living things, that have the capability to create stable complex compounds with most organic and inorganic compounds found in living organisms. The biological activity of these elements is attributed to their ease of ionization by electron loss, transforming into positively charged ions that have a high tendency to bind to chemical sites within living organisms or their surrounding environment (Hammood, 2021). Therefore, heavy metals recovery and removal from effluent streams are crucial for environmental preservation (Saleh *et al*., 2022).

While many metals are thought to be essential for living things, exposure to some metal species over time can be quite harmful (Singh *et al*., 2022). Copper ions are vital trace metal that are necessary for the catalysis of a number of important biological enzymes. However, because copper can stimulate the production of harmful reactive oxygen species, large amounts of this metal ions exposure can damage cells (Pensini *et al*., 2021).

Several conventional treatment technologies have been developed for heavy metals removal of from industrial effluents or wastewater like membranes filtration, nanotechnology treatments, chemical precipitation, ion exchange, electrochemical evaporation and adsorption (Shrestha *et al*., 2021). Unfortunately, these techniques are constrained by their serious shortcomings, which include poor selectivity, costly, incomplete removal, large energy requirements and release hazardous chemical reagents into the environment. Therefore, research into alternatives to the commercially available processes has become necessary due to the need for safer, less costly and more effective ways to remove heavy metals from contaminated water (Barquilha *et al*., 2017).

Biosorption is an emerging technique that uses either live or dead biomass to remove heavy metals from aqueous effluents at a cheap cost and with ease (Benaisa *et al*., 2019.

One of the most promising biosorbent materials with a promising future in biosorption experiments is macro algal dry materials biomass, which has proven to be highly efficient in removing heavy metals from aqueous solutions. This is because it is not affected by heavy metals toxicity, does not require growth media for its maintenance, and has a large surface area (Aranda-García, and Cristiani-Urbina, 2018). Additionally, the presence of many functional groups that operate as metal ligands gives macroalgae their high adsorption capacities, including amino, amide, sulfhydryl, carboxyl, carbonyl, and hydroxyl (Mokone *et al*., 2018).

These biosorbents are usually used in powder form. Nevertheless, the powdered algal biomass has a number of disadvantages in industrial biosorption applications, such as reduction in process efficiency, potential blockage of unit treatment, poor biomass regeneration and challenges in separating from treated wastewater (Benaisa *et al*., 2019).

Thus, these drawbacks can be addressed by cell immobilization, which is the limitation of cell mobility through chemical or physical means. The use of cell immobilization technology in wastewater treatment is becoming importance since it offers a number of benefits over biosorption using free cells, such as enhancing genetic stability, supplying high biomass, cell reuse, great mechanical strength, and resistance to harmful chemicals (Lee *et al*., 2020).

For immobilizing algae, a variety of natural and synthetic polymers are employed as matrices; however, they must satisfy many requirements, such as phototransparency, nontoxicity, preservation of cellular viability, and stability in the culture media (Bouabidi *et al.*, 2019; Nadersha and Hassan, 2022).

Alginate is the most common used polymer as supportive matrix to algae biomass immobilization using entrapment technique, being a polysaccharide found mostly in brown algae cell walls (Dobrincic *et al*., 2020), that is comprised of β-D-mannurono pyranosyl and α-L-gulurono linked by 1–4 linkages (Kumar *et al*., 2024). They readily interact with divalent cations such as Ca(II) to produce robust gels that can retain algae for extended durations (Barquilha *et al*. 2017). The main benefits of alginate gel include its non-toxicity, easy to processing, cost-effective, transparency and permeablity (Hurtado *et al*., 2022).

The goal of this study is to compare copper ions removal efficiency from an aqueous medium between free dried algal cells and alginate-immobilized algal cells via the process of biosorption, in addition to investigative the impact of different initial concentration of copper ions and contact time on biosorption process. Further analysis of the algae in its free and immobilized states before and after the biosorption was done by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) analyses.

Materials and Methods

Collection and preparation biomass of algae

Macro green algae *Enteromorpha intestinali* samples were collected directly from open water ponds near the Shatt Al-Basrah canal, Basrah Province, during the Spring 2023 (Fig. 1). The pre-treatment procedure involved washing algal samples several times with tap water followed by distilled water to remove all impurities and adhering dirt. The algal biomass samples were air-dried at room temperature for a period of three days. After that, the samples were dried in an oven at 60 C° for approximately 24 hours. The dried, non-living biomass particles were ground and passed through a sieve of 63 µm mesh size to obtain a fine powder. The samples were kept in airtight plastic containers at room temperature until further usage (Alobaidi and Alwared, 2023).

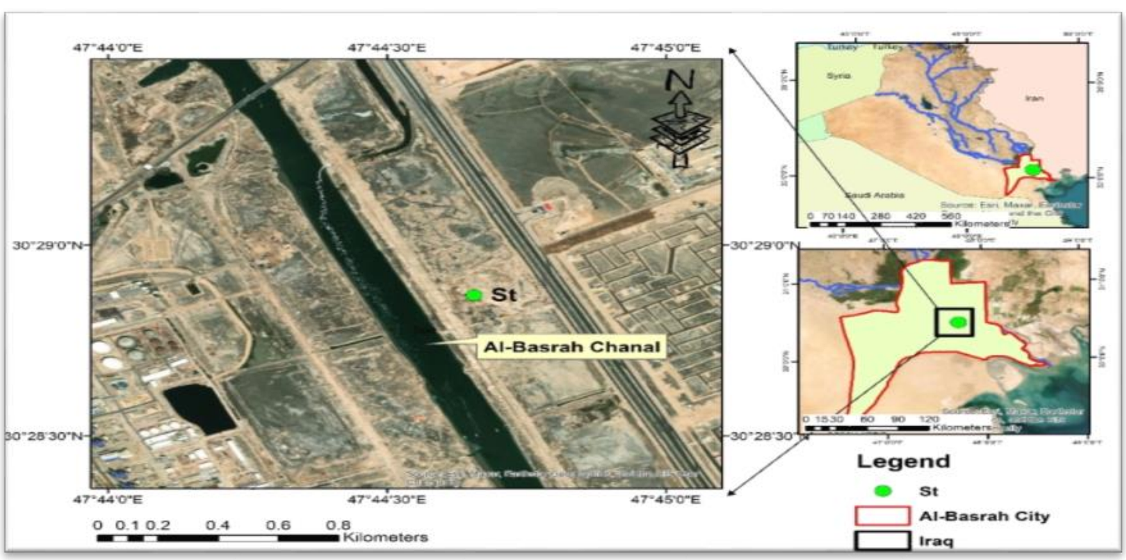


Figure 1. Location of collection of algae samples

Genetic Identification

Algae DNA was extracted using GENEzol ™ DNA Reagent plant kit according to the manufacturer's protocol.

The PCR thermal cycling protocol for amplification and sequencing of ITS region, included an initial denaturtion at 95°C for 5 min; following that 35 cycles of denaturation for 30 seconds at 95°C, primer annealing for 30 seconds at 58°C and elongation for 60 seconds at 73°C, followed by 5 minutes of final elongation at 73°C. The primers given in Table 1 were used to amplify the ITS region via Polymerase chain reaction.

Table 1. Sequences of primers utilized for the amplification the ITS region

Primer	Sequence of primer	Reference
ITS F	Forward: 5́-GGAAGKARAAGTCGTAACAAGG-3́	Cheng *et al*., 2016)
ITS R	Reverse: 5́-RGTTTCTTTCCTCCGCTTA-3́	

Immobilization of algae

Immobilized algae biomass, follow the methodology employed by Abu Al-Rub *et al*. (2004). Up to 0.5g of *E. intestinalis* powdered was mixed in 3% ($^{w}/_{v}$) solution of sodium alginate (NaC6H7O6). Then the mixture was dropped into a 2% solution of calcium chloride (CaCl2.2H2O) via sterile separate funnel, to formed beads with a diameter of 4.0± 0.5mm. Leave the beads to complete gelation in the calcium chloride solution for at least four hours (Fig. 2 A). Then, the beads were rinsed with sterile deionized water. A blank set of alginate beads, prepared using the similar procedures but without algae (Fig. 2 B).

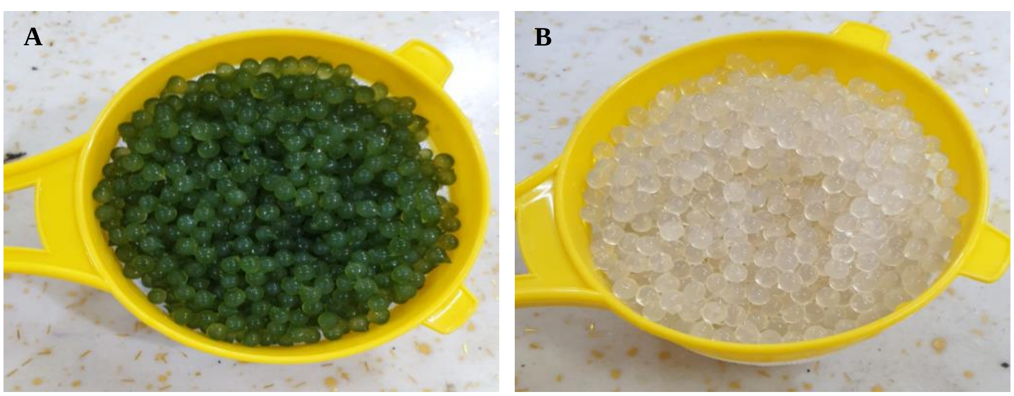


Figure 2. Preparation of A: Alginate-immobilized algae B: Blank alginate beads

Preparation of copper solution

A known quantity of pure salts Cu(NO3)2.3H2O powder was dissolved in a known volume of sterile distilled deionized water to prepared a stock solution of copper ions 1000 mg/L. Serial dilutions 5,10 and 15 mg/L were made and employed.

A biosorption experiment

The efficiency of copper ions removal using dried algae species as biosorbents was assessed in a batch system with initial nickel ion concentrations of 5, 10, and 15mg/ L, a pH of 7 ± 2 and a time of contact (0–8) days at 25°C. The experiments were carried out in 250 ml conical flasks including 100 ml of sterilized growth medium. Different concentrations of copper ions were added to the medium. Separately. Some flasks were inoculated with 5g of alginate-immobilized algae, while others were inoculated with 0.5 g (dry weight) of dried free algae biomass. Additionally, alginate beads without algae were used as a control. The flasks were stirred using orbital shaker for 8 days at 120rpm. The concentrations of copper ion in the culture medium were measured using a flame atomic absorption spectrophotometer.

Removal efficiency analysis

Removal efficiency of the absorbed copper ions by free and alginate-immobilized algae as well as control beads was investigated using the equation below:

Removal efficiency (%) = $\frac{Ci-Cf}{Ci} * 100$

Where, Ci (mg/l) is an initial metal ion concentration, and Cf (mg/l) is a final metal ion concentration (Al Prol et al., 2017).

Characterization of algae biomass

The surface morphology of the dried free and alginate-immobilized algae was probed by means of scanning electron microscopy (SEM,) while, the energy dispersive X-ray spectroscopy (EDX) using to determined algae surface of elemental composition.

Statistical analysis

The results were analyzed statistically using the SPSS Version 20. The two-way ANOVA was employed to analyze the data with a significance level set at 0.05. Additionally, the least significant difference (LSD) test was conducted to determine the extent of variation in a metal ions removal efficiency of the studied algae.

Results and Discussion

Impact of initial copper ions concentration

Relation between an initial copper ions concentrations in solution (5, 10 and 15) mg/l and the biosorption efficiency of *E. intestinalis* alga in its alginate-immobilized and free dried algae as well as alginate beads under optimized conditions of pH 7 ±2 and temperature 25°C are illustrated in the Fig. 3.

Results indicate that the means of removal efficiency for copper ions using immobilized *E. intestinalis* algae were 74.49 %, 66.34 % and 58.38 % for concentrations of (5, 10, and 15) mg/l, respective, free dried algae achieved removal efficiency of 66.51 %, 56.49 % and 50.96 % at the same concentrations. For blank alginate beads, the removal rate were 38.75 %, 33.34 % and 29.59 % for the concentrations of 5, 10, and 15ppm, respectively (Fig. 3).

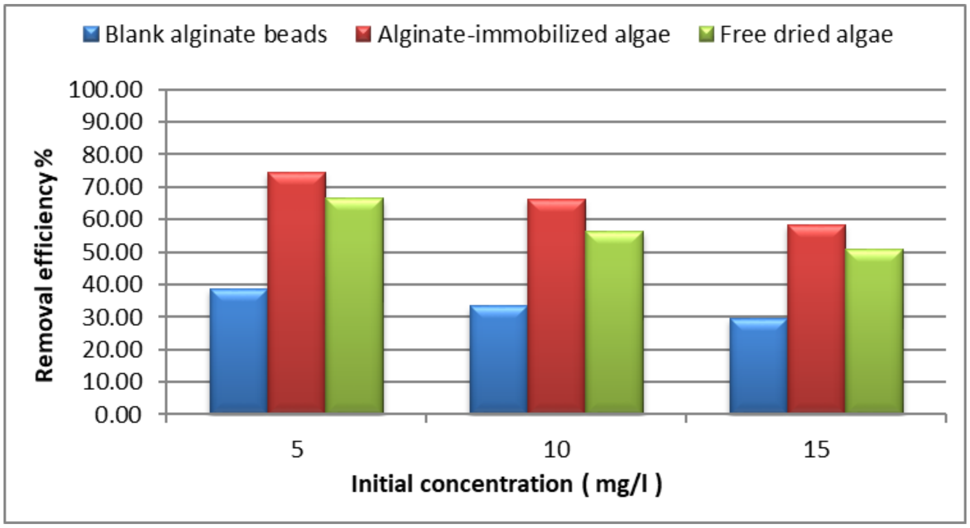


Figure 3. Impact concentration of copper ions to removal efficiency

The results of the study showed that algae efficiency in its immobilized and free states for copper ions removal increases significantly ($P< 0.05$) as the concentration of metal ions in the solution decreases through the process of biosorption (LSD= 2.944). This is attributed to the fact that at higher metal ions concentration, causes the binding sites on the surface of the algae to become saturated, which leads to fewer available active sites for further adsorption (Kumar *et al*., 2006). However, at low concentrations, a number of ions in the solution is equal to or comparable to the number of available active sites on the surface of algae, resulting in a higher biosorption efficiency (El-Naggar *et al*., 2019).

The statistical results showed significant differences ($P< 0.05$) among the studied alga states (LSD= 0.258), where it was found that alga in its alginate-immobilized state achieved the highest removal percentage for all initial concentrations compared to both the free dried algae state and the control alginate beads. This outcome suggests that algae immobilizing enhances its ability to remove pollutants from solution, possibly by improving its stability and increased number of binding sites after the integration cells of algal into alginate beads (Katırcıoglu *et al*., 2008). These results are consistent with the study by Barquilha *et al*, (2017), which demonstrated that alginate-immobilized marine algae *Sargassum sp* have a high absorption capacity for nickel and copper ions compared to the absorption capacity of free algae biomass state.

Impact of contact time

The experiments were conducted to evaluate the impact of contact time in the process of adsorption on algae surface in its immobilized and free dried states Fig. 4.

The highest copper removal rate by alginate-immobilized algae appeared on the fourth, sixth and eighth day of the experiment for the concentrations of (5, 10 and 15) mg/l, respectively (Fig. 4). When using a free dried algae state, the highest removal rate of initial concentration of (5 and 10) mg/l was observed on the second day of experiment, beyond which there was no further removal increase (Fig 4 A,B) while, at initial concentration 15 mg/l, the highest removal efficiency recorded on fourth day of the experiment. (Fig. 4C). The statistical results indicated significant differences ($P< 0.05$) among zero, two and four (LSD=0.577) while, no significant difference ($P> 0.05$) were found among days four, six and eight of the experiment.

Similar experiments were conducted with controls alginate beads, and removal was observed in second day of the experiment at concentrations of 5 and 10 mg/l (Fig. 4 A,B) while, on the fourth day at concentration of 15 mg/l (Fig. 4C).

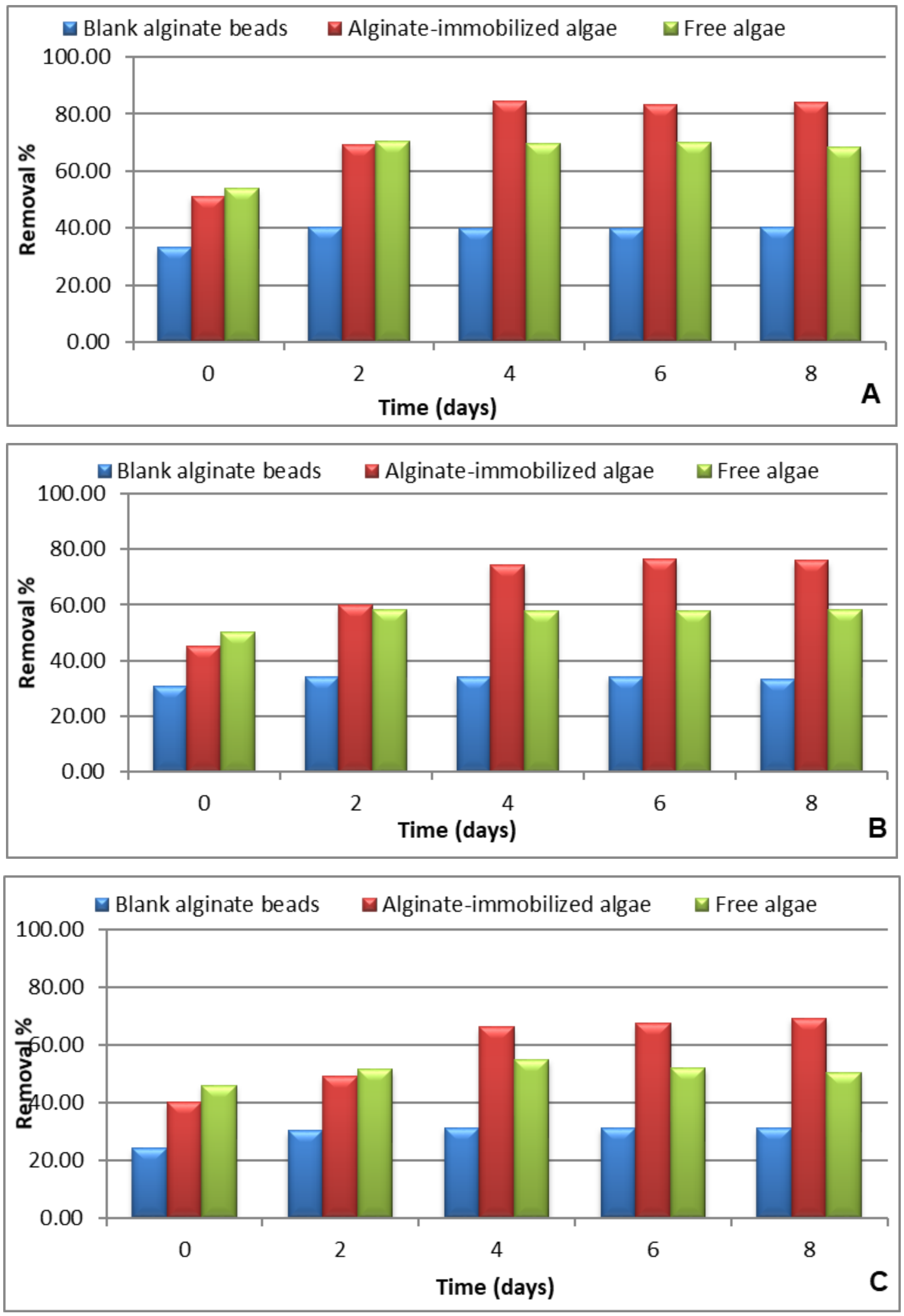


Figure 4. Impact of contact time on copper ions biosorption by *E. intestinalis* at A. 5 mg/l; B. 10 mg/l, and C.15 mg/l

It appears from the results that the copper absorption was enhanced by increasing the contact period until equilibrium was achieved at different time intervals and depending on the algae states. The initial adsorption was elevated, probably due to the extensive surface area of the biosorbent present. Upon exhaustion of the adsorbent capacity at equilibrium, the absorption was regulated by the transfer of the metal ions from the external surface to the internal sites of the biosorbent particles (Verma *et al*., 2006). Nevertheless, the duration required to achieve the equilibrium was significantly related to an initial concentration of copper ions (Jiang *et al*. 2020).

Study results showed that removal rate of copper ions using free dried algae state was faster compared to alginate-immobilized algae. The slow kinetics of immobilized biomass relative to free algae can be attributed to biomass entrapment inside the immobilized matrix, but the binding sites of free algal biomass remain accessible to metal ions. This finding was achieved by (Benaisa *et al*., 2019).

Characterization of biomass

Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX) studies

SEM combined with EDX analysis of the *E. intestinalis* dried biomass in its free and immobilized were performed to investigate the morphology surface of alga prior to and subsequent to the copper metal ions adsorption.

Fig (5A) shows the surface of the dried alga in its free state before exposure to the copper ions, where it appeared in this form as a result of drying and grinding. Meanwhile, The SEM image after absorption of copper ions is presented in Fig. 5B., where algal filaments appeared fragmented and shattered, with additional luminous areas observed on the surface correspond to copper ions adsorbed, as confirmed by the EDX results.

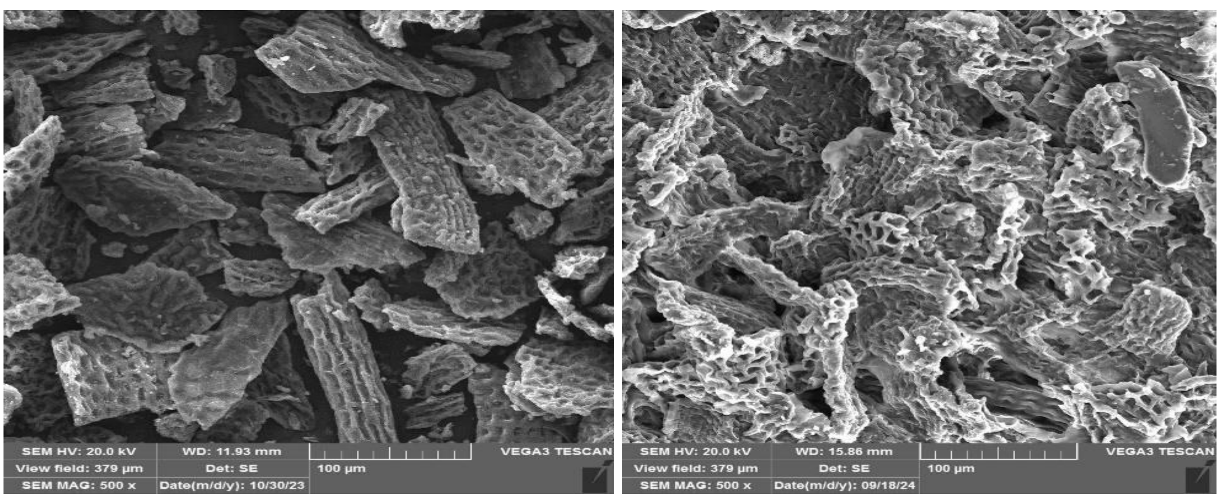


Figure 5. SEM micrographs of free dried *E. intestinalis* A: before treated B: after treated with cooper ions.

The dried alga state after being immobilized with alginate is shown in the SEM (Fig. 6A), where it appeared spherical with almost regular dimensions, with folds visible on the alga surface. These folds are beneficial for adsorption purposes as they allow metal ions to aggregate (Yasir et al., 2022).

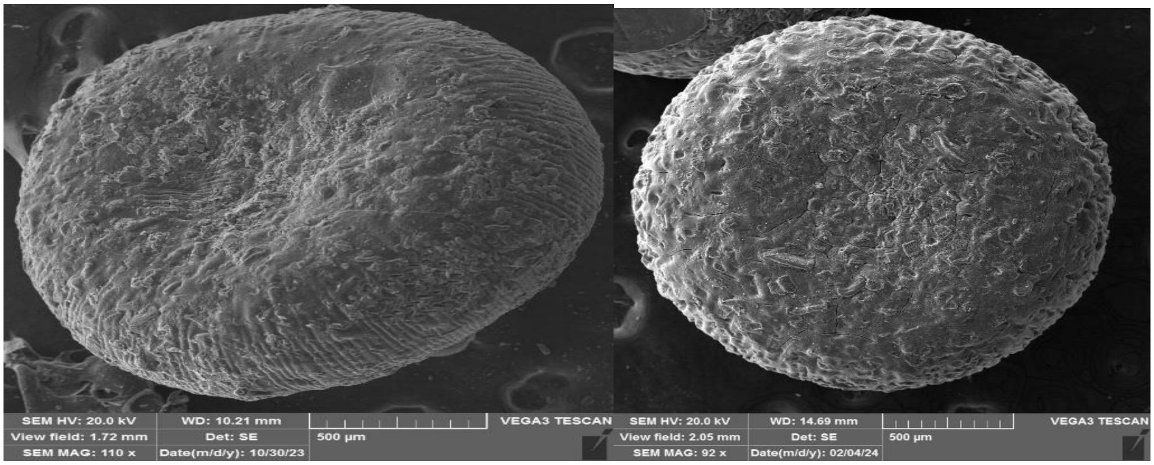


Figure 6. SEM micrographs of alginate-immobilized *E. intestinalis* A: before treated B: after treated with cooper ions.

Meanwhile, (Fig. 6B) shows the alginate-immobilized alga after exposure to copper ions.It can be observed that the surface became rougher, with the appearance of many folds and grooves, which are surface characteristics that are useful for adsorbents to maximize interaction with absorption sites on the biomass (Navarro et al., 2016).

EDX results of free state of dried algae (Fig. 7A) showed the presence of O, C, Mg, P, S, Cl, K, and Ca, which are anticipated elements attributable to the biological characteristics of the algae. In the EDX spectrum analysis after copper adsorption (Fig. 7B), three new peaks corresponding to copper appeared at about 0.98, 8.03 and 8.9 keV. The existence of this element confirmed the sequestration of copper metal by the algae surface.

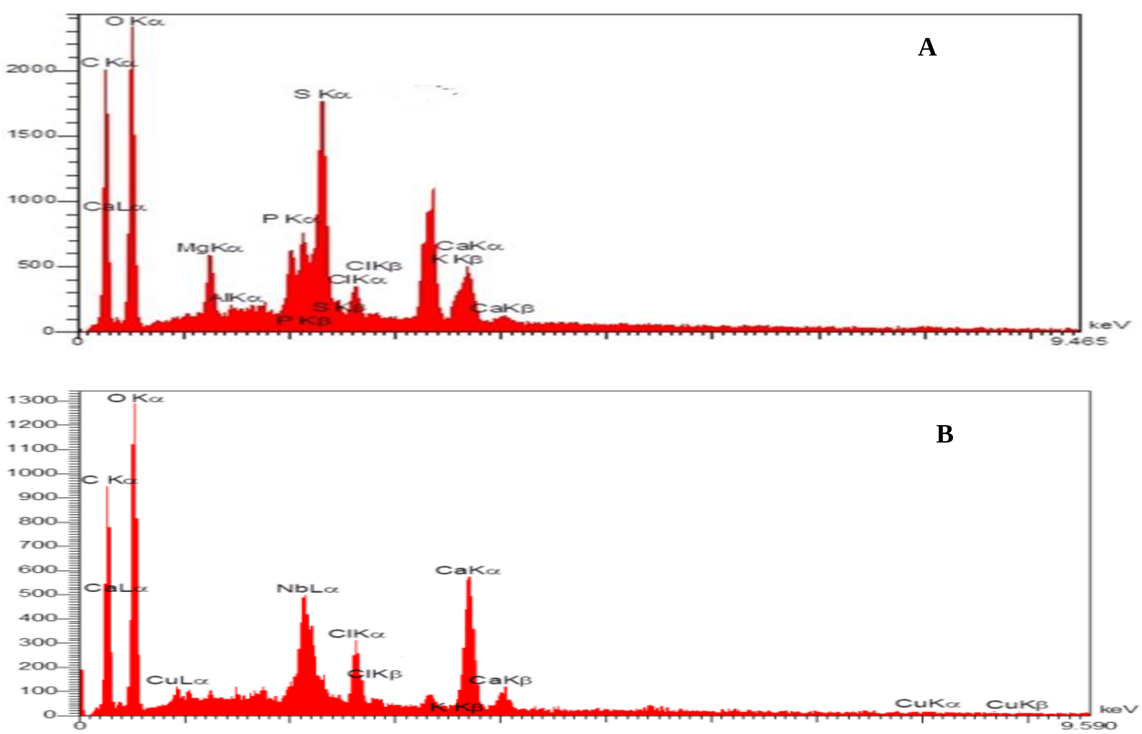
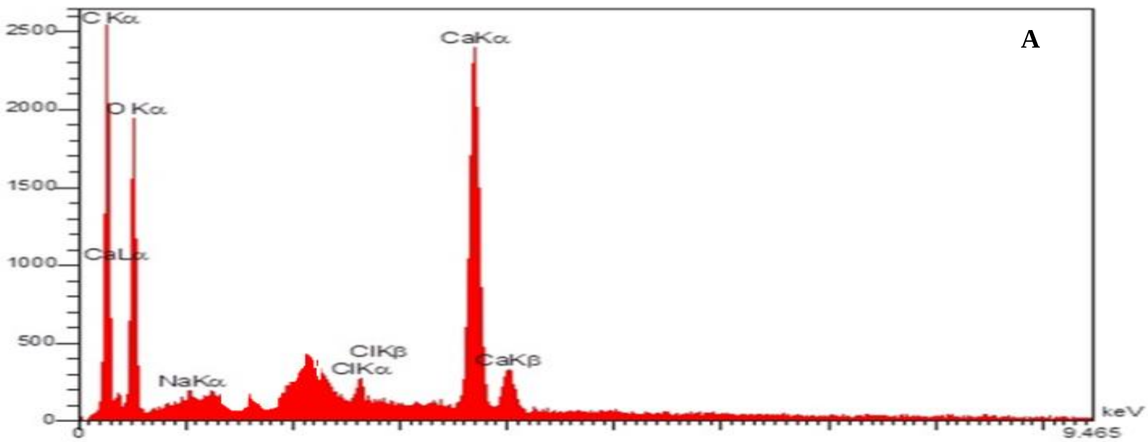


Figure 7. EDX analysis of free dried *E. intestinalis* A: before treated B: after treated with copper ions.

The EDX results for alginate-immobilized alga as control sample showed the presence of C, O, Na, Cl, and Ca as indicated in figure (Fig. 8A). The utilization of $CaCl_2$ as a hardening agent in the formulation of algal beads accounts for the pronounced Ca peak observed in EDX spectra (Ismaiel *et al*., 2022). Furthermore, the appearance of three distinct peaks 0.98, 8.03 and 8.9 keV of copper in EDX spectra of the treated immobilized algal samples (Fig. 8B) further indicates the biosorption process. It demonstrates that algal biomass can effectively sequester copper ions (Vijayaraghavan *et al*., 2018), as well as varying concentrations and peaks for other elements ions. These elements, naturally occurring in the cell walls of algae, are associated with various functional groups (Ahmad *et al*., 2018).



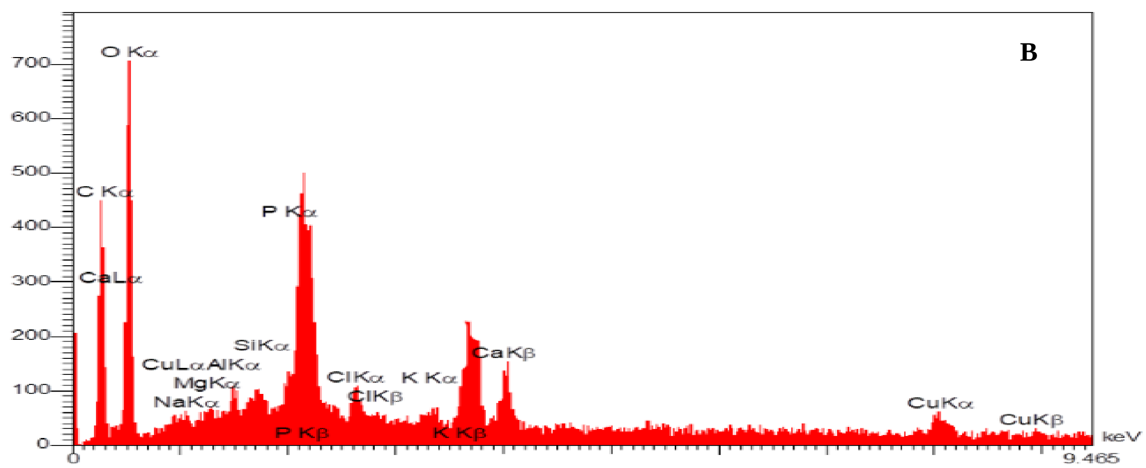


Figure 8. EDX analysis of alginate-immobilized *E. intestinalis* A: before treated B: after treated with copper ions.

Conclusions

In this study, dried *E. intestinalis* algae in both immobilized and free states were used as a biosorbent for the removal of copper metal ions from aqueous medium in batch system, in addition to using alginate beads as a control agent. Results obtained from this work indicated that alginate-immobilized algae improved copper ions adsorption compared to the free algae cells. Factors such as an initial concentration and contact time had a significant impact on biosorption process. A surface of both alginate-immobilized algae and free dried algae was examined using SEM-EDX before and after the biosorption process. Given the high efficiency of alginate-immobilized algae in removing copper, the use of the immobilization process instead of free algae cells can be considered a low-cost and an eco-friendly bioremediation approach compared to expensive physical and chemical treatment options.

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