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REMOVAL OF HEAVY METALS USING BACTERIAL BIO-FLOCCULANTS OF *BACILLUS SP.* AND *PSEUDOMONAS SP.*

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Abstract. Bio-flocculants produced by *Bacillus sp.* and *Pseudomonas sp.* were evaluated as flocculating agents for the removal of Cu (II), Pb (II) and Cd (II) from chemical and textile wastewater industries. Both bio-flocculants were very effective for removal of heavy metals at a dosage not exceeding 0.1 mg/ml. However, the removal efficiency of heavy metals was dependant on initial concentration and type of bio-flocculants. 84.0% of Cu^{2+} and 99.5% of Pb^{2+} were removed from industrial wastewater using *Bacillus sp.* Bio-flocculant resulting residual values of 28.5 and 1.13μg/l respectively in the treated effluent. Lower removal efficiencies of 70.4% for Cu^{2+} and 97.8% for Pb^{2+} occurred using *Pseudomonas sp.* bioflocculant. Nevertheless, *Pseudomonas sp.* bio-flocculant achieved a substantially higher removal efficiency of Cd^{2+} (93.5%) as compared to 72.9% using *Bacillus sp*. Based on these results bio-flocculants are considered as a viable alternative for the treatment of industrial wastewater containing heavy metals.

Keywords: heavy metals, wastewater, bio-flocculants, *Bacillus sp*., *Pseudomonas sp*.

Introduction

Heavy metal pollution is one of the most serious environmental problems that threaten a large number of people. Heavy metals are not biodegradable and tend to be accumulated in organisms and cause numerous diseases and disorders. Extensive damage to human organs, such as liver, kidney, digestion system, and nervous system can be caused by uptake of excess heavy metals (Ozer, Pirincci 2006; He *et al.* 2013). The discharge of heavy metals into the environment due to agricultural, industrial, and military operations and the effect of this pollution on the ecosystem and human health are growing concerns. Recent research in the area of heavy metals removal from wastewaters and sediments has focused on the development of materials with increased affinity, capacity, and selectivity for target metals (Gadd 1990; Gadd, White, 1993; Totura 1996). The use of microorganisms to sequester, precipitate, or alter the oxidation state of various heavy metals has been extensively studied (Gadd 1990; Gadd, White, 1993; Macaskiey 1990; Rittle *et al.* 1995; Shen, Wang 1993). Industrial wastes contain toxic and hazardous substances, most of which are detrimental to human health (Gana *et al.* 2008; Ogunfowokan *et al.* 2005; Rajaram, Ashutost 2008). Heavy metals from industrial processes are of special concern because they produce water or chronic poisoning in aquatic animals (Ellis 1989). More researchers are focusing their attention on industrial wastewater treatments (El-Sheekh *et al.* 2005; Gao *et al.* 2009; Liu *et al.* 2009; Lu *et al.* 2005; Wang *et al.* 2007) and on drinking water treatment (Liu *et al.* 2009) using microbial bio-flocculants. The use of bio-flocculants in wastewater treatment seems to be an economical alternative to physical and chemical means (Vijayalakshmi, Raichur 2003).

Biological materials or dead bacterial cells (Zheng *et al.* 2008), algae (El-Sheekh *et al.* 2005), protozoans (Rehman *et al.* 2008), yeasts, fungi (Guangyu, Thiruvenkatachari 2003) and plants (Heredia, Martin 2009) have been shown to play significant roles in heavy metal removal and recovery. Amongst bio-remediants, bio-flocculants have gained increasing attention since they are environmentally-friendly, biodegradable and non-toxic (Shih *et al.* 2001). Bio-flocculants contain various organic groups, which are responsible for binding metals, such as uronic acids (containing a carbonyl and a carboxylic

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acid function) (Aguilera *et al.* 2008; Lu *et al.* 2005; Wu,Ye 2007), glutamic and aspartic acid in the protein component (Dignac *et al.* 1998) or galacturonic acid and glucuronic acid in the polysaccharide component (Bender *et al.* 1994). The fact that these bio-flocculants have higher efficiencies at low metal concentrations makes them very attractive for the removal of heavy metals from industrial effluents/wastewaters (Kotrba *et al.* 1999). Feng *et al.* (2013) used microbial flocculant GA1 (MBFGA1) to remove Pb^{2+} ions from aqueous solution. The removal efficiency of Pb^{2+} reached up to 99.85%. Moreover, Lin, Harichund (2011b) recorded that up to 90% of Pb^{2+} was removed by bio-flocculant produced by *Pseudomonas sp*. CH8. Bio-flocculants produced by *Pseudomonas* sp. CH6 and *Herbaspirillium* sp. CH13 flocculated 78% of Hg^{2+} and 66% of Cd^{2+} from the metal solutions respectively.

In order to mitigate the metal pollution in Egyptian industrial wastewater, metal-tolerant bacterial species that are capable of producing bio-flocculants have been isolated from industrial sludge. The removal efficiency of heavy metals (Cu^{2+}, Pb^{2+} and Cd^{2+}) present in industrial wastewater using bacterial bio-flocculants of *Bacillus sp.* and *Pseudomonas sp.* was investigated at different initial concentrations.

1. Materials and methods

1.1. Wastewater characteristics

Industrial wastewater containing heavy metals was collected from chemical and textile manufacturing companies (Shoubra El-Kheima – Qaluobyia Governorate, Egypt). The samples were collected in sterilized plastic bottles. Copper (Cu), lead (Pb) and cadmium (Cd) concentrations were determined by GBC Atomic Adsorption Spectroscopy (AAS) (Savanta AA). pH was measured using HANNA HI 9024. pH values of the wastewater ranged between 6.4–7.9. The concentration of Cu, Pb and Cd are highly fluctuated and varied (47±1–482.6±2.5 μg/l), (85±1.5–304.3±1.5 μg/l) and (34.4±0.66–321±6 μg/l) respectively.

1.2. Isolation and identification of bacteria

Bacillus sp. and Pseudomonas sp. bacteria species resistant to heavy metals were isolated from industrial sludge harvested from pipe effluent of chemical and textile industry. *Bacillus sp.* and *Pseudomonas sp.* were selected for preparation of bacterial bio-flocculants (Duguid *et al.* 1975; Nanda *et al.* 2011). Serial dilution and pour plating method using nutrient agar, blood agar and MacConky agar (Himedia Company) were used. Strains were maintained in agar slants containing nutrient agar. The colony morphology, physiological and biochemical characteristics of different strains were assessed. Every week, isolated strains were transferred to a new medium in order to keep metabolic activity and check the purity by microscopic examination process. The growth of *Bacillus sp.* took place on blood agar with the following biochemical characteristics, large Gram-positive rod, given positive results with indole, urease, nitrate and negative with catalase, citrate and oxidase. A *Pseudomonas sp.* bacterium is Gram negative rod gave positive results with motility, ornithine, catalase, citrate and oxidase.

1.3. Production of bio-flocculants

The selected bacterial species (*Bacillus sp.* and *Pseudomonas sp.*) were cultivated in a 250 ml Erlenmeyer flask containing 30 ml YMPG medium (0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone, 1% glucose and 2% agar at pH 7) at 28°C, 220 rpm for 20 h. A portion amounting to 0.7 ml of the cultivated bacterial strains was inoculated into 70 ml of production medium (0.5% yeast extract, 0.5% polypeptone, 2% ethanol, 1% glycerol, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, and 0.2% $CaCO_3$) at the above conditions for 72 h (Nakata, Kurane 1999). Bio-flocculants were recovered from the supernatant after centrifugation (4000 rpm) for 15 min and precipitated by adding 2 volumes of ethanol at 4 °C overnight. The pellet was centrifuged at 4000 rpm for 15 min and dried in desiccators containing anhydrous cobalt chloride at room temperature under reduced pressure (Kurane *et al.* 1994; Lin, Harichund 2011a).

1.4. Experimental set-up

Erlenmeyer flasks contained 150 mL of wastewater with different concentrations of heavy metals (Cu^{2+}, Pb^{2+} and Cd^{2+} ions) and 15.0 mg of bio-flocculants (0.1 mg/ml). The flasks were kept, under constant agitation (120 rpm), at room temperature for 24 hrs. Bio-flocculants were separated from the medium after 24 hrs and residual metal concentrations in the inlet and outlet were determined by GBC Atomic Adsorption Spectroscopy (AAS) (Savanta AA). The percentage of removed heavy metals (Cu^{2+}, Pb^{2+} and Cd^{2+}) was calculated as follows,

$$Removal\,\% = \left\langle {C_0 - C_f}\big/{C_0}\; x\,100 \right\rangle,$$

where C_0 and C_f are the initial and final concentrations of metal ion in wastewater in μg/L.

1.5. Statistical analysis

All experiments were performed in triplicate and the results were expressed as the means±SD. T-tests were used to examine the statistically significant differences ($p < 0.001$) for heavy-metal removal after bio-flocculants treatment using SPSS version 17. The results of each experiment were assumed to be independent with different variance.

2. Results and discussion

2.1. Lead (Pb^{2+}) removal

The results presented in Table 1 and Figure 1 show the bio-flocculants of *Bacillus sp.* and *Pseudomonas sp.* is very effective for removal of lead (Pb^{2+}) from industrial wastewater. However, the removal efficiency of lead (Pb^{2+}) using *Pseudomonas sp.* was significantly affected at increasing the initial concentration from 85±1.5 to 304.3±1.5 µg/l. The residual values of Pb^{2+} in the treated water were increased from 0.32±0.82 to 9.2±0.29 µg/l at increasing the initial concentration from 85±1.5 to 304.3±1.5 µg/l respectively. This was not the case for bio-flocculants of *Bacillus sp.* where the removal efficiency of Pb^{2+} was not largely affected at increasing the initial concentration from 85±1.5 to 304.3±1.5 µg/l. The removal efficiency slightly changed from 99.4% to 99.7% at increasing the initial concentration from 85±1.5 to 304.3±1.5 µg/l respectively. This indicates that bio-flocculants of *Bacillus sp.* has a higher capability of adsorption of Pb^{2+} as compared to *Pseudomonas sp.* Similar findings are recorded by Lin, Harichund (2011a) who found that bio-flocculant produced by *Bacillus sp.* CH15 was capable of removing Pb^{2+} (87%) and Cr^{2+} (86%). The main mechanism removal of Pb^{2+} by bio-flocculants is mainly due to charge neutralization and adsorption bridging. Guo, Yu (2014) found that the removal efficiency of Pb^{2+} reached 94.7 % using MBFR10543. Fourier transform infrared spectra analysis indicated that functional groups, such as –OH, C = O, and C – N, were existed in MBFR10543 molecular chains, which had strong capacity for removing Pb^{2+}. The maximum bio-floccution activity (95%) was recorded by *Achromobacter sp.* TL-3 bacteria for Pb^{2+} removal (Batta *et al.* 2013). Moreover, Feng *et al.* (2013) showed that the results of Pb^{2+} adsorption by microbial flocculant GA1 could be described by the Langmuir adsorption model, and being the monolayer capacity negatively affected with an increase in temperature and the adsorption process could be described by pseudo-second-order kinetic model.

Table 1. The efficiency of bio-flocculants of *Bacillus sp.* versus *Pseudomonas sp.* for removal of Lead (Pb^{2+})

Bioflocculants	*Bacillus sp.*		*Pseudomonas sp.*	
Metal initial concentration (µg/l)	Residual values (µg/l)	%R	Residual values (µg/l)	%R
85±1.5	0.47±0.06	99.4	0.32±0.82	99.6
142.7±2	0.47±0.15	99.7	2.5±0.35	98.2
286±1	1.5±1.5	99.5	5.2±0.87	98.2
287±1	1.4±0.15	99.5	6.4±0.4	97.8
304.3±1.5	1.8±15	99.4	9.2±0.29	97.0

Note: %R*: percentage removal

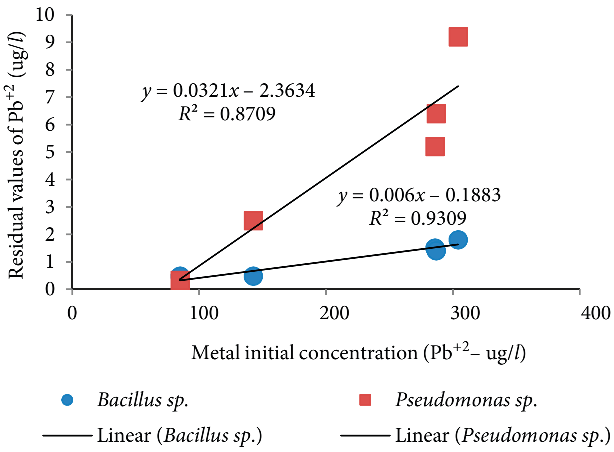

Fig. 1. Relationship between the initial concentration and residual values of Lead (Pb^{2+})

2.2. Cupper (Cu^{2+}) removal

Similar trends were observed for removal of Cupper (Cu^{2+}) ions from industrial wastewater using bio-flocculants of *Bacillus sp.* and *Pseudomonas sp.* as shown in Table 2 and Figure 2. The reduction in Cu^{2+} concentration was substantially higher using *Bacillus sp.* bio-flocculant as compared to *Pseudomonas sp.* Nevertheless, the removal efficiency of Cu^{2+} was highly deteriorated at increasing the initial concentration from 47±1 to 482.6±2.5 µg/l. Bio-flocculant of *Bacillus sp.* had the highest copper removal percentage (87.2%) than *Pseudomonas sp.* (68.1%) at initial concentration of47±1µg/l. The removal efficiency of Cu^{2+} was significantly increased from 68.1% to 75.8% at increasing the initial concentration from 47±1 to 482.6±2.5 µg/l using *Pseudomonas sp.* This indicates that the efficiency of *Pseudomonas sp.* is initial concentration dependant. On the contrary, the removal efficiency of Cu^{2+} was slightly affected at increasing the initial concentration from 47±1 to 482.6±2.5 µg/l where the removal efficiency dropped from 87.2% to 84.1% respectively. The bio-flocculant adsorption/participates is mainly depends on the

Table 2. The efficiency of bio-flocculants of *Bacillus sp.* versus *Pseudomonas sp.* for removal of Cupper (Cu^{2+})

Bioflocculants	*Bacillus sp.*		*Pseudomonas sp.*	
Metal initial concentration (µg/l)	Residual values (µg/l)	%R*	Residual values (µg/l)	%R*
47±1	6±1	87.2	15.4±0.7	68.1
58.9±1.5	6.7±1.5	88.6	17.2±0.6	71.2
113.7±1.5	17.3±1.5	84.8	43.3±0.9	62.6
191±1.5	36±1	81.2	71.6±1.4	62.5
482.6±2.5	76.7±1.5	84.1	117.1±1.5	75.8

Note: %R*: percentage removal

available hydroxyl, carbonyl and carboxyl groups which induces very high binding capacity. The negative charge groups could react with the positively charged site of heavy metals present in the wastewater, in this case, the metals can approach sufficiently close to each other so that attractive forces become effective. Chemical groups in the bio-flocculants act like a bridging agent of metals complexes and reduce inter-metals distances through the ionic bonds mechanism, metals adsorbed onto one bio-flocculant molecular chain, and they could be adsorbed simultaneously by other chains, leading to the formation of three-dimensional flocs, which were capable of rapid biosorption process. Shuhonga *et al.* (2014) found that exopolysaccharide (EPS) from *Arthrobacter* ps-5 have strong biosorption capability, up to 169.15 mg/g of Cu^{2+}, 216.09 mg/g of Pb^{2+} and 84.47 mg/g of Cr^{6+}, respectively.

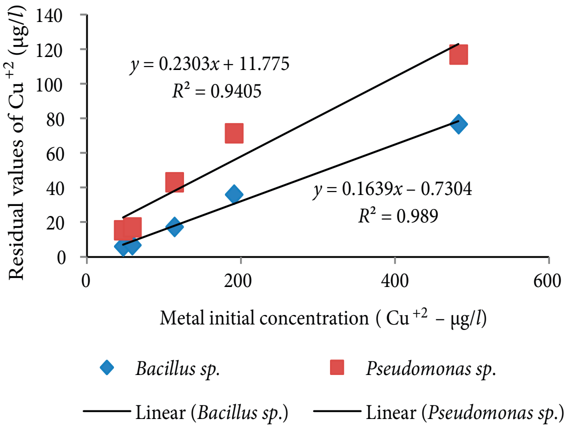

Fig. 2. Relationship between the initial concentration and residual values of Cupper (Cu^{2+})

Table 3. The efficiency of bio-flocculants of *Bacillus sp.* versus *Pseudomonas sp.* for removal of Cadmium (Cd^{2+})

Bio-flocculants	*Bacillus sp.*		*Pseudomonas sp.*	
Metal initial concentration (µg/l)	Residual values (µg/l)	%R*	Residual values (µg/l)	%R*
34.4±0.66	6.4±0.53	81.4	1.8±0.15	94.8
38.3±0.64	10.3±0.7	73.1	1.4±0.08	96.3
67.2±0.91	21.2±1	68.5	4.1±0.95	93.9
272±2.1	78.5±1.8	71.1	19.2±0.92	92.9
321±6	81.9±0.65	74.5	21.2±1.2	93.4

Note: %R*: percentage removal

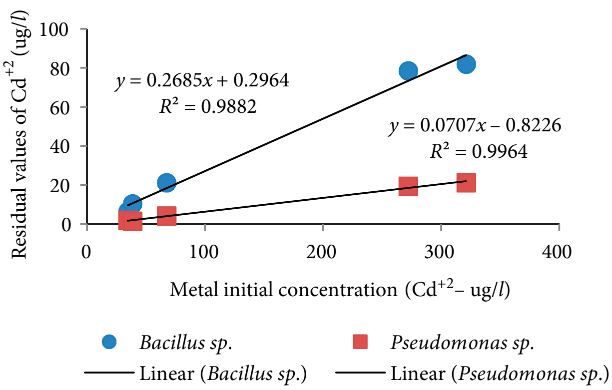

Fig. 3. Relationship between the initial concentration and residual values of Cadmium (Cd^{2+}).

2.3. Cadmium (Cd^{2+}) removal

The results for Cadmium (Cd^{2+}) revealed that *Pseudomonas sp.* achieved higher removal efficiency as compared to *Bacillus sp.*, bio-flocculants at the same initial concentrations (Table 3 and Fig. 3). Moreover, the removal efficiency of Cd^{2+} using *Pseudomonas sp.* bio-flocculants was slightly affected (96.3–93.4%) at largely fluctuated the initial concentration from 38.3±0.64 to 321±6 µg/*l*. likely, the removal efficiency of Cd^{2+} using *Bacillus sp.* bio-flocculants remained unaffected at a level of 74% at increasing the initial concentration from 38.3±0.64 to 321±6 µg/*l*. This indicates that both *Bacillus sp.*, and *Pseudomonas sp.* bio-flocculants are very efficient for removal of Cd^{2+} from industrial wastewater. Nevertheless, the residual values of Cd^{2+} in the treated effluent were quite low using *Pseudomonas sp.* as compared to *Bacillus sp.* (Table 3). This metal biosorption potential of *Bacillus sp.*, and *Pseudomonas sp.* could probably be attributed to the role of bio-flocculant produced by it. Ion uptake by the range of bacterial component, such as cell wall component and extracellular polysaccharide, plays important roles in controlling heavy metal pollution in the treatment processes. Bio-flocculant producing bacteria such as *B. subtilis* WD 90, *B. subtilis* SM 29, and *E. agglomerans* SM 38 were reported to absorb the heavy metal by the cell component and the biopolymer (Saithong, Poonsuk 2002). Shamim, Rehman (2012) reported that *Klebsiella pneumonia* CBL-1 that isolated from heavy metal laden industrial wastewater was capable to remove 54% and 82% cadmium from the industrial effluents after 4 and 8 days of incubation at room temperature.

2.4. Comparison between the efficiency of *Bacillus sp.* and *Pseudomonas sp.* bio-flocculants for removal of Pb^{2+}, Cu^{2+} and Cd^{2+} at the same initial concentration

The results in Figure 4 show comparison between the efficiency of *Bacillus sp.* and *Pseudomonas sp.* bio-flocculants for removal of Pb^{2+}, Cu^{2+} and Cd^{2+} at the same initial concentration. Available data revealed that *Bacillus sp.* was very effective for removal of Cu^{2+} and Pb^{2+} from industrial wastewater resulting residual values of 28.5 and 1.13 µg/*l* in the treated effluent. Lower removal efficiencies of 70.4% for Cu^{2+} and 97.8% for Pb^{2+} were occurred using *Pseudomonas sp.* flocculants. Nevertheless *Pseudomonas sp.*

bio-flocculants achieved a substantially higher removal efficiency of Cd^{2+} (93.5%) as compared to 72.9% using *Bacillus sp.* bio-flocculants. Bio-flocculants cause aggregation of particles and cells by bridging or charge neutralization, colloid entrapment and double layer compression (Salehizadeh, Shojaosadati 2001). Shuhonga *et al.* (2014) found that exopolysaccharide (EPS) from *Arthrobacter* ps-5 have strong biosorption capability, where infrared spectrometry analysis demonstrated that the groups of O—H, C═O, C—O—C and C═O—C of the EPS involved in metal biosorption process and were the main functional groups for binding metal ions. Initial pH of the culture medium is known to play a key role in determining the electric charge of the cells together with the oxidation potential that affects the nutrient absorption and enzymatic action (Xia *et al.* 2008).

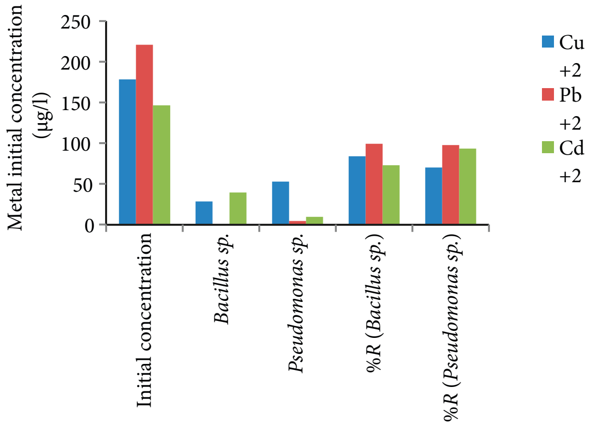

Fig. 4. Comparison between the efficiency of *Bacillus sp.* and *Pseudomonas sp.* bio-flocculants for removal of Pb^{2+}, Cu^{2+} and Cd^{2+} at the same initial concentration

Conclusions

- Bio-flocculants produced by bacteria isolated from industrial sludge are capable of removing heavy metals simultaneously and effectively however, the removal efficiencies were initial concentration dependant.
- Bio-flocculants produced from *Bacillus sp.* and *Pseudomonas sp.* with a concentration 0.1 mg/ml provided a maximum removal efficiency of 99.5% and 97.9% for Pb^{2+} & 83.8% and 68% for Cu^{2+} respectively.
- The removal efficiency of Cd^{2+} by the bio-flocculants originated from *Pseudomonas sp.* was significantly higher (93.5%) than those achieved (72.9%) using *Bacillus sp.*
- These results demonstrate that bio-flocculants *Bacillus sp.* and *Pseudomonas sp.* would serve as a potential candidate for bioremediation of industrial wastewater containing heavy metals.

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