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Full Length Research Paper

Isolation and characterization of heavy metal removing bacterial bioflocculants

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Thirteen metal-tolerant bacteria capable of producing metal-removing bioflocculants were isolated from an industrial effluent sample. *Pseudomonas* sp. was found to be the pre-dominant species among the isolates (8 out of 13), followed by *Herbaspirillium* spp. (4) and one *Paenibacillus* sp. The flocculating activity of bioflocculants produced by these microorganisms was assayed using the kaolin clay. The heavy-metal-removal efficiency was determined using atomic absorption spectrometer before and after mixing the bioflocculant with the heavy metal solutions. Bioflocculants exhibit different flocculating abilities of removing kaolin clay in the presence of different heavy metals. Bioflocculants produced by *Pseudomonas* sp. CH_9 possessed the highest flocculating activity (1.8) compared to the remaining bioflocculants. The flocculating activities of CH_{11} and CH_{13} increased to 0.95 and 0.87 in the presence of Pb^{2+} and to 0.89 and 0.98 in the presence of Hg^{2+} respectively from 0.015 in the presence of Ca^{2+} in the standard kaolin clay assay. Up to 90% of Pb^{2+} was removed by *Pseudomonas* sp. CH_8 bioflocculants. Seventy-eight percent of Hg^{2+} and 66% of Cd^{2+} was removed by *Pseudomonas* sp. CH6 and *Herbaspirillium* sp. CH_{13} bioflocculants respectively. Most of the bioflocculants demonstrated a higher percentage of heavy-metal removal at low concentrations. This study demonstrates that microbial bioflocculants have potential to be used as an alternative bioremedial tool for industrial effluents and wastewater treatments which are co-contaminated with heavy metals.

Key words: Bioflocculant; heavy metals, *Pseudomonas* sp., *Herbaspirillium* sp., *Paenibacillus* sp., Industrial effluent.

INTRODUCTION

Water is a scarce resource in South Africa with 98% of the national water has been allocated (NWRS, 2004). Security of water supply has become a key strategic issue for continued and sustained economic growth (Turton and Ashton, 2008). The quality of South Africa's water resources is also declining as a result of industrial and urban conurbations (Coetzee et al., 2006) thereby becoming unfit for human and industrial consumption (Neytzell-De Wilde, 1992; Oberholster and Ashton, 2008). All pollutants and effluents now need to be treated before being discharged into waters.

Heavy metal pollution of freshwater arising from mines and other industries is a cause for concern in South Africa (Coetzee et al., 2006; Cobbing, 2008). The metals tend to sediment to the bottom of the water where they concentrate (Davies et al., 1991) and are capable of accumulating in the tissues of aquatic biota. Even at low concentrations, the composition, distribution and diversity benthic organisms are affected (Geydu-Ababio et al., 1999; Jarup, 2003; Hemme et al., 2010). Removal of toxic heavy metals from industrial waste waters is essential from the standpoint of environmental pollution control (Guangyu and Thiruvenkatachari, 2003).

There are several reports on potential of various species of bacteria, fungi, algae and plants (Keskinkan et al., 2004; Kiran et al., 2007a, b; Mamba et al., 2009; Veglio and Beolchini, 1997) to absorb metals. A number of heavy-metal removing bacterial bioflocculants have also been studied (He et al., 2003; Jang et al., 2001;

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Kaewchai and Prasertsan, 2002; Noghabi et al., 2007; Salehizadeh and Shojaosadati, 2003; Wu and Ye, 2007) due to them being environmentally friendly, biodegradable and non-toxic. The fact that these bioflocculants have higher efficiencies at low metal concentrations makes them very attractive for the removal of heavy metals from industrial effluents/wastewaters (Kortrba et al., 1999).

In order to mitigate the metal pollution in South Africa water systems, 13 metal-tolerant bacterial species including *Pseudomonas* sp., *Herbaspirillium seropedica* and *Paenibacillus* sp. that are capable of producing bioflocculants have been isolated from one industrial effluent. The flocculating activities of microbial bioflocculants produced by these bacteria in the removal of kaolin clay and various heavy metals are reported in this study.

MATERIALS AND METHODS

Isolation and identification of bioflocculant producing bacteria

Bioflocculant producing microorganisms were isolated from a local industrial filtered sludge effluent sample. This effluent sample was plated out onto nutrient agar (Merck) plates containing various concentrations (1 to 1000 ppm) of different heavy metals (Zn^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}) at 37℃ overnight. The microorganisms that grew on nutrient agar containing the highest metal concentrations were randomly isolated based on their morphology. Pure cultures were obtained using four way streaks on nutrient agar plates.

Production of bioflocculants

The above isolates were cultivated in a 250 ml Erlenmeyer flask containing 30 ml YMPG media (0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone, 1% glucose and 2% agar at pH 7) at 28℃, 220 rpm for 20 h (Nakata and Kurane, 1999). A 0.7 ml portion 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% $MgSO_4 . 7H_2O$, 0.2% NaCl, and 0.2% $CaCO_3$) at the above conditions for 72 h (Nakata and Kurane, 1999). Bioflocculants were recovered from the supernatant after centrifugation (4000 x g) for 15 min and precipitated by adding 2 volumes of ethanol at 4℃ overnight. The pellet was centrifuged at 4000 x g for 15 min and dried in a desiccator containing anhydrous cobalt chloride at room temperature under reduced pressure. Productivity of the bioflocculants was expressed in terms of the dry weight after ethanol precipitation of the culture supernatant (Kurane et al., 1994).

Flocculating and heavy-metal removing activities of bacterial bioflocculants

The potential of bacterial bioflocculants for removing heavy metals and organic/inorganic contents (flocculating activity) was assessed. The determination of the above activities was followed by the modified method of Nakata and Kurane (1999) and Gao et al. (2009). One millilitre of bacterial bioflocculant (1000 ppm) was mixed with 9 ml of kaolin solution. The mixture was vortexed for 30 s and rested for 5 min. The upper layer of the solution (3 ml) was then removed for further analyses. The flocculating activity was calculated based on OD_{550nm} as described by Kurane et al. (1994). The heavy metal concentrations were also measured using ICP-OES. One millilitre of water (Millipore Elix purification system, 17 megaΩ) was substituted for the bioflocculant in the control in all experiments.

Flocculating activity and heavy metal removal efficiency of bacterial bioflocculants

The efficiency by which bacterial bioflocculants removed heavy metal was determined using the modified method described by Nakata and Kurane (1999) using heavy metals without kaolin clay. Different bioflocculant concentrations ranging from 100 to 5000 ppm were suspended in the various concentrations of heavy metal solutions for 5 min. The heavy metal concentrations were also measured using Atomic Absorbance Spectrometer (Perkin Elmer AAnalyst, 200).

Statistical analysis

All experiments were performed in triplicate and the results were expressed as the means ± SD. The correlation between the flocculating activities and heavy-metal removal efficiency of bioflocculants in the presence of various metals was determined using Pearson correlation coefficient (SPSS version 15).

Identification of heavy metal-removing bioflocculant producing bacteria

The bioflocculant producing isolates that were capable of removing heavy metals were identified in accordance with Bergy's manual of systematic bacteriology (Sneath et al., 1996) confirmed by 16S rDNA sequencing (Marchesl et al., 1998) and compared with known sequences available in GENBANK (NCBI). For the long–term preservation, the bacterial isolate was stored in 40% glycerol at -70°C.

RESULTS

Thirteen bioflocculant producing bacterial isolates, capable of removing heavy metals, were isolated from an industrial effluent sample. These isolates were identified as *Pseudomonas* sp. (8 isolates including one *Pseudomonas putida* and one *Pseudomonas aeruginosa*), *Herbaspirillium seropedica* (4 isolates) and 1 *Paenibacillus* sp. (Table 1) designated as CH1-13 based on the biochemical tests and 16S rRNA sequencing. *Herbaspirillium* sp. CH10 produced the highest amount of crude bioflocculant (4.14 gl^{-1}) and *Paenibacillus* sp. CH11 produced the least quantity (0.29 gl^{-1}). All isolates were able to replicate in the nutrient agar containing 1000 ppm of Zn^{2+}, Cd^{2+}, Pb^{2+}, but not with Hg^{2+}. Six isolates (five *Pseudomonas* sp. and one *H. seropedica*) could tolerate the presence of mercury at 100 ppm in the media (Table 1).

Figure 1 shows the flocculation activities of bacterial bioflocculants using the kaolin clay assay. Bioflocculants produced by *Pseudomonas* sp. CH_9 possessed the highest flocculating activity (1.8) compared to the remaining bioflocculants when the standard kaolin clay assay was used. Bioflocculants of *Paenibacillus* sp. CH11 and *Herbaspirillium* sp. CH13 had very little

Table 1. Mass of crude bioflocculant produced from bioflocculant producing bacteria.

Isolates	Bioflocculant producing bacteria	Mass of bioflocculant (g per litre)	Growth at 100 ppm Hg^{+2}
CH_1	*Pseudomonas* sp.	1.47	No
CH_2	*Pseudomonas* sp.	0.80	Yes
CH_3	*Pseudomonas* sp.	0.80	No
CH_4	*Pseudomonas* sp.	1.60	Yes
CH_5	*Pseudomonas* sp.	0.53	No
CH_6	*Pseudomonas* sp.	1.47	Yes
CH_7	*Herbaspirillium* sp.	0.27	No
CH_8	*Pseudomonas* sp.	2.93	Yes
CH_9	*Pseudomonas* sp.	2.94	Yes
CH_{10}	*Herbaspirillium* sp.	4.14	No
CH_{11}	*Paenibacillus* sp.	0.27	No
CH_{12}	*Herbaspirillium* sp.	0.67	No
CH_{13}	*Herbaspirillium* sp.	0.40	Yes

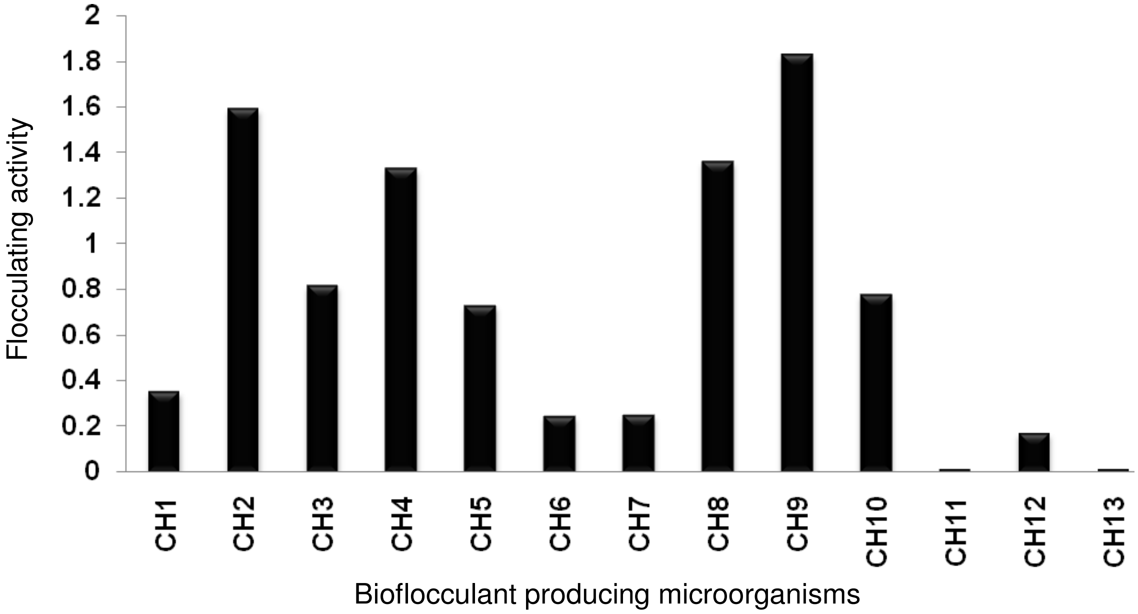

Figure 1. Flocculation activities of bacterial bioflocculants (10000 ppm) using kaolin clay assay. All results were present as the means (n = 3).

flocculating activities on kaolin clay in the presence of Ca^{2+}.

Microbial bioflocculants in this study exhibited different flocculating abilities to remove kaolin clay in the presence of different heavy metals. The flocculating activities of CH_{11} and CH_{13}, which showed low activities as shown in Figure 1, increased to 0.95 and 0.87 in the presence of Pb^{2+} (1000 ppm) (Figure 2A) and to 0.89 and 0.98 in the presence of Hg^{2+} (100 ppm) (Figure 2C), respectively. On the other hand, bioflocculants produced by CH_2, CH_4, CH_8 and CH_9 preferred forming flocculating complexes with kaolin clay and Ca^{2+} (Figure 1) compared to other metals (Figure 2). In the presence of Cd^{2+}, microbial bioflocculants displayed least flocculating activities (< 0.2) compared to that in the presence of other metals (data not shown).

The flocculating activity of microbial bioflocculants increases with a decrease in bioflocculant concentration in the presence of Pb^{2+}, Zn^{2+} and Hg^{2+} as shown in Figure 2. There are strong correlations ($r = 0.70\text{-}0.76$; $p < 0.001$) between the flocculating activities of bioflocculants in the presence of Pb^{2+}, Zn^{2+} or Hg^{2+}. All microbial bioflocculants in this study demonstrate metal sorption potential with varying levels of efficiencies (Figures 3). Up

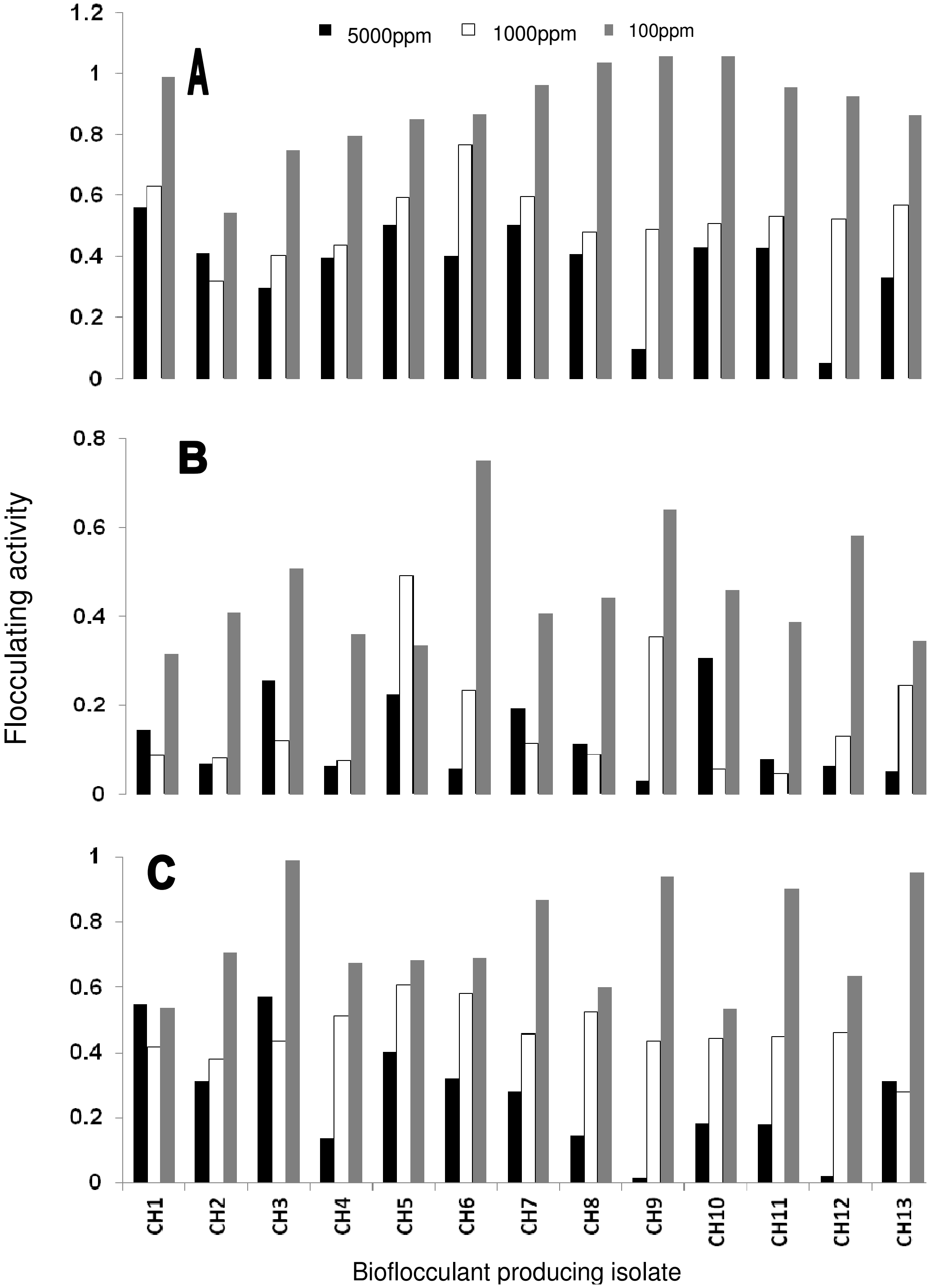

Figure 2. The effect of bioflocculants concentrations on the flocculation activity in the presence of different metal (a) Pb^{2+}; (b) Zn^{2+} and (c) Hg^{2+}.

to 90% of Pb^{2+} was removed by bioflocculant produced by *Pseudomonas* sp. CH_8 (Figure 3). Bioflocculant produced by *Pseudomonas* sp. CH_6 and *Herbaspirillium* sp. CH_{13} flocculated 78% of Hg^{+2} and 66% of Cd^{+2} from the metal solutions respectively (Figure 3). All bacterial bioflocculants in this study possessed less efficiency to bind Zn^{2+}. Less than 50% of Zn^{2+} was removed through the flocculation process.

The efficiency of heavy-metal removal by bioflocculants is metal-concentration as well as bioflocculant-concentration dependent (Figures 4 and 5). Most of the bioflocculants demonstrated a higher removal percentage

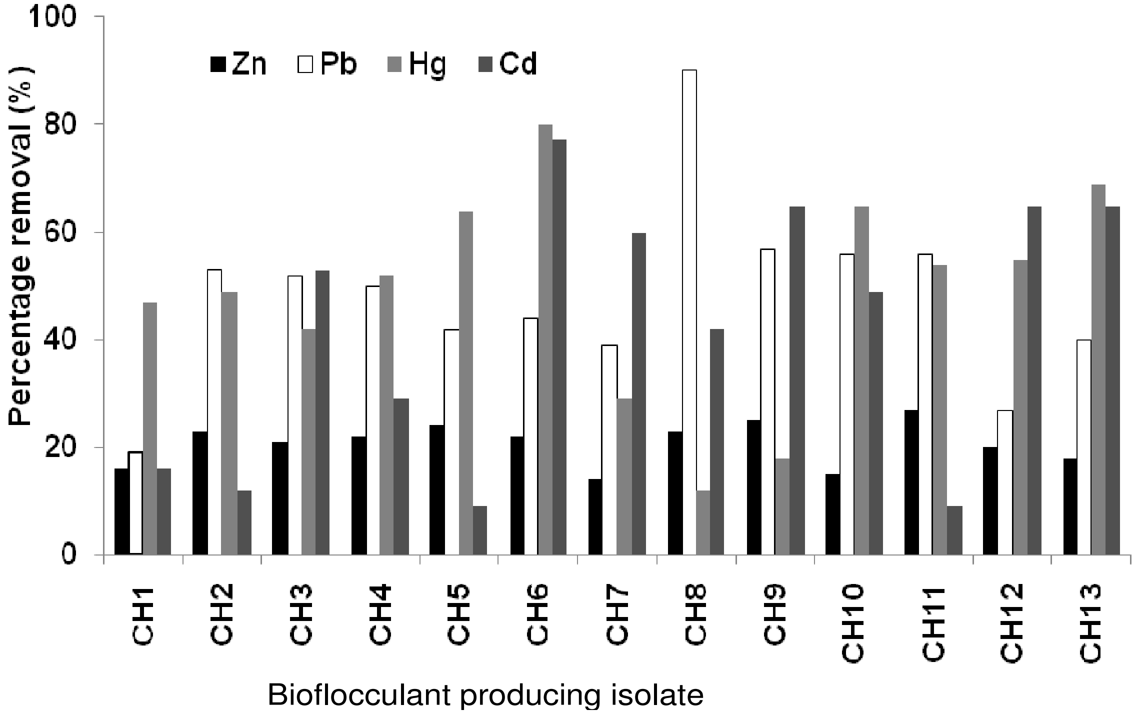

Figure 3. Percentage of heavy metal (1 ppm) removal 5 min after mixed with 10000 ppm bacterial biofloculant.

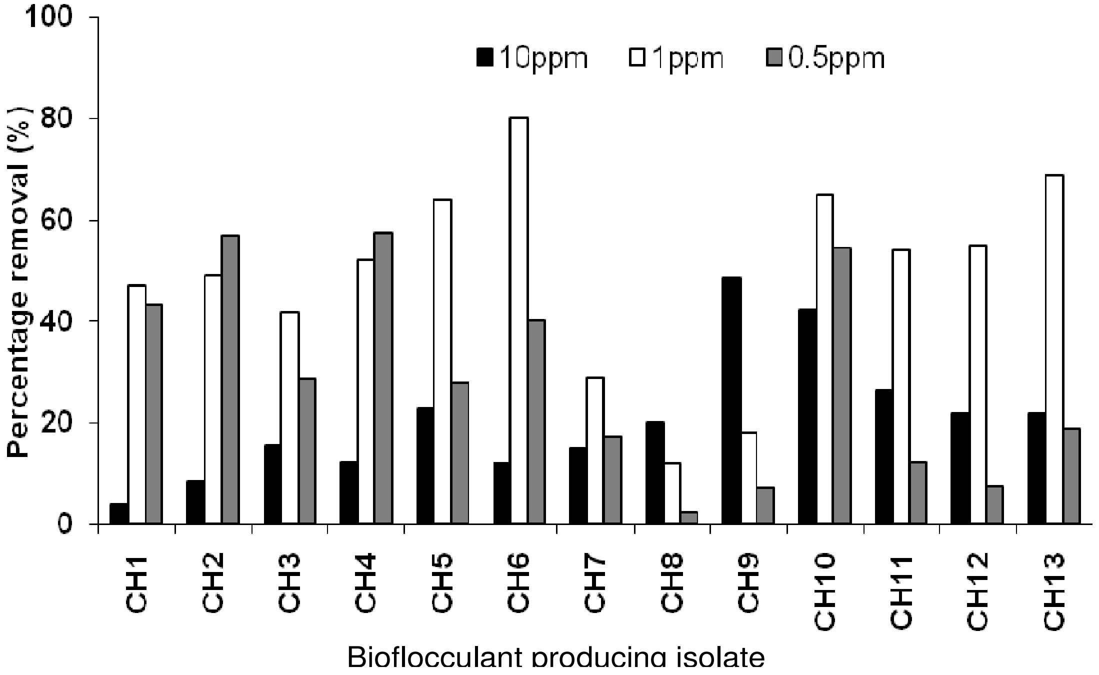

Figure 4. The effect of metal concentrations on the percentage of Hg^{2+} removal using 10000 ppm bacterial bioflocculant.

at 1 ppm of Hg^{2+} compared to the values at 10 and 0.5 ppm (Figure 4). Bioflocculants produced by CH_6 removed 80% of Hg^{2+} at 1 ppm compared to 9 and 38% at 10 and 0.5 ppm, respectively. Bioflocculants produced by CH_9, however, demonstrated a better ability to remove Hg^{2+} at higher concentrations. The presence of low bioflocculant concentrations in the assay solution produced better results in removing heavy metals especially Pb^{2+} and Zn^{2+}

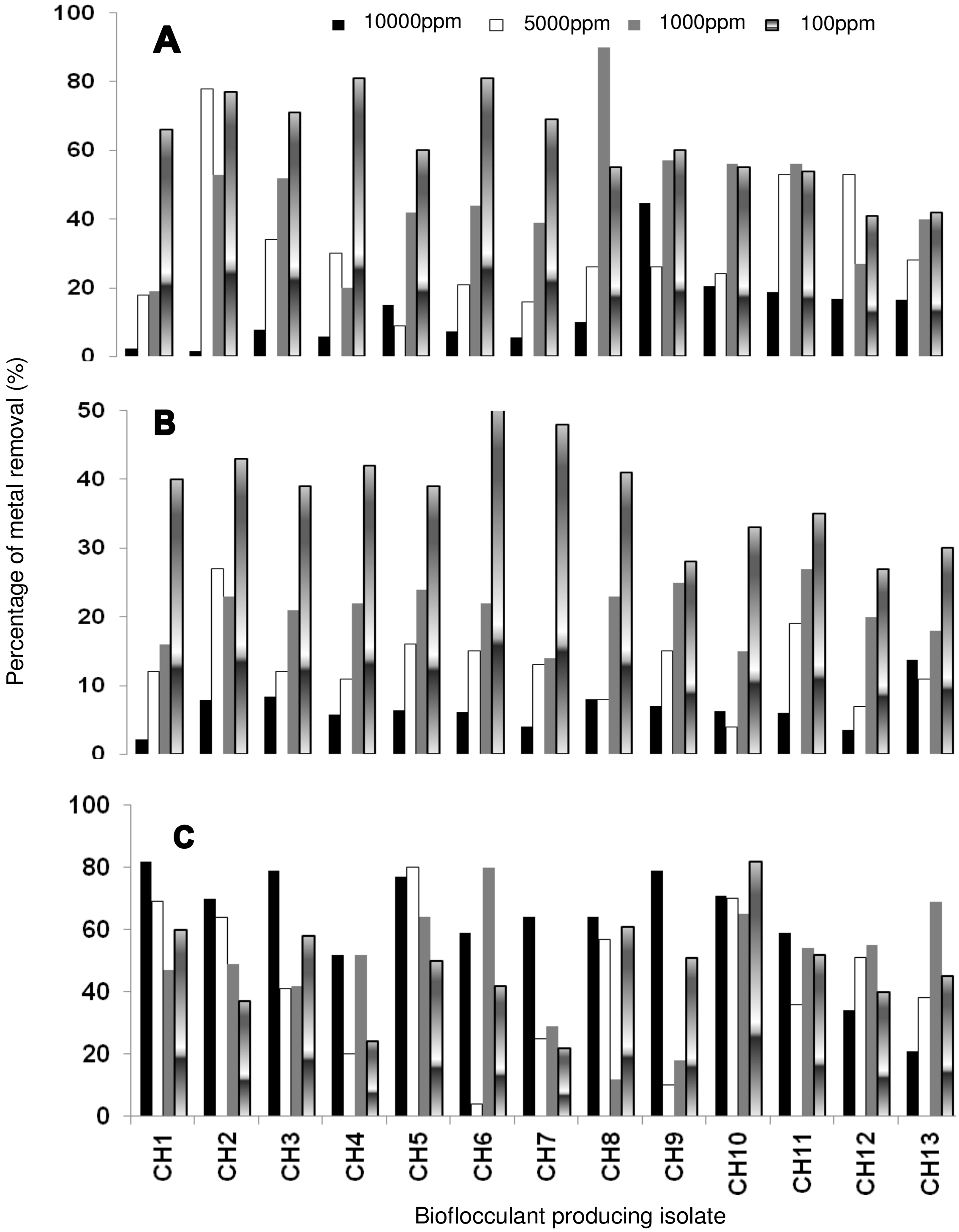

Figure 5. The effect of bacterial bioflocculant concentrations on the percentage of metal removal. (A) Pb^{2+} (1 ppm), (B) Zn^{2+} (1 ppm) and (C) Hg^{2+} (1 ppm) removal.

as shown in Figures 5A and B. However, a reverse trend was also observed for some bioflocculants (CH_1, CH_3 and CH_5) in bio-absorption of Hg^{+2} from the solutions.

There are significant differences ($p < 0.05$) between the percentages in removing different heavy metals by bacterial bioflocculants. There is a strong correlation ($r = 0.81$; $p < 0.001$) of the percentages of Zn^{2+} and Pb^{2+} removal by the bioflocculants. There is also a strong correlation ($r = 0.68$; $p < 0.001$) between the flocculating activities of bioflocculants (Figure 2) and the percentages of Zn^{2+} removal by bioflocculants (Figure 5). A moderate correlation ($r = 0.37$; $p < 0.001$) between the flocculating activities and the percentages of heavy-metal removal by bioflocculants in the case of Pb^{2+}.

DISCUSSION

Thirteen bacterial isolates were isolated from an industrial effluent sample containing heavy metals. These bacterial isolates belong to *Pseudomonas* sp. (8 isolates), *Herbaspirillium* sp. (4 isolates) and one *Paenibacillus* sp. *Pseudomonas* sp. was found to be a predominant species in this study and was commonly isolated as bioflocculant producing bacteria (Kachlany et al., 2001; Noghabi et al., 2007; Wang et al., 1997). Both *Paenibacillus* and *Herbaspirilla* species have been commonly localized on and within roots and aerial parts of plants with the role of nitrogen fixation in growth promotion (Gyaneshwar et al., 2002; Yoon et al., 2003). *Paenibacillus* bioflocculants have been reported in efficiently harvesting algal cells (Yoon et al. 2003). Metal-removing exopolysaccharide produced by *Paenibacillus* sp. was also documented in the literature (Morillo et al., 2006). As our best knowledge, it is the first report that *Herbaspirillium* sp. produces bioflocculants that possess heavy-metal biding capacity. With increasing problems in heavy metal pollution in natural environments, micro-organisms have to adapted and thus become metal tolerant to toxic concentrations of heavy metals (Avezzu et al., 1995; Hemme et al., 2010) through biosorption process (Davis et al., 2003) or biotransformation that is dissimilatory reduction of metal ions (Lee et al., 2000; Hemme et al., 2010). These additional abilities allow scientists to use microorganisms or their bioproducts for industrial effluent treatments contaminated with heavy metals (Salehizadeh and Shojaosadati, 2003).

The bioflocculating activity was best demonstrated with the use of kaolin clay as the suspended particles in the presence of Ca^{2+} (Nakata and Kurane, 1999). In the present study, bacterial bioflocculants exhibit different levels of flocculating ability by varying heavy metals. There are significantly different ($p < 0.001$) on the flocculating activities of bioflocculants in the presence of different metals. Salehizadeh et al. (2000) and Xia et al. (2008) also demonstrate the ability of various metals to stimulate the flocculating activities of bacterial bioflocculants. Yokoi et al. (1997) and Lu et al. (2005) demonstrated that Fe^{2+} and Zn^{2+} respectively instead of Ca^{2+} activated the flocculating activities of bioflocculants produced by *Enterobacter* sp. Wu and Ye (2007) suggest that the presence of cation(s) affects flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups by forming bridges between particles. However, the presence of metal is not absolutely essential for bacterial biofloculating activities. Bioflocculants produced by *Citrobacter* sp. TKF04 (Fujita et al., 2000) and *Bacillus* sp. F19 w (Zheng et al., 2008) were capable of flocculating kaolin clay without metals.

In addition, the impact of heavy metals on the bioflocculating activity is also not clear. Similar to our findings, Salehizadeh et al. (2000) showed that an increase in Al^{3+} and Fe^{2+} concentration resulted in a decrease in the flocculating activities of bioflocculant produced by *Bacillus* sp. As-101. The same bioflocculant possessed a better activity by increasing Ca^{2+} and Mg^{2+} concentrations.

The mechanisms of flocculation are not entirely clear in biological systems (Esser and Kues, 1983). Wu and Ye (2007) propose that the composition of bacterial bioflocculants plays a major role in their flocculating activities. Liao et al. (2001) suggest that type and physical characteristics of extracellular polysaccharide affect bioflocculation. In general, bioflocculants have been found to have a net negative charge. The presence of uronic acid of polysaccharide assists the metal uptakes (Horan and Eccles, 1986; Kaplan et al., 1987; Kong et al., 1998; Salehizadeh and Shojaosadati, 2003; Lu et al., 2005; Wu and Ye, 2007; Aguilera et al., 2008). Aguilera et al. (2008) propose that the presence of uronic acid in bioflocculants allows for adhesion of microorganisms to surfaces and is involved in the uptake of metallic ions. Carboxylic and sulfate groups present in acidic exopolysaccharides works as a non-specific ion exchange material which may convey chelating property (Valls and De Lorenzo, 2002). The protein component containing multiple carboxyl groups such as glutamic and aspartic acid (Dignac et al., 1998) or the presence of galacturonic acid and glucuronic acid (Bender et al., 1994) are also important in the process of flocculation with kaolin clay and heavy metals. The efficiency to reduce the surface charge density by adsorption of the bioflocculant and the particles such as the heavy metals (Levy et al., 1992; Hantula and Bamford, 1991) becomes crucial. Differences in affinity of metals for bioflocculants are due to charge density, attractive interaction, and types of conformation of polymer with adsorbed ions (Morillo et al., 2006). Increasing bioflocculant as well as heavy metals concentrations might result in lower flocculating activities due to the repulsion of charge. The current knowledge will not be able to predict the effectiveness of bioflocculants in their flocculating and metal-removing activities in a real case scenario.

Biosorptions of heavy metals by a novel acidic polysaccharide produced from microorganisms have been reported (Kong et al., 1998; Salehizadeh and Shojaosadati, 2003). Noghabi et al. (2007) reported that bioflocculant produced by *Pseudomonas fluorescence* removed 70% mercury, 30% zinc and 45% cadmium. Bioflocculant from the three thermotolerant isolates, *E. agglomerrans* SM 38, *Bacillus subtilis* WD 90 and *B. subtilis* SM 29, adsorbed nickel and cadmium up to 90 and 85% respectively (Kaewchai and Prasertsan, 2002). Bioflocculants produced in this study also demonstrated their capacity to remove different heavy metals with varying levels of efficiency. Most of the bioflocculants in this study possessed better efficiency in removing Pb^{2+} (90%) and least in Cd^{2+} (< 20%). Our results also show that bioflocculants exhibited a better efficiency of for removal of heavy metals at lower bioflocculant concentrations (1 ppm) as described by others (Das and Santra, 2007). There is a strong correlation between the flocculating activities of bioflocculants and their ability to remove heavy metals indicating that these microbial bioflocculants can remove heavy metals and solid suspension simultaneously. South Africa, similar to the rest of the world, is facing an increasing problem in the heavy metal pollution in our natural environments (Coetzee et al., 2006; Cobbing 2008). Higher efficiencies in removing heavy metals at the low bioflocculant concentrations make them very attractive in the treatment of industrial effluents/wastewaters.

Conclusion

This study has shown that bacterial bioflocculants are capable of removing suspension particle such as kaolin clay as well as heavy metals simultaneously and effecttively. Bacterial bioflocculants in this study also demonstrate their optimal capacity using lower concentrations. Therefore microbial bioflocculants have the potential as an alternative use for the removal and recovery of toxic metals economically and effectively from industrial effluents /wastewaters.

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