International Journal of Scientific Research Studies

ISSN(print): Applied, ISSN(online): Applied

Volume 01 Issue 01 September 2024

Page No: 01-10

Immobilization of *Chlorella vulgaris* in Alginate Beads and its Application in Heavy Metal Removal from Industrial Wastewater

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ABSTRACT

Heavy metal ions have been one of the most serious wastewater pollution problems. The physicochemical parameters of the industrial wastewater including the heavy metals "Cd, Pb, Zn, Ni, Cu, Co, Fe and Mn" for a petrochemicals plant were measured. The marine green algae Chlorella vulgari was used as efficient, eco-friendly and low cost biosorbent for heavy metals ions removal from industrial wastewater. pH, nitrates, Temperature, sulphates, orthophosphate, and heavy metals including Pb, Fe Cu, Cr, Zn, Cd, Ni and Mn were measured. According our results, the biosorption efficiency was 20% after 15 min of water treatment, while after 60 min the efficiency reached to 84.5% meanwhile after 120 min, the efficiency rate remained stable or was slightly lower than 60 min at biomass weight 1 g/L. Fresh biomass of the micro-green alga C. vulgari used as low cost, efficient, and eco-friendly biosorbent for some heavy metals' removal.

KEYWORDS: Chlorella vulgaris, heavy metals, biosorption, and Immobilization.

ARTICLE DETAILS

Published On:
09 September 2024

Available on:
https://ijsrs.org/

INTRODUCTION

Clean and good quality water resources are essential to all living organisms for their growth, reproduction and survival. The waste drainage, domestic, agricultural, industrial, mining, manufacturing and power generation had a negative impact on water quality, biota and Human needs **(Gad *et al*., 2011).** Heavy metals are elements with atomic weights ranged from (63.5 - 200.6) and a basic gravity greater than 5.0. Industrial waste water is the main source of heavy metals contamination (**Shanab *et al*., 2012, Mosleh *et al*., 2012).** In recent years, heavy metal pollution in wastewater has become a big concern. Heavy metals are not biodegradable, thus they accumulate in living tissues as a result of transportation and transformation, resulting in long-term sustainability detrimental impact on the water environment and human health **(Deng *et al.*, 2008; Harabawy and Mosleh, 2014).**

Increasing urbanization and industrialization have caused HMs to reach dangerously harmful levels in the environment, implying that HM enrichment in many ecosystems is directly linked to human activity **(Lasat, 2000; Estrella and Garcia, 2009; Mofeed and Mosleh, 2013)**. Heavy metals (HM) are released to the water environment through, agricultural chemical, (pesticides, fertilizers), industrial activities (painting, petroleum refining), mining activities, smelting, car exhausts, battery manufacturing and pigments**)** wastes **(Lesmana *et al.*, 2009; Ardila *et al*., 2017; Mosleh *et al*., 2021; 2023)**. They can endanger ecosystems and public health because of their high mobility, lack of degradation and high capacity to accumulate within all living beings. Heavy metals can participate in biological reactions that ruin vital processes within cells, tissues and organs promoting disease even it was low at the environment **(Mosleh *et al.*, 2006; Mosleh, 2013; Jinsong and Paul Chen, 2014)**. In an ecological way, however, any metal or metalloid that pollutes the environment or cannot be decomposed biologically is considered a pollutant (and is therefore bio accumulated**)** it's possible to classify it as an HM **(Estrella and Garcia, 2009)**. Some of these metals are essential micronutrients for plant growth (e.g., zinc, copper, manganese, nickel, and cobalt), whereas others have unknown biological functions and are poisonous such as Cd, Pb and Hg **(Gaur and Adholeya, 2004; Mosleh and Mofeed, 2014).** Mercury, cadmium, copper, zinc, lead, and nickel, among other harmful heavy metals, are well-known freshwater and marine contaminants **(Mehta and Gaur 2005; Singh *et al*., 2007**). Certain heavy metals such as cobalt, copper, iron, manganese, molybdenum, vanadium, strontium and zinc are needed by traces for the activities of living organisms. However, high amounts of these metals may be toxic **(Mutawie, 2013; Mosleh *et al*., 2014; Ibrahim, 2016**).

While arsenic, cadmium, chromium and copper are widely used in industrial processes and have toxic effect on human health**. (Mead, 2005; Onalo, 2015)**. Arsenic listed by the USEPA as the second most frequent pollutant of great importance as it continues to pollute water bodies across the globe. Cadmium is not vital and it has no advantage for the environment, it is only harmful to the ecosystem. It's used in nickel–cadmium batteries, plastics and pigment manufacturing. According to **(Onalo, 2015)** the existence of cadmium in the water bodies can lead to dangerous situations in fish including the alteration of steroid hormones, contractions of the skeletal and longitudinal body, and collapse of the vertebrae. Chromium is popular metal in our environment that is richly distributes on earth **(Mofeed and Mosleh, 2013; Mofeed, 2017; Nashwa, 2018; Bayader *et al* ., 2018).** Burning coal and oil is a rich source of chromium in our environment as it released through drainage and fertilizer**s (Bayader *et al*., 2018).** The oxidation state for chromium such as Cr^{+3}, Cr^{+6} are toxic for all living organisms and human health **(Mohanty and Kumar, 2013; Bayader *et al*., 2018; Mosleh *et al*., 2023).** Also copper is an essential trace element, which is needed by biological systems for the activation of some enzymes during photosynthesis where at higher concentrations it has adverse effects on the human health **(Mosleh *et al*., 2021**).

Several methods exist for absorbing metals from the environment, such as precipitation, ion exchange or sorption and these are physical- chemical processes that are very active in removing metal from water but costly. While the conventional treatment methods for extracting biologically polluted heavy metals or wastewater effluents include: exchange of ions, filtration of membranes, reduction of contaminants, chemical precipitation, adsorption of activated carbon, treatment of nanotechnology, electrochemical removal and advanced oxidation **(Nashwa, 2018; Mosleh *et al*., 2021, 2023)**.

Additionally, there are biological processes that use live or dead, free or immobilized cells of bacteria, fungi and algae or plant tissues, as cells have carbohydrates and polypeptides in their walls with hydroxyl, aldehydes, ketones, amines, phosphates and carboxyl groups responsible for metal caption adsorption and chelation **(Abdel-Aal and Mofeed, 2020, Liliana *et al*., 2017; Mosleh *et al*., 2023)**.

Unfortunately, many of these conventional processes are limited due to significant disadvantages, which are low selectivity, high cost, incomplete removal, high energy consumption or high toxic waste generation. Therefore, the need for safe, cheaper, and more effective methods for extracting heavy metals from contaminated water needed research into low-cost alternative methods to those available commercially**.** Biosorption is using of living organism **(Nashwa, 2018)**, mainly microorganism (fungi, bacteria, algae and yeast) as bio sorbents. It's an alternative technique for extracting heavy metals from waste water due to many advantages such its low cost and high efficiency **(Handojo Djati Utomo *et al*., 2016).** The biosorption mechanism; there are many ways for metal uptake by microbial cell **(Ahalya *et al*., 2003)**. They can be classified according to different criteria depend on metabolism in dependent according to the location where metal removed from solution. It may be as: extra cellular accumulation. Biosorption is a physiochemical property of biological material that causes contaminants, primarily HMs, to be removed from wastewater by ionic or covalent bonding **(Zeraatkar *et al*., 2016; Salama *et al*., 2019).** Metal biosorption from aqueous solutions has the potential to be a useful wastewater treatment system. It focuses on biological materials ability to absorb heavy metals ions from wastewater through metabolically mediated or physicochemical uptake pathways (which can include living or dead microorganisms and their components, seaweeds, and so on) **(Fard *et al*., 2011; Nashwa, 2018)**. Several studies have shown that the key advantages of microorganisms extracting heavy metals from wastewater over traditional treatment methods include; lower cost, better performance, high quality, reducing chemical and/or biological sludge, bio sorbent regeneration, and the potential for metal recovery **(Nashwa, 2018)**.

Algae are a wide category of eukaryotic organisms ranged from unicellular like Chlorella to multicellular like giant kelp and to huge brown algae that can grow up to 50 meters. When compared to other photosynthetic organisms, this group of living organisms is characterized by their high productivity per unit area. Algae can absorb nitrate, phosphorus and heavy metals leading to an enhancement of the water quality **(Davis *et al*., 2003; Li *et al*., 2010).** The benefits of algal biosorbents can compensate for the drawbacks of commercial resins, which have reduce sorption efficiency at lower metal concentrations in wastewaters **(Eccles, 1999).** The majority of studies focus on the removal efficiency of metals by algal dry materials biomass, meaning that dead cells may absorb more metals than living cells **(Mehta and Gaur, 2005).**

Lower cost, improved performance and high efficiency are the key advantages of using such microorganism **(Lombi *et al*., 2001; Ghada, 2017; Mosleh *et al*., 2021, 2023**). The principle of waste water treatment using plant species for purification contaminated water is generally referred to as phytoremediation. In the absorption and accumulation of different heavy metals various plant species have been identified and checked for their characteristics, thereby inactivating or Trans locating metals. This is seen as a modern and highly promising technology for the recovery of contaminated sites and is cheaper than physicochemical approaches.

The aim of the present study was to evaluate the feasibility of fresh microalgae *Chlorella vulgaris* to remove selected heavy metals from wastewaters.

MATERIALS AND METHODS

Microalgal biomass and stock solution

Chlorella vulgaris isolated species of green microalgae obtained from National Institute of Oceanography and Fishery, Egypt. Algal biomass grew using Bold basal medium (BBM). *Chlorella vulgaris* was maintained in batch cultures containing 100 mL of Bold basal medium (BBM) (**Bischoff and Bold, 1963**), under continuous aeration, at 22 ±1 ºC, illumination of $\mu mol.m^{-2}s^{-1}$ and placed on an orbital shaker (150 rpm). Every week the subcultures were made by adding 20 mL of one-week-old suspension into 100 mL of fresh growth medium.

Harvesting of microalgae

Harvesting of the algal biomass culture was done by centrifugation at 4000 rpm for 10 min. The recovered biomass was washed in deionized water before being centrifuged again. Samples were dried in oven at 60 °C until the humidity reached the lowest level, and finally stored at 4°C for further use.

Collection and preparation

Water samples were collected from industrial drainage of Petrochemicals plant; the samples were kept in the dark and transported to the laboratory, where the chemicals investigations were done. Industrial wastewater samples were mixed well, and filtrated through millipore filtration system (Millipore Comp. 0.22) and stored at 4°C to be used for chemicals analysis.

Physico-chemical analysis

The filtered industrial wastewater sample was taken in a polyethylene bottle in order to directly determine the physico-chemical parameters (Temperature, pH, Nitrite –N, Nitrate-N, Ammonia-N, and total dissolved salts, Chlorides, Sulfate, Reactive Silica, Hardness, Total Carbon, Alkalinity and Ortho-phosphate) as soon as reaching the laboratory, according to standard methods followed by American Public Health Association APHA, (1995).

The concentrations of heavy metals in the samples were determined using atomic absorption spectrophotometry (**kumar *et al*., 2008**). Standard operating parameters were set and the hollow lamps for Cd, Zn, Cu, Mn, Pb, Fe, Co and Ni (Analytikjena Model Nova 350) were used as radiation source and fuel was air acetylene. All the standard and samples were run in triplicate (**Kunkel, 1973**).

Immobilization of *C. vulgaris* in alginate beads and its application in heavy metal removal.

Immobilization of microalgae have been used to facilitate evaluation of its biosorption capacity to heavy metals from aqueous solution, by using separating funnel packed with immobilized *C. vulgaris* cells in sodium alginate beads. sodium alginate solution (4%) was made by dissolving 4 g sodium alginate (Sigma-Aldrish) in 100 ml distilled water and vigorously mixing for 30 minutes at 60 °C with continuous stirring for improved solubility (Kumar and Saramma, 2012; Mosleh et al 2023). After cooling, 1 g (1 % W/V) was added and stirred for 5 minutes at room temperature. The beads were made by dropping the cell suspension and sodium alginate combination into a cold sterile 2.5% $CaCL_2$ solution at room temperature in sterile conditions under gentle stirring. The beads were spherical with an average diameter of 1.5 mm 0.2 mm and a diameter of 1.5 mm 0.2 mm. The resulting spherical beads were rinsed multiple times with autoclaved distilled water to remove unreacted $CaCL_2$ from the surface, and then kept overnight at 4°C in autoclaved distilled water to stabilize and harden the beads by the same procedure, sodium alginate beads without incorporation of the *C. vulgaris* biomass are also prepared and used as control. The beads were immersed in 0.2 M HCl buffer (pH 7.2) for storage and kept at 4 °C until needed. The experiment was carried out in a 100 mL separating funnel (Simax glass) containing alginate algal beads. The separating funnel's effluent wastewater was removed. The effluent was collected in 5 ml at a flow rate of 3 $ml.min^{-1}$ on a regular basis (every 30 minutes for up to 5 hours) and examined using inductively coupled plasma-atomic emission spectroscopy. The absorption of *C. vulgaris* for metals ions was determined by the difference in the concentration of the heavy metals before and after absorption.

Biosorption Experiment

The scientific objective of this experiment was to evaluate the adsorption efficiency of *Chlorella vulgaris* as algae biosorpent depending on contact time and concentrations of the algal biosorpent. The biosorption experiments were carried out in batch mode, were, in different concentrations (0.2, 0.4, 0.6 0.8, and 1 $g.L^{-1}$) of the algae biosorpent which were added to 100 ml of the industrial wastewater in 250 ml conical flask for designed intervals period of 15, 30, 60 and 120 min on an orbital shaker at 150 rpm. The temperature adjusted at 23±1°C and pH 7.5 before adding adsorbent. The suspended solids were separated out with GF/C filter. Heavy metal ion concentrations were then measured in the filtrate water. The residual concentrations of the tested heavy metals (Cd, Zn, Cu, Mn, Pb, Fe, Co and Ni) in the filter were determined using atomic absorption spectrophotometry (**Kunkel, 1973**). The percentage removal and metal uptake efficiencies (Biosorption %) of all adsorbents were determined with following expressions (**Hashim and Chu, 2004**).

Statistical analysis

All the biosorption experiments were conducted in triplicates to substantiate the results. The data shown are the mean are means ± standard deviations. Data were analyzed by Student's T test for independent samples. Analysis was performed using the SPSS 14.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA), and the minimum significant level was set at 0.05.

RESULTS

Data presented in (fig. 1-A) showed that the treatment of aqueous solution containing Mn with immobilized *C. vulgaris* biomass in sodium alginate-beadsremoved 84.8% of metal ion at an initial concentration of 0.6 g/L of algal biomass after 120 min.

Also fig. (1-A) showed the amount of Mn adsorbed was 0.030 and 0.047 mg/L by *C. vulgaris* at concentration 0.5 g/L after 15 min and 120 min respectively. However with the highest amount of biomass 0.6 mg/L the amount of metal adsorbed reached to 0.057 mg/L and 0.069 mg/L respectively after the same time.

The initial concentration of Cd was (0.060 mg/L) as shown in (fig. 1-B) indicated that the treatment of aqueous solution containing immobilized *C. vulgaris* beads was 40 % and 48.3% after 120 min at concentration 1 and 4 mg/L of immobilized biomass respectively. The bioremoval of Cu was shown in fig. (1-C) the initial concentration of Cu was 0.090 mg/L. The removal% of Cu by fresh beads of *C. vulgaris* was at concentration 0.5 g/L of algal solution after 60 and 120 min was 21.1% and 11% respectively. While the removal percentage by fresh *C. vulgaris* beads at concentration 0.6 g/L after 60 and 120 min was 51% and 40% respectively. Data listed in fig. (1-D) According to biomass concentration of immobilized *C. vulgaris* showed the initial concentration of Ni 0.140 mg/L. Fig (1-D) presented the initial concentration of Fe 0.472 mg/L. The removal percentage at 1 mg/L by beads of *C. vulgaris* was 47% at 120 min. Meanwhile the percentage reached to 53.3 % at concentration 0.4 g/L within the same time. For Co metal ion data presented in fig. (1-E) The initial concentration of Co was 0.075 mg/L and the efficiency was 1.7 mg/L by immobilized *C. vulgaris* at concentration 0.5 g/L after 120 min and the efficiency was 38 mg/L at concentration 0.6 g/L of fresh algal biomass after the same time.

Fig. (1-F) showed Alginate beads of *C. vulgaris* was able to adsorb Zn from industrial effluent media after 60 min at concentration 0.5 g/L and 0.6 g/L was 5.8 mg/L and 101.9 mg/L respectively. Also data presented in figs (1-H) showed The initial concentration in industrial effluent waste water for metal ion Pb was 0.165 mg/L the biosorption efficiency by *algal biomass of C. vulgaris* beads reached to 1.2 mg/L and 145.7 mg/L at concentration 0.5 g/L and 6 mg/L respectively after 120 min.

DISCUSSION

Technologies for immobilization and matrices used entrapment is a technique for immobilizing cells that involves trapping them in a three-dimensional gel lattice made of natural (agar, cellulose, alginate, carrageenan) or synthetic (polyacrylamide, polyurethane, polyvinyl, polypropylene) polymers**. (Bashan, 2010; Hameed and Ebrahim, 2007; *Liu et al*., 2009; Mosleh *et al*., 2021).** Microalgae were immobilized by encapsulation, as defined by immobilized microalgae have been used in a variety of bioprocesses, including the removal of heavy metal ions**.** Wastewater treatment systems, which often require heavy metals from liquid effluents, appear to be one of the most promising applications for immobilized microalgae **(Eroglu *et al*., 2015, Mosleh *et al*., 2021, 2023).** Alginate has been widely used in the immobilization of algal and other forms of biomass. Alginate is a water-soluble sodium salt derived from algae. When calcium replaces sodium,ionic cross-linking between carboxylic acid groups occurs giving a gelatinous substance **(Mehta and Gaur, 2005)**. According to some research, immobilized cells have a higher metal sorbing capacity than free cells **(Barquilha *et al*., 2017; Rangsayatorn *et al*., 2004; Mofeed 2020)**. Beads can be used in several biosorption /desorption cycles to remove metal ions from the medium, suggesting that immobilization may be an efficient and suitable technique for biosorbent reuse **(Ahmad *et al*., 2018; Mosleh *et al*., 2021).** *Chlorella* sp. was common and effective species for the immobilization and nutrient removal purposes **(Lau *et al*., 1998 and 1997**). Algal cells immobilized on polysaccharide gels have been used to remove nitrate, phosphate, and heavy metal ions from their aqueous environment as an alternative to conventional physicochemical waste water treatment technologies **(Chen *et al*., 2005; Bayramoğlu *et al*., 2006; Abdel Hameed, 2006)** reported that the efficiency of the immobilized beads over the free cells. Immobilization tends to increase the accumulation of metal by biomass **(Aksu, 1998)**. Because of the increased permeability of the cell wall, immobilized cells are more efficient than free cells at removing metal from biomass **(Brouers *et al*., 1989; Mosleh et al., 2021)**. Several factors, including type and biomass concentrations, have a major impact on the biosorption of metals from aqueous solutions. Physico-chemical factors **(Park *et al*., 2010)** like temperature, pH, dissolved oxygen, contact time and initial metal concentration **(Li and Tao, 2015)**.

Chlorella sp. immobilized in alginate beads may be used for cadmium bioremediation processes at low concentrations of the metals, since the presence of viable micro-algae biomass increases the alginate's removal capability **(Christian Valdez *et al*., 2019)**. Immobilized cells more effective than free cells for metal removal by biomass due to increase in the cell wall permeability **(Brouers *et al*., 1989).** It is clear that, the biosorption capacity of immobilized beads of *C. vulgaris* for Zn, Ni, and Co metal ions was higher than immobilized beads of *S. obliquus* during the entire period of our work. While the biosorption of Mn, Cd, Cu, Fe and Pb by *C. vulgaris* beads was lower than other overall biosorption potential of other metal ions within waste water. Accordingly Mn, Fe ,Cu and Cd were the most adsorbed metal ions into surface of immobilized *C. vulgaris* beads . It was investigated also by (**Valdez *et al*., 2019; Mosleh *et al*., 2021).** The treatment corresponding to alginate beads with immobilized

C. vulgaris biomass is estimated to be the best for Cd elimination. At 120 minutes, it was discovered that the micro-algae improves its metal removal potential at low metal concentrations, achieving a removal percentage of 59.67 % with *Chlorella sp*., which is substantially higher than the removal offered without *Chlorella* sp. (55.56 %). This result is had compliance with our investigation for maximum capacity of Cd removal by immobilized beads of *C. vulgaris* reached to 49% after 120 min at concentration 4 mg/L. The biosorption efficiency for Pb metal ion was in a small scale when compared to other metal ions of the present study within effluent waste water. Whereas another study by **(Abdel Hameed, 2006***)* reported that Pb removal by the immobilized beads of *C. vulgaris* was 92% However, lead removal was mainly caused by the alginate beads matrix with only a slight contribution by *Chlorella vulgaris*. The green alga *C. vulgaris* is often used to study adsorption of heavy metals **(Hans and Urbach, 1983; Wong *et al*., 1979)**. The uptake efficiencies of Mn, Cu, Cd, Fe and Pb by alginate beads of green micro algae *S. obliquus* was higher than beads of green microalgae *C. vlgaris* within petrochemical effluent after 120 min, however in case of Zn , Co and Ni *S. obliquus* efficiency was the lowest in comparison to the overall biosorption potential of other metal ions (Mn, Cu, Cd, Fe and Pb) within waste water after the same time. the result by **(Ardila *et al* ., 2017, ~~Heham et al., 2021~~)** investigated the immobilized *S.obliquus* had the ability to adsrorb Cr metal ions from tannery wastewater, with removal levels of up to 35.3 % . Immobilized cells more effective than free cells for metal removal by biomass due to increase in the cell wall permeability **(Brouers *et al*., 1989).** Another study by **(Aksu, 1998)** reported that immobilization appears to increase the amount of metal accumulated by biomass**.** it's worth mentioning that biosorbtion efficiency for Pb,Zn and Co metal ions by two immobilized beads of green microalgae *Chlorella vulgaris* and *Scendsmous obliquus* within petrochemical industrial effluent had the lowest capacity/or efficiency of biosorption in comparison to the overall biosorption potential of other metal ions (Fe, Mn, Cu ,and Cd) after the same time, during the whole period of investigation.

CONCLUSION

Many studies have tried various methods for removal of heavy metal from industrial wastewater. Through the obtained results, testing the green micro-alga *Chlorella vulgaris* as a biosorbent proved its superior ability to remove heavy metals from aquas industrial wastewater that negatively adversely affect the environment. Where, during the first 15 min contact time the biosorption efficiency was about to 50%, however after 60 min. only the efficiency reached to 95.8%. The results proved differences in the ability to remove different heavy metals by bio-accumulation in algal cells, where the highest results were for lead, cadmium and iron, followed by copper and nickel, then came the rest of the heavy metals (manganese, cobalt and zinc, in order). Which makes us take into account types of heavy metals to be removed from wastewater so that we can choose the appropriate adsorbent in order to obtain the best removal efficiency. So, we recommend using *C. vulgaris* as low-cost, eco-friendly and efficient biosorpent to eliminate some heavy metals from the petrochemical's industrial effluent wastewater.

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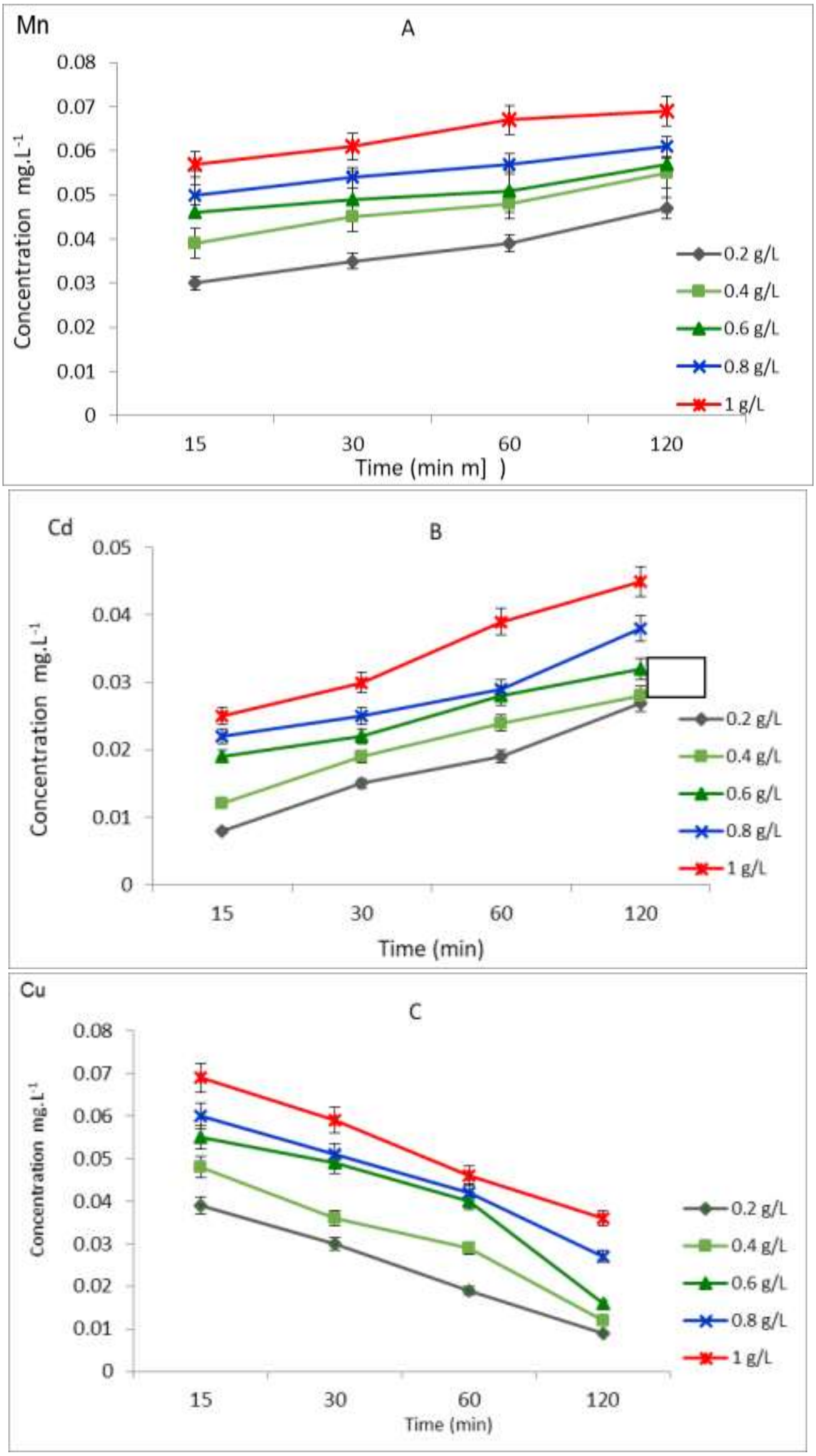

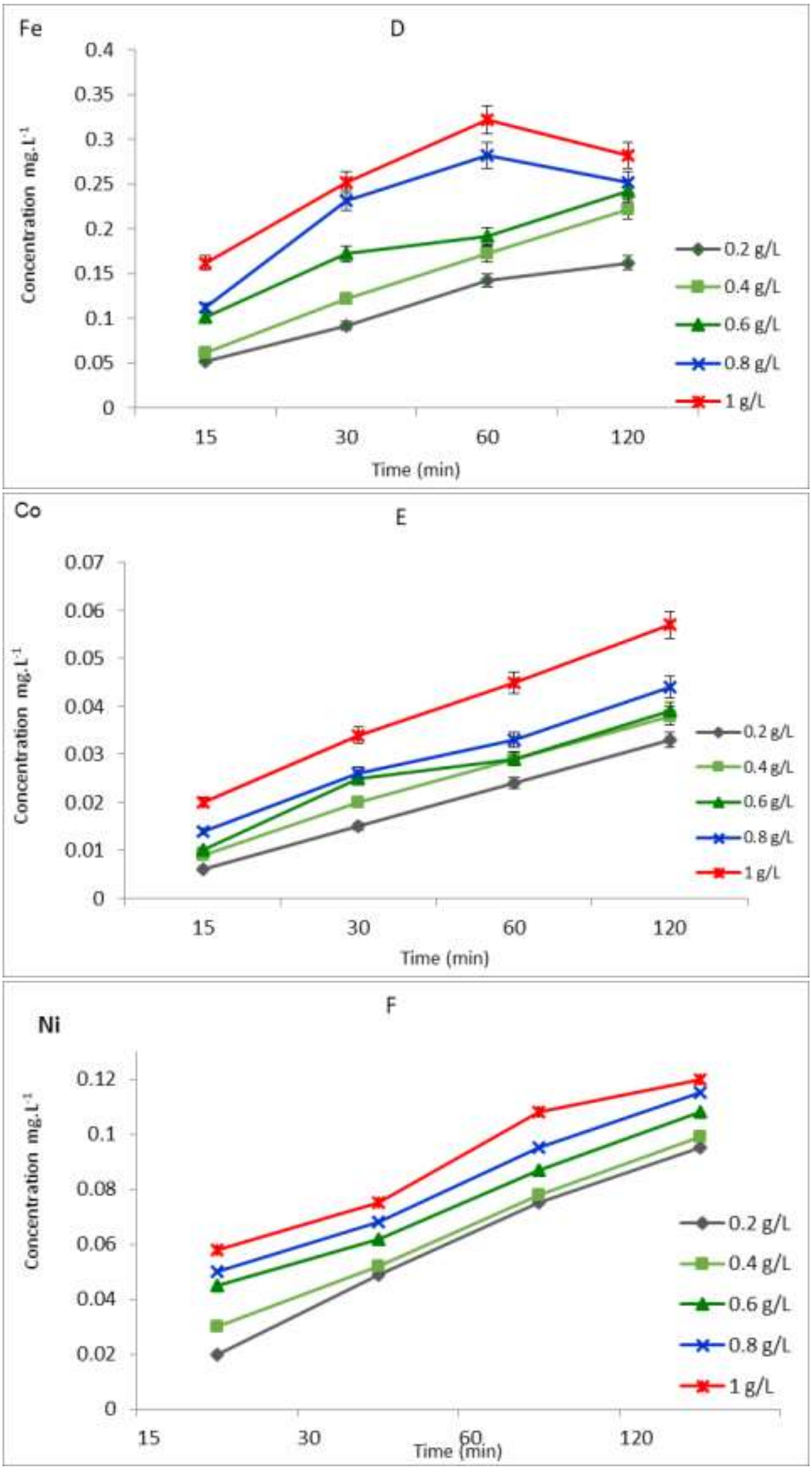
Fe
D
Concentration mg.L-1
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
15
30
60
120
Time (min)
0.2 g/L
0.4 g/L
0.6 g/L
0.8 g/L
1 g/L
Co
E
Concentration mg.L-1
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
15
30
60
120
Time (min)
0.2 g/L
0.4 g/L
0.6 g/L
0.8 g/L
1 g/L
F
Ni
Concentration mg.L-1
0.12
0.1
0.08
0.06
0.04
0.02
0
15
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60
120
Time (min)
0.2 g/L
0.4 g/L
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0.8 g/L
1 g/L

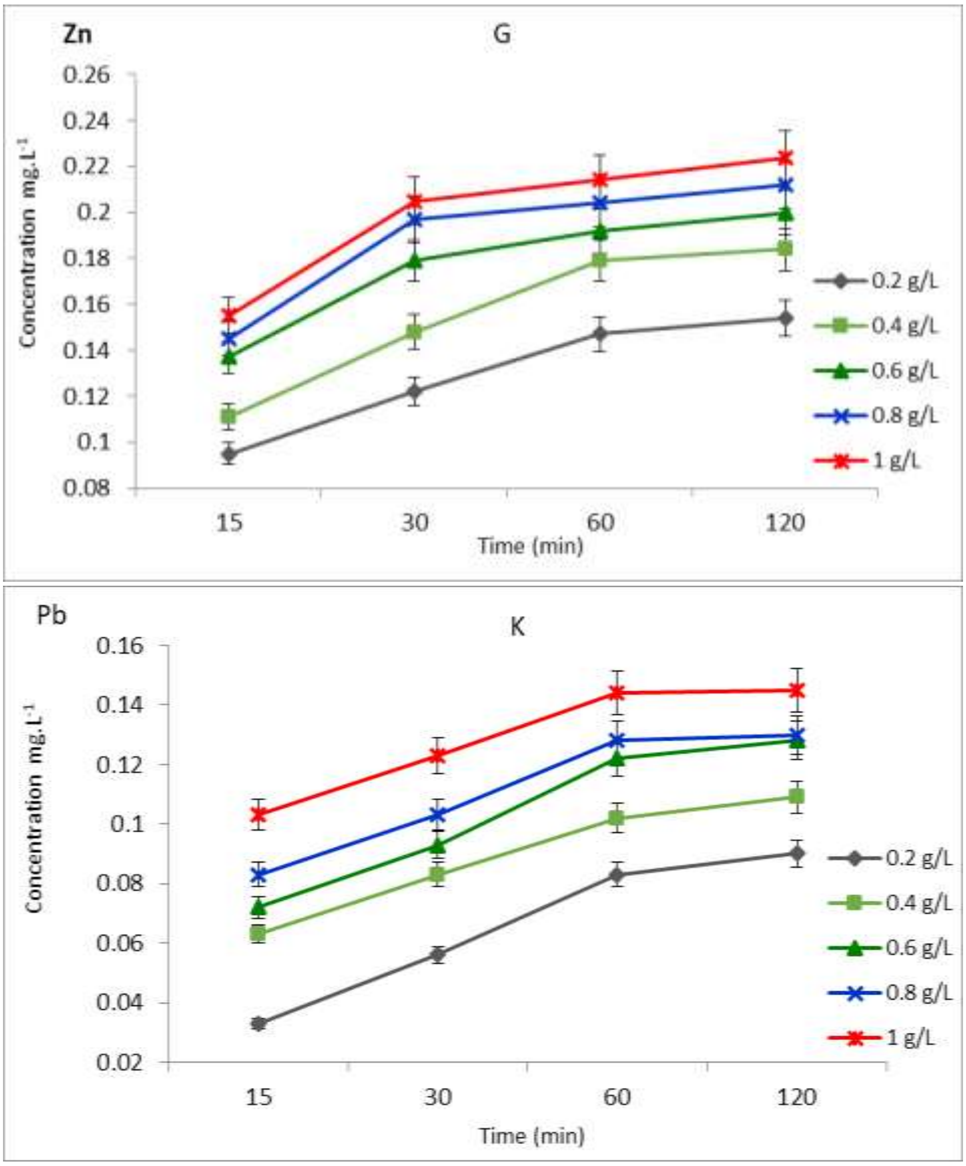

Fig (1): Immobilization of *Chlorella vulgaris* biomass in alginate beads and its application in heavy metals removal from industrial wastewater (A- Mn, B- Cd, C-Cu, D- Fe, E- Co, F- Ni, K- Zn, and G- Pb