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Assessment of bioremediation potential of *Microcystis aeruginosa* for removal of cadmium and lead ions from aqueous matrices

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Abstract

Capability of cyanobacterium, *Microcystis aeruginosa*, in the removal of cadmium and lead ions was investigated which was obtained after isolation from wastewater stabilization pond effluents. Influence of operational parameters including metal concentrations, pH, temperature and biomass dosage on removal efficiency was investigated. Also, applicability of closed reflux techniques for the digestion of cyanobacterial pellet was studied. A multi-phase washing method was developed in order to attain more elevated desorption efficiency. 1-5 washing cycles were repeated consecutively by the addition of new EDTA solution (4mM), stirring on mixer and centrifugation for each cycle. Maximum removal efficiency occurred at pH 7, temperature 28°C and biomass dosage of 0.2g for Cd^{2+} and corresponding values of 6, 25°C and 0.1g for Pb^{2+}. In all tested concentrations of Cd^{2+} and Pb^{2+}, extracellular absorption overweighed intracellular uptake with highest ratio of the former to the latter of 172 for $Cd^{2+}$and 143 for Pb^{2+}. EC_{50} values attained for $Cd^{2+}$and Pb^{2+}were 20 mg L^{-1} and 15 mg L^{-1}, respectively. The closed-reflux digestion method was found as a suitable choice for cyanobacterial pellet digestion. Three-stage consecutive washing procedure devised in our study gave desorption efficiencies of 92 and 86 percent for Cd^{2+} and Pb^{2+} respectively as compared with values of 52 and 44 percent for Cd^{2+} and Pb^{2+}, respectively obtained by the conventional washing procedure. This investigation verifies the possibility of employing *M. aeruginosa* for removal of Cd^{2+} and Pb^{2+} from aqueous and wastewater solutions.

Keywords: Bioremediation, Cadmium, Lead, *Microcystis aeruginosa*

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Introduction

The increment of population, urbanization, industrialization and their consequences such as massive production of wastewater, and the release of untreated wastes into freshwater resources have led to increased seriousness of environmental pollution concerns. (Renuka *et al.*, 2015). Wastewaters and sewage effluents have considerable amounts of heavy metals and other materials that may cause toxicity in humans (Al-Lahham *et al*., 2007). Although low concentrations of metals such as zinc, copper and iron are required for metabolic activities, other metals such as arsenic, cadmium and nickel are dangerous even in low amounts (Alavian Petroody *et al*.**,** 2017). Cadmium and lead as toxic heavy metals can jeopardize human health with consequences such as kidney injury, hepatic dysfunction and cancer (Benguendouz *et al*., 2017). Removal of heavy metals from aqueous solutions using traditional methods such as chemical precipitation, chemical oxidation or reduction, activated carbon, ion exchange, filtration, electrochemical treatment, membrane-based technologies and evaporation recovery may be associated with demerits such as inefficiency or high cost, generation of waste materials accompanied by disposal complications (Ahluwalia and Goyal, 2007). These limitations have contributed to increasing attention for utilization of biosorption technology for heavy metal removal from aqueous solutions (Onyancha *et al*., 2008). Algae properties including omnipresence in aquatic ecosystems and high affinity for binding to metals makes them suitable candidates as biosorbents of heavy metals (Jin *et al*., 2012). Cyanobacteria are preferred over other microorganisms for application as heavy metal biosorbent due to having large surface area, greater mucilage volume, high binding ability and uncomplicated nutritional demands (Gupta and Rastogi, 2008). Efficacy of some cyanobacteria such as *Dunaliella*, *Spirulina*, *Nostoc*, *Anabaena* and *Synechococcus* for heavy metals removal has been cited (Abdel-Aty *et al.*, 2013).

The purpose of present study was evaluation of cyanobacterium *Microcystis aeruginosa* with regard to biosorption capability and determination of optimum operational conditions for Cd^{2+} and Pb^{2+} removal by this cyanobacterium.

Materials and methods

Chemicals

Analytical grade chemicals were used and solutions were prepared with deionized water. Lead nitrate and cadmium nitrate (Merck Company, ultrapure grade) were used for preparation of 100 mg L^{-1} Pb^{2+} and Cd^{2+} stock solutions. Working solutions were obtained by dilution of stock solutions. HNO_3 and NaOH solutions were used for pH adjustment.

Samples were collected from effluents of wastewater stabilization pond systems located in the city of Fooladshahr, Isfahan Province, Iran. This treatment system consisted of an

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anaerobic pond followed by a facultative pond and finally a maturation pond.

Cyanobacteria identification

Collected samples were preserved using formaldehyde solution. Identification was carried out using an inverted microscope at 400× magnification using Prescott (1962) and Desikachary (1959) references.

Isolation of cyanobacteria

To obtain monocultures of cyanobacterial strains, 1mL of sample was inoculated into test tubes containing Blue Green-11 (BG-11) broth (pH 7) prepared as recommended by Rippka *et al*. (1979). Tubes were maintained at ambient temperature under irradiance of 60 µmol $m^{-2}s^{-1}$ and agitation speed of 110 rpm for ten days. After this period, a relatively pure culture of cyanobacterium was procured through repeated streaking onto BG-11 agar. Isolated cultures were identified by microscope.

Evaluation of growth was conducted by measuring optical density at 655nm (Menamo and Wolde 2015). Finally, cyanobacterial cells were gathered and thoroughly washed with distilled water and utilized in biosorption experiments.

Molecular identification

Pure cultures were centrifuged at 12000 rpm for 5 minutes to obtain cyanobacterial pellets. DNA extraction was performed by kit QIAGEN, following the manufacturer's instructions. To find presence of *M. aeruginosa*, amplification of cyanobacterial 16S rRNA gene sequences was conducted using designed primers as already suggested (Nubel *et al*., 1997): CYA106F (5'-GGG GAA TYT TCC GCA ATG GG 3') and CYA781R (5'- GAC TAC TGG GGT ATC TAA TCC CAT T 3'). PCR mixture comprised of 0.2 µL of each primer, 2mM $MgCl_2$, 0.2 mM dNTP mix, 2.5 U Go Taq Flexi DNA polymerase, 2.5 µL of 10* Go Taq Flexi PCR buffer and 150 ng of template DNA and was diluted to a final volume of 25 µL. Thermocycling program was run at 95°C for 5 minutes, 30 cycles at 95°C for 30s, 50°C-60°C for 30 s and 72°C for 1 minute and a final extension step at 72°C for 7 minutes. All DNA products were subjected to gel electrophoresis for visualization on molecular imager system. Obtained sequences were submitted to GenBank DNA database and searched using blast function.

Acute toxicity tests

Determination of heavy metals toxicity was performed by monitoring of growth inhibition test in accordance with algal growth inhibition test standards (OECD 1984). Experiments were carried out in 96 well microtiter plates with final volume of 200 µL at ambient temperature. Exponential growth phase cells were harvested and added to wells containing BG-11 medium. Then, metals solutions were dispensed in wells to reach intended final concentrations of 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50 mg L^{-1}. Microplates were incubated inside a growing chamber under irradiance of 60 µmol $m^{-2}s^{-1}$

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photons m s on a rotary shaker. Growth rate was assessed by measuring optical density at 650nm wavelength (OECD, 1984).

Biosorption experiments

Addition of cyanobacterium biomass to 250 mL flasks having different concentrations of Pb^{2+} and Cd^{2+} solution (2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50 mg L^{-1}) was attempted, followed by mixing of flasks at 110 rpm speed at ambient temperature and under cool-white fluorescent bulb for eight days. Blank samples were composed of cells incubated under the same conditions without the addition of Cd^{2+} or Pb^{2+} solution. Cyanobacterial biomass was sequestered from reaction mixtures by centrifugation at 6000 rpm for 15 minutes at intended intervals. The supernatant was acidified and refrigerated until analysis by atomic absorption spectroscopy.

Influence of initial metal concentration

To study a more extended concentration range, bioremediation potential of isolated *M. aeruginosa* in varying initial metal concentrations of Pb^{2+} and Cd^{2+} (2, 4, 6, 8, 10, 15, 20, 25, 30, 40 mg L^{-1}) was investigated.

Effect of pH

Influence of pH values ranging from 3 to 9 on removal efficiency was determined at constant concentrations of 10 mg L^{-1} of Pb^{2+} and Cd^{2+} solutions and given biomass quantity. pH adjustment was performed using dilute HCl -and NaOH normal solutions.

Effect of absorbent dosage

To ascertain variation of removal efficiency as a function of culture density, different biomass values of 0.05, 0.1, 0.15, 0.2, 0.4 and 0.5 g were employed at constant concentrations of 10 mg L^{-1} of Pb^{2+} and Cd^{2+} solutions.

Influence of temperature

Effect of varying reaction mixture temperatures (10°C-40°C) on removal efficiency of Pb^{2+} and Cd^{2+} solution of 10 mg L^{-1} was examined to obtain optimum temperature for biosorption.

Measurement of supernatant residual heavy metal

Residual concentration of lead and cadmium was measured by a flame atomic absorption spectrophotometer instrument (PerkinElmer AAnalyst700). Operational conditions including wavelength 283.3, lamp current 25mA and spectral bandwidth 0.7nm were used for lead determinations. Cadmium analysis was conducted under conditions of wavelength 228.8, lamp current 10mA and spectral bandwidth 0.7nm.

For comparison, both undigested and digested supernatant were examined. The hot plate digestion technique and closed reflux digestion method were used for decomposition of supernatant. Details of the procedure are the same as those that will be stated for cyanobacterial pellet digestion.

Cyanobacterial pellet digestion methods

Cyanobacterial pellet remaining after centrifugation was digested in order to

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measure intracellular lead and cadmium. Two digestion techniques including hot plate digestion following EPA Method 200.3 (US EPA, 1991) and closed reflux digestion method were applied.

Hot plate digestion procedure consisted of mixing of cyanobacterial pellet with a mixture of HCl and HNO_3 at 90°C on a hot plate. Addition of acid continued until the appearance of a clear solution. Final digested pellet was diluted to a determined final volume and analyzed by atomic absorption spectrophotometry.

In closed reflux digestion method, a defined volume of cyanobacterial pellet was introduced into a digestion tube. Then, HNO_3 and HCl mixture was poured into the tube, placed in a heating reactor programmed at temperature 105°C for 2h. Acids were added at determined intervals till digestion was complete. Final volume was analyzed by atomic absorption spectrometry. Total metal removal by cyanobacterial cells was obtained using the following equation (1) (Rangsayatorn *et al*., 2002).

(1): $q=(C_i-C_f)\ V/M$

Where q= total metal sorption (mg g^{-1}); M= dry mass of algae (g); V= volume of metal solution (L); C_i= initial metal concentration (mg L^{-1}); C_f= final metal concentration (mg L^{-1}).

Desorption with single- stage and multi-stage washing

Metal-impregnated biomass was generated by contacting appropriate amount (0.2g for Cd^{2+} and 0.1g for Pb^{2+}) of biomass with a defined volume of 10 mg L^{-1} metal solutions. Mixing and centrifugation stages followed. Then, cyanobacterial biomass was exposed at 20 mL of 0.02M EDTA solution for 15 minutes. The mixtures were centrifuged and metal concentration in the final solution was measured by flame atomic absorption spectrometry.

In addition to the single-phase washing method mentioned, multi-phase washing procedure was also tested for achievement of best washing method. Multi-phase washing is the same as single-phase method only with this difference that addition of new EDTA solution (4mM), stirring on mixer (3 minutes for every cycle) and centrifugation was repeated consecutively so that 1,2,3,4,5 washing cycles were attained. Consequently, the most efficient washing procedure was selected for the following experiments. For comparison, desorption efficiency was calculated by the formula:

Desorption efficiency=amount of metal ion desorbed/amount of metal ion absorbed×100

Statistical analysis

Results attained in triplicate were shown as mean±standard deviation in tabular or graphic formats. Analysis at p value less than 0.05 was considered statistically significant. All statistical analyses were conducted by SPSS v.18.0 (IBM Corp., Armonk,NY,USA) program.

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Results

Morphological identification

Colorless mucilage, spherical cells with the presence of gas vesicles within whole cell volume were observed. Cell diameters sized from 4 to 7µm. Colonies were irregular in outline, lobate with distinct holes in older colonies. Cells were densely and irregularly agglomerated with irregularities at margin. Diffuse mucilaginous margin slightly overlapping cell agglomerations was also found.

16SrRNA-based identification of cyanobacterial isolate

Amplification and sequencing of 16S r RNA gene were used for molecular identification. The sequence was analyzed at NCBI server using BLAST tool and corresponding sequences were downloaded. Utilizing internal primers, sequences belonging to isolated cyanobacterium exhibited 98 percent similarity with *M. aeruginosa*. The isolate is identified as *M. aeruginosa* on the basis of 16S rRNA gene sequence analysis.

Growth measurements

Potency of isolated *Microcystis aeruginosa* for Pb^{2+} and Cd^{2+} removal was studied at various initial metal concentrations for an exposure time of 8 days. Growth pattern of cyanobacterial biomass was monitored by determination of optical density values in the exponential growth phase. Results regarding growth rate for different concentrations of Pb^{2+} and Cd^{2+} were graphed in Figs. 1 and 2. Data showed decreased absorbance values associated with increasing metal concentrations. Lead concentration ranging from 2 to 6 mg L^{-1} was found not to cause significant influence on growth rate as compared to control samples (p>0.05), but higher lead concentrations exhibited a considerable influence on growth rate (p<0.05). Growth rate was insignificantly affected by 2, 4, 6, 8 mg L^{-1} of cadmium in comparison to control samples. (p>0.05). On the contrary, cells exposed to cadmium concentrations higher than 8 mg L^{-1} (10-50 mg L^{-1}) showed significant fall in growth rates (p<0.05). EC_{50} values obtained for cadmium and lead were 20 mg L^{-1} and 15 mg L^{-1}, respectively.

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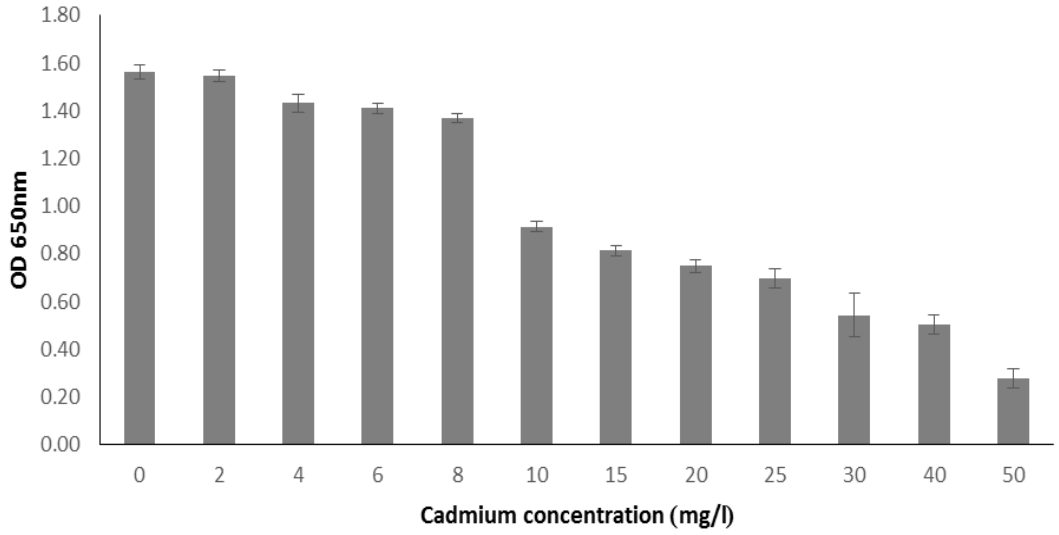

Figure 1: Effect of different concentrations of cadmium on growth of *Microcystis aeruginosa.*

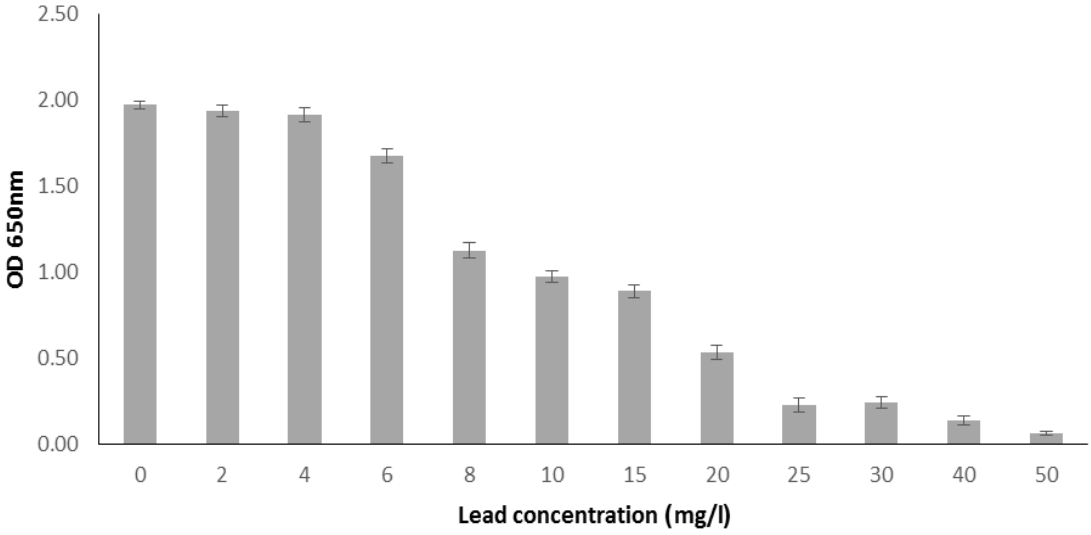

Figure 2: Effect of different concentrations of lead on growth of *Microcystis aeruginosa.*

Contribution of initial metal concentration to removal efficiency and metal uptake was investigated for an exposure time of eight days. Total metal removal, intracellular uptake and extracellular fraction for Pb^{2+} are shown in Table 1.

Elevated metals concentrations were coincident with increased metal uptake, so that the increment of the initial concentration of cadmium from 2 to 30 mg L^{-1} coincided with enhancement of metal uptake from 0.905 to 9.74 mg g^{-1}. This trend also was true for Pb^{2+} with uptake capacity of 0.905 and 7.15 mg g^{-1} for 2 and 30 mg L^{-1} respectively on the first day of exposure.

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Table 1: Removal of Pb^{2+} by *Microcystis aeruginosa* as bioadsorption and bioaccumulation at different initial concentrations of pb^{2+} .

Pb^{2+}	Biosorption (mg g^{-1})	Days							
		1	2	3	4	5	6	7	8
2ppm	Removal	0.908±0.14	0.902±0.12	0.894±0.08	0.891±0.11	0.872±0.15	0.812±0.18	0.793±0.32	0.764±0.13
	Adsorbed	0.857±0.11	0.841±0.15	0.865±0.12	0.872±0.15	0.844±0.18	0.791±0.21	0.775±0.32	0.753±0.18
	Accumulated	0.051±0.03	0.06±0.02	0.029±0.008	0.019±0.005	0.028±0.009	0.021±0.004	0.018±0. 005	0.011±0.007
4ppm	Removal	1.12±0.16	1.32±0.12	1.48±0.17	1.66±0.15	1.75±0.18	1.86±0.21	1.65±0.31	1.52±0.14
	Adsorbed	0.96±0.11	1.11±0.08	1.21±0.12	1.32±0.17	1.38±0.13	1.42±0.14	1.34±0.25	1.28±0.011
	Accumulated	0.16±0.05	0.21±0.03	0.27±0.06	0.34± 0.08	0.37±0.04	0.44± 0.11	0.31±0.05	0.24±0.09
6ppm	Removal	1.75±0.17	1.94±0.22	2.36±0.32	2.68± 0.28	2.48± 0.42	2.35± 0.21	2.28±0.27	2.22±0.17
	Adsorbed	1.47±0.13	1.58±0.18	1.84±0.25	2.11±0.31	2.32±0.31	2.21±0.16	2.17±0.14	2.12±0.14
	Accumulated	0.28±0.04	0.36±0.08	0.52±0.11	0.57±0.04	0.16±0.08	0.14±0.03	0.11±0.05	0.10±0.03
8ppm	Removal	2.18±0.45	2.57±0.21	2.83±0.11	3.29±0.52	3.18±0.18	2.97±0.32	3.11±0.37	3.15±0.52
	Adsorbed	1.87±0.28	2.18±0.18	2.37±0.13	2.75±0.45	2.69±0.24	2.56±0.41	2.73±0.19	2.98±0.41
	Accumulated	0.31±0.07	0.39±0.09	0.46±0.0.03	0.54±0.13	0.49±0.12	0.41±0.05	0.38±0.04	0.17±0.13
10ppm	Removal	2.85±0.51	3.15±0.42	3.64±0.72	4.32±0.34	4.91±0.61	5.21±0.61	5.32±0.74	5.14±0.21
	Adsorbed	2.37±0.19	2.54±0.17	2.85±0.18	3.34±0.27	3.85±0.45	4.11±0.34	4.17±0.55	4.08±0.16
	Accumulated	0.48±0.15	0.61±0.08	0.79±0.11	0.98±0.18	1.06±0.28	1.10±0.14	1.15±0.38	1.06±0.13
15ppm	Removal	3.42±0.32	3.91±0.52	4.23±0.41	4.82±0.51	5.24±0.51	5.48±0.48	5.82±0.64	6.34±0.32
	Adsorbed	2.85±0.18	2.97±0.64	3.15±0.33	3.42±0.62	3.62±0.51	3.72±0.23	3.93±0.33	4.41±0.47
	Accumulated	0.57±0.12	0.94±0.25	1.08±0.13	1.4±0.24	1.62±0.51	1.76±0.14	1.89±0.52	1.93±0.11
20ppm	Removal	4.73±0.23	5.32±0.42	5.92±0.62	6.18±0.34	6.72±0.21	7.22±0.34	7.11±0.27	6.94±0.14
	Adsorbed	3.82±0.41	4.21±0.54	4.52±0.53	4.67±0.78	5.14±0.13	5.38±0.62	5.22±0.61	5.17±0.52
	Accumulated	0.91±0.18	1.11±0.13	1.40±0.61	1.51±0.31	1.58±0.64	1.84±0.41	1.89±0.53	1.77±0.64
25ppm	Removal	5.23±0.42	5.84±0.65	6.17±0.21	6.82±0.71	7.34±0.52	7.85±0.65	7.21±0.65	6.23±0.41
	Adsorbed	4.18±0.18	4.62±0.31	4.85±0.43	5.12±0.62	5.46±0.41	5.92±0.41	5.85±0.82	5.76±0.32
	Accumulated	1.05±0.0.17	1.22±0.18	1.32±0.52	1.70±0.43	1.88±.0.11	1.93±0.16	1.36±0.15	0.47±0.12
30ppm	Removal	7.15±0.62	8.14±0.54	9.62±0.32	11.34±0.42	13.54±0.82	14.82±0.31	14.64 ±0.64	14.75±0.91
	Adsorbed	6.84±0.12	7.75±0.18	9.18±0.62	10.85±0.27	12.96±0.15	14.11±0.65	14.07 ±0.48	14.52±0.95
	Accumulated	0.31±0.11	0.39±0.07	0.44±0.17	0.49±0.11	0.58±0.16	0.71±0.14	0.57±0.17	0.23±0.08
40ppm	Removal	7.11±0.34	7.36±0.24	8.52±0.52	9.82±0.18	10.48±0.65	11.57±0.41	11.38±0.33	10.64±0.18
	Adsorbed	6.54±0.52	6.72±0.19	8.41±0.14	9.65±0.34	10.17±0.34	11.22±0.72	11.14±0.14	10.52±0.42
	Accumulated	0.57±0.18	0.64±0.22	0.11±0.06	0.17±0.05	0.31±0.09	0.35±0.18	0.24±0.58	0.12±0.13
50ppm	Removal	7.11±0.34	7.36±0.24	8.52±0.52	9.82±0.18	10.48±0.65	11.57±0.41	11.38±0.33	10.64±0.18
	Adsorbed	6.54±0.52	6.72±0.19	8.41±0.14	9.65±0.34	10.17±0.34	11.22±0.72	11.14±0.14	10.52±0.42
	Accumulated	0.57±0.18	0.64±0.22	0.11±0.06	0.17±0.05	0.31±0.09	0.35±0.18	0.24±0.58	0.12±0.13

Variation of metal removal on different exposure days was also examined. Reaction mixtures containing low cadmium or lead concentrations showed highest value of metal uptake on the first day of exposure. On the other hand, cultures with higher metal concentrations reached maximum uptake on the following days of exposure.

The ratio of extracellular cadmium to intracellular fraction was variable in different cadmium concentrations. The ratio increased with initial cadmium concentration with the highest value of 172 occurring in cultures in contact with the concentration of 50 mg L^{-1} cadmium. Differences between bioadsorbed fractions and bioaccumulated parts were statisticallysignificant for all concentrations of cadmium and lead all through the exposure period (p<0.05).

Influence of pH

Variation of uptake rate due to pH change ranging from 3 to 9 is rendered in Fig. 3. A pH increase from 3 to 7 was accompanied by boosted absorption capacity. The use of higher pH values viz. 8 and 9 led to decreased uptake rates. Influence of acidic and alkaline pH on removal capacity was significant (p<0.05).

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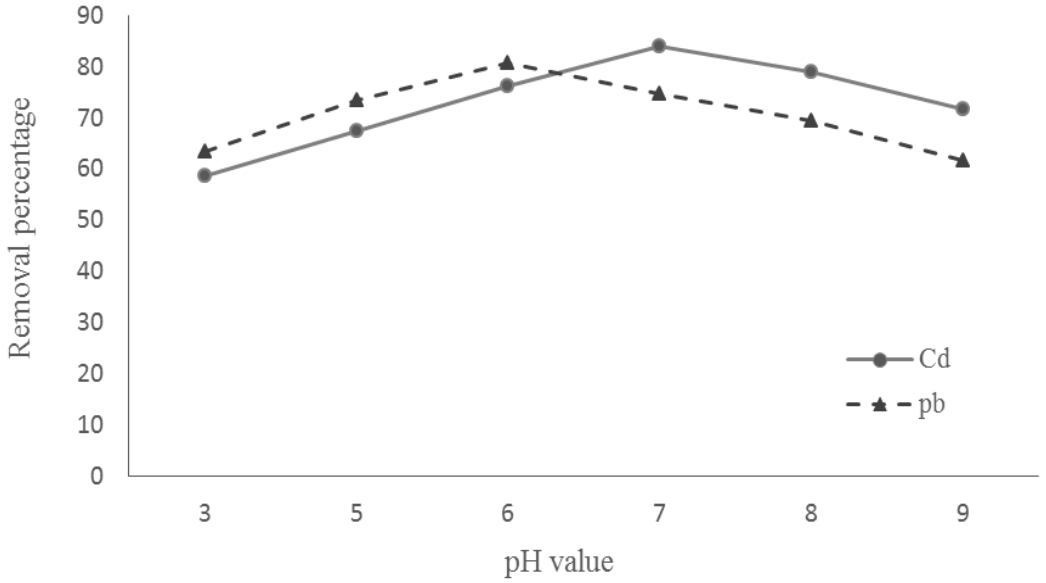

Figure 3: Influence of pH on removal efficiency of cadmium and lead solutions of 10 mg L^{-1} by *Microcystis aeruginosa.*

Effect of biomass dosage

Biosorption efficiency variation derived from employment of different biomass dosages for cadmium is presented in Fig. 4. Increase of sorbent quantity resulted in higher efficiency with optimum values of 0.2g and 0.1g for cadmium and lead, respectively. The addition of biomass values beyond the cited values was not found to bring about a considerable change in that a plateau was reached.

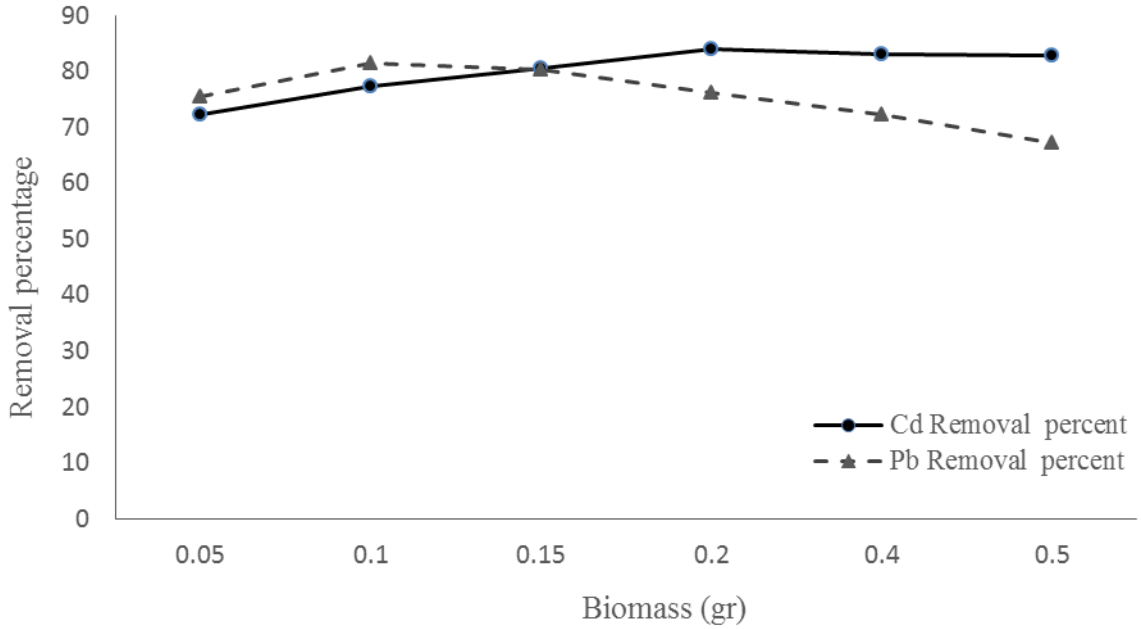

Figure 4: Effect of biomass dosage of *Microcystis* on removal efficiency of cadmium and lead solutions of 10 mg L^{-1} by *Microcystis aeruginosa*.

Influence of temperature

Effect of temperature ranging from 10°C to 40°C on removal efficiency of Cd^{2+} and Pb^{2+} (10 mg L^{-1}) was studied. Removal efficiency of Cd^{2+} and Pb^{2+} at lowest temperature (10°C) was

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significantly different from efficiency obtained at temperatures of 25^0C and 30°C (p<0.05). Maximum percentage removal of Pb^{2+} and Cd^{2+} took place at temperatures of 25 °C and 30°C (Fig. 5).

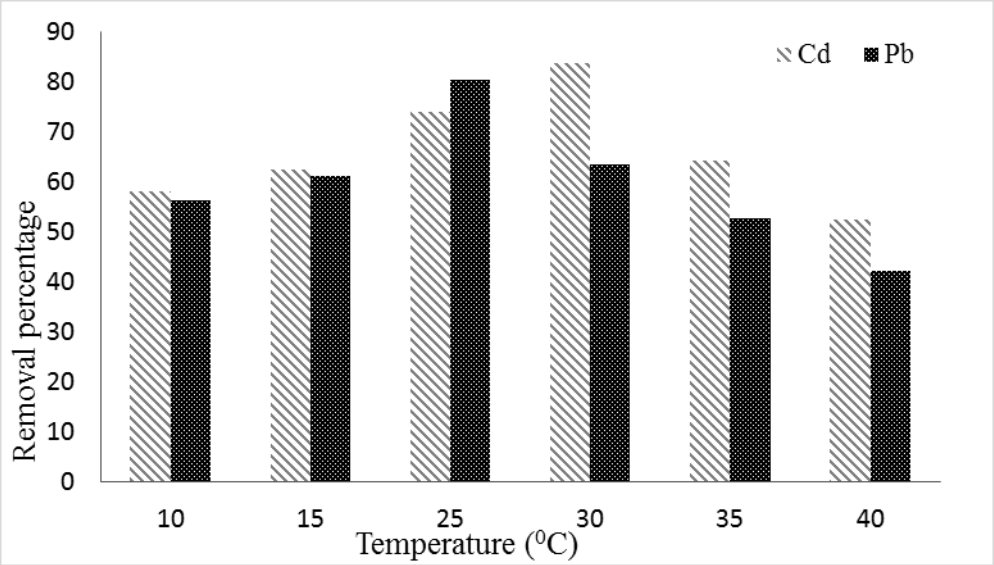

Figure 5: The effect of temperature on removal efficiency of cadmium and lead solutions of 10 mg L^{-1} by *Microcystis aeruginosa.*

Desorption efficiency

Desorption efficiency of Cd^{2+} and Pb^{2+} (10 mg L^{-1}) by conventional washing method was only 52 and 44 percent, respectively. Multi-phase washing method developed in our study resulted in improved desorption efficiency. Desorption efficiency values of Cd^{2+} and Pb^{2+} (10 mg L^{-1}) obtained from the first washing cycle were only 24 and 18 percent, respectively. An increasing trend of efficiency with successive washing was observed. Better results were derived from two washing cycles with efficiency values of 67 and 51 percent for Cd^{2+} and Pb^{2+}, respectively. Application of three washing cycles was accompanied by attainment of values of 92 and 86 percent for Cd^{2+} and Pb^{2+}, respectively. Employment of washing for four and five stages contributed to marginal improvement of desorption efficiency, henceforth, utilization of three washing cycles was selected as the optimum situation. Comparison of findings showed that a three-stage washing method with dilute EDTA solution was more efficient than using concentrated EDTA solution for a single-stage washing.

Digestion of supernatant

In order to determine the necessity of digesting the supernatant used for determination of residual heavy metal, both undigested and digested supernatants were analyzed by atomic absorption spectrophotometry. The two digestion methods including hot plate digestion following EPA method and closed reflux digestion were compared. Undigested samples showed values significantly lower than digested ones (p<0.05), even heavy metal content of some undigested samples was not detectable by flame atomic absorption

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technique. Supernatant residual metal values obtained in the two digestion procedures were comparable (p>0.05).

Cyanobacterial pellet digestion

Suitability of the two digestion techniques viz. the hot plate method and closed-reflux procedure for cyanobacterial pellet decomposition was assayed. Values procured using the methods mentioned were in good agreement with each other and either of the methods was found suitable for cyanobacterial pellet digestion (p>0.05) (Table 2).

Table 2: Comparison of hot plate digestion method and closed reflux digestion technique for determination of intracellular Pb^{2+} on the fifth day of exposure.

Pb Standard solutions (mg L^{-1})	Intracellular Pb (mg g^{-1}) by Hotplate digestion	Intracellular Pb (mg g^{-1}) by Closed reflux digestion
2	0.028±0.009	0.034±0.007
4	0.37±0.04	0.24±0.02
6	0.16±0.08	0.21±0.05
8	0.49±0.12	0.53±0.15
10	1.06±0.28	0.98±0.19
15	1.62±0.51	1.72±0.55
20	1.58±0.64	1.47±0.52
25	1.88±0.11	1.95±0.16
30	0.58±0.16	0.51±0.11
40	0.31±0.09	0.42±0.04
50	0.24±0.09	0.18±0.03

Discussion

Growth inhibition effects of low concentrations of both metals were not detected. In a similar study by E. Torres *et al*. (1998) cadmium solution exhibited inhibitory effects on growth of marine diatom *Phaeodactylum tricorunutum* (EC_{50}=22.39) only in concentrations higher than 5 mg L^{-1}. These findings matched the results of Rzymski *et al*. (2014) that cited negligible influence of low concentrations of cadmium and lead on *M. aeruginosa*. Attained EC_{50} values were higher than values reported previously for some species. Concentration of 0.06 mg L^{-1} cadmium completely arrested growth of *Anabena inequalis* (Les and Walker, 1984). In comparison, the reported cadmium EC_{50} value for cyanobacterium *Spirulina platensis* was 17.28 mg L^{-1} (Rangsayatorn *et al*., 2002). EC_{50} values obtained showed that *M. aeruginosa* is resistant to lead and cadmium toxicity, that is, it can be regarded as a heavy metal-tolerant cyanobacterium. Regardless of containing high EC_{50} values and consequently considerable tolerance against cadmium, *Dunaliella salina*, was not able to remove cadmium efficiently in an investigation conducted by Folgar *et al.* (2009). Thus, this point must be stressed that a high EC_{50} value does not necessarily mean high removal capability.

Direct relationship between initial metal concentration and removal capacity (mg g^{-1}) are in agreement with the investigation of Monteiro *et al*. (2011) who pointed out the direct

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association between Zn concentration and uptake capacity of microalga *Scenedesmus obliquus*. This behavior can be attributed to the role of metal concentrations in the enhancement of the mass transfer driving force required for ions diffusion and consequent improved metal uptake. Metabolism-independent biosorption was dominant in all prepared cadmium and lead concentrations. The priority for the bioaccumulation process for heavy metal removal by *M. aeruginosa* is in accordance with findings of Bi *et al.* (2016) that revealed marked surface-adsorbed cadmium in comparison with bioaccumulated fractions. Similar conclusions were drawn in the study of Folgar *et al*. (2009) who found low contribution of intracellular uptake in total cadmium removal. Investigations performed by Miranda *et al*. (2013) also displayed the very significant role of surface adsorption for Pb^{2+} removal by cyanobacterium *Chroococcus multicoloratus*. Results incongruous to our observations have been rendered in the work of Sisman-Aydin *et al*. (2013) where extracellular removal was only the main mechanism on the first day and the following days were characterized by the dominance of intracellular uptake.

The priority for surface adsorption could be attributed to the existence of exterior polysaccharide layers on cyanobacteria walls that could provide appropriate circumstances for binding of heavy metals via adsorption. A similar explanation has been presented by Singh *et al.* (1998) who compared capsulated and decapsulated *Microcystis* cells for biosorption, demonstrating a considerable contribution of the external layers in biosorption. In addition to the reasons mentioned, high metal concentration can lead to cyanobacterium cell damage followed by the arrest of metabolically-mediated removal and also increased dissemination of intracellular cadmium.

The effect of pH on bioremediation can be described as follows; the lower the pH, the less the uptake rate of cadmium. Rzymski *et al.* (2014) concluded that maximum removal efficiency of Cd^{2+} by *M. aeruginosa* was attainable at a neutral pH value. Remarks consistent to our data are also found in a study by Rangsayatorn *et al.* (2002) who reported pH 7 as the optimum value for cadmium removal by *Spirulina platensis*. A similar trend was observed for lead solutions. Most removal percentage of 10 mg L^{-1} Pb^{2+} solution was obtained at pH 6.

Of course, unsuitability of an acidic pH for bioremediation is not a general rule. Sun *et al.* (2014) stated coincidence of pH 2.5-2.6 to maximum biosorption of antimony by *Microcystis*. Also, a study of Malkoc and Nuhoglu (2013) on removal of chromium by *Ulothrix zonota* demonstrated highest biosorption at pH 1. Comparison of these inconsistent observations demonstrated varying optimum pH values for biosorption of different metals by the same algal species, as mentioned above.

It must be pointed out that lower pH can lead to an increase in metal toxicity because most metals are found as free metal ions at low pH. Also, affinity of

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H^+ ions to bind to algal cells can restrict surface subjected to metal ions adsorption. Higher pH effects can be attributed to generation and precipitation of insoluble metal hydroxides or anionic hydroxide complexes before biosorption can take place.

Data related to biomass quantity parameters were indicative of considerable contribution of biomass dosage to biosorption efficiency. Higher available attaching points resulting from increased surface area of sorbent could justify higher efficiency associated with increased sorbent concentrations. Aggregation of algal biomass and consequent decreased surface area for absorption could highlight trends obtained at higher dosage. Positive influence of increased temperature can be attributed to boosted interaction of metal ions and active sites and thinned boundary layer around adsorbent groups. Also, enlargement of algae vents at higher temperature and ensuing increment of surface area for sorption, diffusion and penetration of metal ions can also be considered. Although removal of Pb^{2+} and Cd^{2+} at different temperatures was different, they occurred more and less without approaching zero. Temperature increment beyond optimum value was demonstrated not to favor biosorption due to damage to and consequent loss of sorption sites.

The conventional washing method was not adequate to strip surface-bound metals completely, but multi-phase washing ameliorated desorption efficiency. Several arguments can be cited for this observation. Firstly, some of eluent solution potency may be spent for sequestration of surface-bound competitive cations such as H^+ which can lead to incomplete removal of surface-bound metal cations in the single-stage conventional washing procedure. Secondly, some metal cations forming strong binding with functional groups may not be separated easily by the one–stage washing procedure, but consecutive washing procedures may weaken the binding gradually and release metal cations.

Results demonstrated that acidification of supernatants for preservation until analysis time is not only sufficient, but digestion is also necessary to break binding between metal cations and organic materials available in the supernatant. Polysaccharides and other metabolites produced and released by cyanobacteria in the supernatant, are mostly organic in nature and form strong binding with metal cations. Employment of the closed reflux digestion technique for supernatant digestion was found to be useful especially when low volumes of supernatant are limiting factors for the use of the hot plate digestion technique. Results showed that the use of the closed-reflux digestion technique by thermal reactor can be poised as a candidate for cyanobacterial pellet digestion. Lower possible environmental pollution of cyanobacterial pellet, decreased acidic solution requirement, shortened digestion time and digestion under pressure and high temperature are some

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of advantages of the closed-reflux digestion over the hot plate method.

Cyanobacterium *M. aeruginosa* showed high tolerance and potency for removal of lead and cadmium from aqueous solutions. Surface adsorption was the main mechanism for removal of tested metals. This property is noticeable for biosorbent commercialization, because removal efficiency is not considerably dependent on the metabolic processes of the organism.

The closed reflux digestion technique can be utilized for the decomposition of cyanobacterial pellet and the supernatant. Also, a multi-phase washing procedure is more efficient than the conventional washing method and must be addressed in biosorption experiment.

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