An alternative approach of toxic heavy metal removal by *Arthrobacter phenanthrenivorans*: assessment of surfactant production and oxidative stress

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The present study demonstrates the removal of three toxic heavy metals (lead, cadmium and nickel) by the bacterial strain *Arthrobacter phenanthrenivorans* from polluted water. The removal efficiency of heavy metals was recorded to be highest in case of lead (79.91%), followed by nickel (47.62%) and cadmium (34.05%). Furthermore, surfactant production by *A. phenanthrenivorans* was optimized, which is useful for different purposes like metal and oil removal from water. Partial characterization of the semi-purified surfactant was done for thermal stability, pH tolerance and metal ions sensitivity. Antioxidant enzyme activity (catalase and superoxide dismutase) and stress marker–malondialdehyde level were also determined in the presence of these three heavy metals.

Keywords: *Arthrobacter phenanthrenivorans*, oxidative stress, surfactant production, toxic heavy metals.

HEAVY metal contamination, especially lead, nickel and cadmium in the environment is posing a worldwide threat. With progressive industrialization, the amount of polluting toxic metal is increasing at an alarming rate. Heavy metal contamination in the environment reduces bacterial growth, biomass as well as diversity. According to Murthy *et al.*[1], toxic metals can disrupt normal physiological functions in three ways: blocking the active site of an enzyme, displacing the essential metal ions and modifying the conformation of a protein. Amongst the heavy metals, lead, cadmium and nickel are the most potent ones. Goldstein[2] reported that higher concentrations of lead affect the liver, kidney and nervous system. Cadmium is a toxic metal that disrupts various body functions, like renal dysfunction and bone weakness[3,4]. Nishijo *et al.*[5] found that people living in cadmium-contaminated areas of Japan are more susceptible to heart failure, cerebral attack and nephrosis.

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Biosurfactants are surface-active amphiphatic molecules which are widely used for different purposes like wastewater treatment and food fermentation processes. Surfactant produced by microbes is of interest due to its diversity, large-scale production and environment-friendly nature. However, some researchers have reported that these secondary metabolites might have function in nutrient transport, host–microbe interaction and self-protection[6]. Biosurfactant plays a crucial role in heavy metal remediation and oil removal from water bodies. Leakage of oil in marine environment destroys natural biodiversity and causes mortality among fishes and birds. Thus, monitoring of bacterial surfactant production and characterization is important from the environment point of view.

Heavy metals induce the production of reactive oxygen species (ROS) like O_2^- and OH^- in microbes[7]. Bacteria produce different types of antioxidant enzymes like catalase (CAT) and superoxide dismutase (SOD), under stress conditions to overcome the situation. Lemire *et al.*[8] reported increased production of ROS in bacteria upon exposure to Cu, Fe and Ni. Measuring bacterial stress profile is important in response to heavy metal remediation. Different parameters like CAT, SOD, malondialdehyde (MDA), glutathione S-transferase (GST), reduced glutathione (GSH), etc. are considered to be the biomarkers for stress profile. Among several parameters, MDA is most crucial; it is produced by the lipid peroxidation process. Heavy metals like lead, nickel and cadmium exert toxic effect on bacterial cell wall, destroy it and produce MDA. To minimize the effect of toxic metals (reduction of lipid peroxidation), several endogenous enzymes such as CAT and SOD are expressed immediately. Bacterial cell integrity is important for heavy metal absorption. Thus, measurement of stress profile in the presence of such heavy metals is necessary to monitor the removal efficiency.

In recent years, the removal of toxic metals from water poses a great challenge. Several types of chemical and mechanical treatments have been established, but these

are not cost-effective. Microbial accumulation efficiency might be an alternative option. Therefore, the aim of the present study was (i) screening, isolation and characterization of heavy metal-tolerant bacterial strains, (ii) quantification of absorption ability by atomic absorption spectroscopy, (iii) optimization and characterization of surfactant, and (iv) evaluation of stress response.

Materials and methods

Isolation of heavy metal-tolerant bacteria and determination of minimum inhibitory concentration

Several bacterial strains were isolated from the polluted river water behind Dasna Devi Mandir, Ghaziabad, Uttar Pradesh, India. Metal tolerance levels of the selected bacterial strains were checked on tryptone soya agar (pH 7.0) plates containing lead, cadmium and nickel, in the form of lead nitrate, cadmium chloride and nickel chloride, at different concentrations ranging from 100 to 400 ppm. The bacterial strain showing the highest tolerance level was selected and minimum inhibitory concentration was calculated.

Characterization and identification of the selected bacterial isolate

Colony morphology of the selected bacterial strain B2 was observed visually in TSA plates. The bacterial isolate was further characterized by various biochemical tests, viz. catalase, oxidase, amylase, indole, citarate and urease. Identification of the selected bacterial strain was done following the method of Banerjee *et al.*[9].

Quantification of metal removal by atomic absorption spectroscopy

Metal removal efficiency by this isolated bacterial strain was carried out using atomic absorption spectroscopy (model Spectra AA55). Removal capacity was determined following the method of Ahemad and Malik[10].

Effect of culture parameters on surfactant production

Production of surfactant was carried out according to the method of Batta *et al.*[11]. Optimization of surfactant production was done at different temperatures (10–50°C), pH levels (5.0–8.0) and incubation periods (24–72 h). Flocculating activity was checked by mixing 1 ml of cell-free supernatant with 9 ml kaolin clay solution and 3 ml of 1% $CaCl_2$. The mixture was kept undisturbed for 5 min. The optical density (OD) of the upper clear solution was measured at 550 nm. Kaolin clay solution without culture supernatant was taken as control. The activity was calculated using the following formula

$$\text{Flocculating activity (\%)} = A - B/A \times 100,$$

where A and B represent the OD of control and sample at 550 nm respectively.

Extraction and partial characterization of surfactant

The bacterial strain was cultured in the above-mentioned media for 72 h at continuous shaking mode, followed by centrifugation (8000 *g*) for 10 min to collect the supernatant. Surfactant precipitation was done following the method of Ugbenyen and Okoh[12].

Thermal stability, pH tolerance and metal ions effect on surfactant activity

Partial characterization of the surfactant was done according to the method of Ugbenyen and Okoh[12]. In order to check the thermal stability, the surfactant was heated at different temperatures, ranging from 30°C to 80°C, for 20 min and flocculating activity was checked following the above-mentioned method. Flocculating activity at different pH levels (pH 4–10) was determined by varying the pH of the surfactant using 0.1 M NaOH or HCl. The effect of different metals (Na^+, Mg^{++}, Fe^{++}, K^+ and Zn^+) on surfactant activity was assayed by substituting $CaCl_2$ solution (already described in flocculating activity assay method) with the above-mentioned metal chloride salt.

Oxidative stress profile of the selected bacterial strain

Oxidative stress profiles of the selected bacterial strain were determined in TSA broths (pH 7.0) containing 400 ppm of lead, cadmium and nickel, separately. Then 16 h cultured bacterial cells were collected by centrifugation (8000 *g* for 10 min), washed twice using 20 mM PBS (pH 7.0) and re-suspended in the same buffer. In order to collect the cytoplasomic content, cells were disrupted by sonication (30 sec pulse and 30 sec cooling, for 3 min) at 4°C, centrifuged (10,000 *g* for 20 min) and the supernatant was collected for further studies.

Determination of malondialdehyde level

MDA level was assayed following the method of Draper and Hadley[13], with some modifications. Briefly, 1 ml of the supernatant was mixed with 2 ml of TBA reagent (20% thiobarbituric acid, 2.5 N HCl and 0.5% TBA), stirred gently and heated for 20 min in a boiling water bath.

After cooling, the supernatant was collected by centrifugation at 500 *g* for 10 min and absorbance was determined at 532 nm using a spectrophotometer (Beckman, DU 730). MDA equivalent content in the sample was calculated using an extinction coefficient of 1.56×10^5/M/cm.

Determination of superoxide dismutase activity

SOD activity was assayed according to the method of Ewing and Janero[14]. Briefly, 25 µl of the supernatant was carefully mixed with 200 µl of reaction mixture containing 50 mM phosphate buffer, 0.1 mM EDTA, 98 mM NADH and 62 µM NBT (pH 7.4) in a microtitre well. To initiate the reaction, 20 µl of another mixture containing 33 µM PMS, 50 mM phosphate buffer and 0.1 mM EDTA (pH 7.4) was added carefully. Absorbance was recorded at 560 nm using the micro-plate reader (Bio-Rad, Model 680, USA).

Measurement of catalase activity

CAT activity was determined following the method of Aebi[15]. In brief, 20 µl of culture supernatant was mixed with 980 µl of H_2O_2 phosphate buffer (2 mM H_2O_2 in phosphate buffer) in 1 ml cuvette, stirred rapidly, and absorption was measured at 240 nm using a spectrophotometer (Beckman, DU 730) for 90 sec at 15 sec intervals. Decreased absorption of H_2O_2 at 240 nm indicated enzyme activity.

Results and discussion

The colony of the bacterial strain B2 was yellowish-white in colour with irregular margins. The bacterial strain was Gram-positive, rod-shaped and showed positive reaction for catalase, nitrate, amylase, citrate and indole production, and negative reaction for urease and oxidase. 16S rDNA sequence analysis identified the bacterial strain as *Arthrobacter phenanthrenivorans*, acc. no. KM402759 (Figure 1). Batta *et al.*[11] isolated *Achromobacter* sp. TL-3 from effluent-treated water, which can tolerate lead nitrate up to 1500 ppm. However, in the present study, minimum inhibitory concentration against lead, cadmium and nickel was recorded to be 1200, 900 and 800 ppm respectively. Due to great affinity for various metal ions, microorganisms actively accumulate metals[16] and store them in different places like cell wall, carbohydrate and protein polyphosphate complex[10].

Table 1 shows the ability of the selected bacterial strain, *A. phenanthrenivorans* B2, to remediate heavy metals. Removal efficiency of lead was recorded to be highest (79.91%), followed by nickel (47.62%) and cadmium (34.05%). It was observed that lead concentration in the culture medium decreased gradually (control 180.398 mg) with increase in incubation period (day 1, 108.449 mg; day 2, 99.195 mg and day 3, 36.22 mg). Similarly, in control, nickel concentration in the medium was 84.9 mg, which after three days of incubation, became 44.47 mg. Cadmium removal by the bacterial strain was slow (control 178.1 mg – day 3, 117.65 mg). Kafilzadeh *et al.*[17] have reported the elimination of lead by *Bacillus* sp. (89.66%), *Pseudomonas* sp. (87.97%) and *Corynebacterium* sp. (86.64%). Whereas *Bacillus thuringiensis* OSM29 was reported to be an efficient bacterium for removal of nickel, cadmium and copper[18].

Optimization of surfactant production by *A. phenanthrenivorans* B2 was carried out at different culture parameters, like temperature, pH and incubation period. Figure 2 *a* demonstrates that surfactant production is highest after a 48 h period of incubation (49.33%). Similarly, maximum surfactant production was recorded at pH 8.0 (56.19%), followed by 9.0, 7.0 and 6.0 (50.81%,

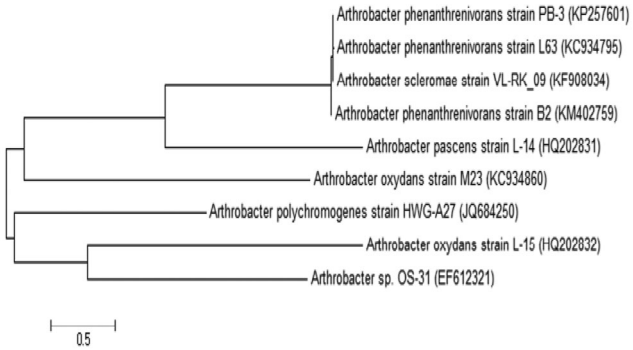

Figure 1. Phylogenetic relationship of the bacterial strain *Arthrobacter phenanthrenivorans* B2 with other close homologous strains available in the NCBI database.

Table 1. Quantification of heavy metals removal (%)

Sample	Lead (mg)	Amount of lead absorbed (mg)	Removal (%)
Control	180.398	0.0	0.0
Day 1	108.449	71.94	39.88
Day 2	99.195	81.20	45.01
Day 3	36.22	144.17	79.91
	Cadmium (mg)	Amount of cadmium absorbed (mg)	
Control	178.1	0.0	0.0
Day 1	119.17	58.93	33.08
Day 2	118.51	59.59	33.45
Day 3	117.65	60.65	34.05
	Nickel (mg)	Amount of nickel absorbed (mg)	
Control	84.9	0.0	0.0
Day 1	48.92	35.98	42.37
Day 2	45.98	38.92	45.84
Day 3	44.47	40.43	47.62

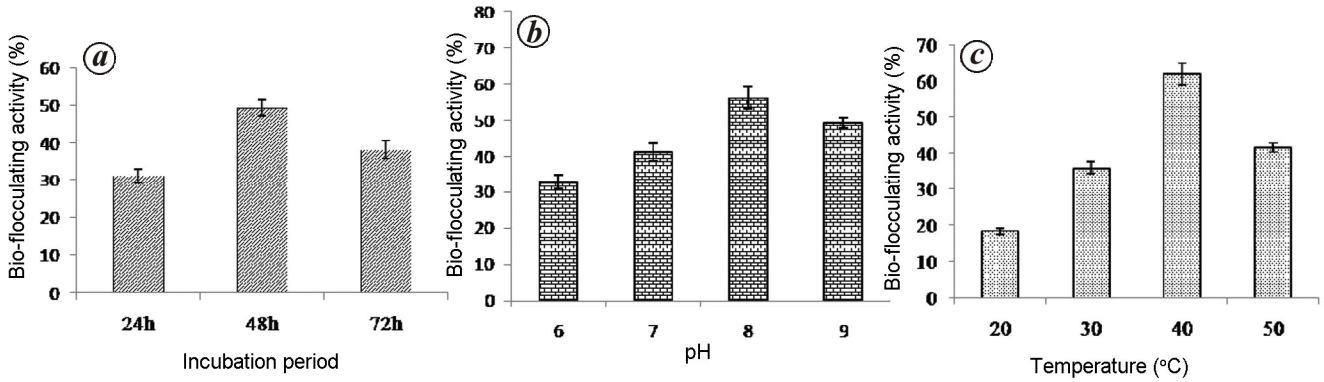

Figure 2. Surfactant production (%) by *A. phenanthrenivorans* B2 at different culture parameters. ***a–c***, Surfactant production at different pH levels, incubation periods and temperatures respectively. Data are presented as mean ± SEM in vertical bars; $n = 3$ replicates.

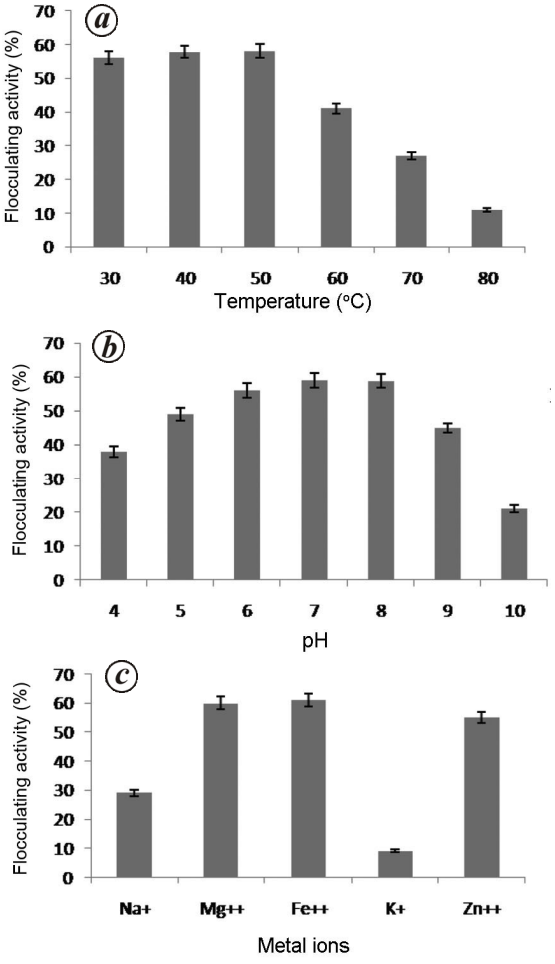

Figure 3. Characterization of surfactant at different physical conditions. ***a–c***, Effect of temperature, pH and metals ion on surfactant activity respectively. Data are presented as mean ± SEM in vertical bars; $n = 3$ replicates.

41.19% and 32.83% respectively, Figure 2 *b*). Figure 2 *c* demonstrates that the bacterial strain *A. phenanthrenivorans* B2 exhibits highest surfactant production at 40°C (61.94%). In the present study, surfactant produced by the bacterial strain *A. phenanthrenivorans* B2 was partially characterized at different temperatures, pH levels and in the presence of metals ions (Figure 3). Surfactant stability was recorded to be highest in the temperature range 30–50°C (Figure 3 *a*). pH levels 6–8 were observed to be the most suitable environment (Figure 3 *b*). Figure 3 *c* indicates that divalent cations are better for surfactant activity. Several studies have reported the applications of surfactant in various fields, such as oil removal in petroleum industries, water treatment and pharmaceutical sectors[19]. According to Deschenes *et al.*[20], biosurfactants are more effective than chemical surfactants like SDS in polycyclic aromatic hydrocarbon solubilization.

MDA level was highest in the nickel group compared to other groups (Figure 4 *a*). However, we detected a high level of MDA in control compared to the cadmium sample. This may be due to the long incubation period (16 h). This observation is also supported by elevated levels of SOD and CAT in the cadmium-treated sample. SOD activity was highest in the cadmium-treated group, followed by nickel and lead (Figure 4 *b*). On the other hand, bacterial strain *A. phenanthrenivorans* B2 exhibited highest CAT activity in the lead-treated group. Nickel and cadmium groups also showed significant ($P < 0.001$) CAT activity compared to the control (Figure 4 *c*). High concentration of metal exerts stress through generation of ROS. Increased CAT and SOD activities in the cadmium group indicate an effort to fight against the harmful free radicals that emerge during oxidative stress to compensate the internal environment of the cell. Similarly, in case of lead and nickel-treated groups, CAT and SOD activities also showed an increase as the stress is relatively higher than the control group, as indicated by the corresponding MDA level, which again supports the hypothesis that increased oxidative stress enhances the activity of antioxidant enzymes to maintain cellular integrity. Stress profile of this bacterial strain indicates that it might be effective for heavy metal removal, even at higher concentrations.

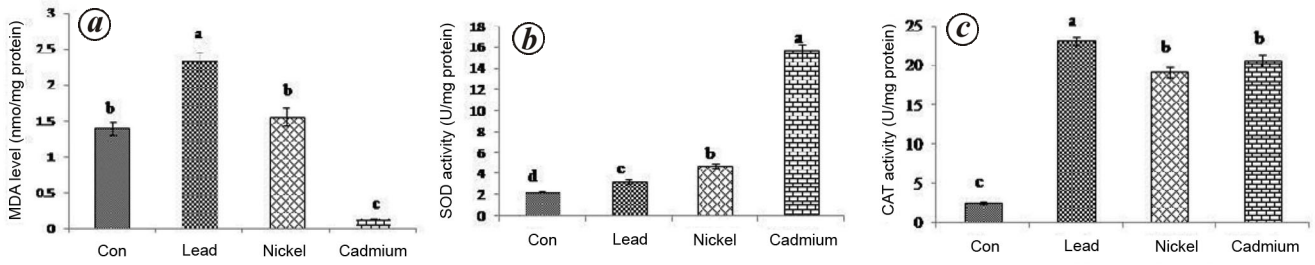

Figure 4. Antioxidant status of the bacterial strain *Arthrobacter phenanthrenivorans* B2. ***a*–*c***, Malondialdehyde (MDA) level, superoxide dismutase (SOD) activity and catalase (CAT) activity respectively. Data are presented as mean ± SEM in vertical bars; $n = 3$ replicates. Different small letters on the error bars indicate significant ($P < 0.05$) differences in the values of a particular variable under different stress conditions.

Conclusion

The present study reports the bioremediation of toxic heavy metals by a potent bacterial strain, *A. phenanthrenivorans*. Till date, several types of mechanical and chemical procedures have been introduced for removal of toxic metals from water, but these are costly, time-consuming and are not environment-friendly. Bioaccumulation properties and surfactant production ability of the microorganisms can be applied for water treatment on a small scale. Most of the reported bacterial species showing heavy metal accumulation belong to the genera *Pseudomonas*, *Bacillus* and *Staphylococcus*. To the best of our knowledge, there have been no previous reports of heavy metal accumulation by *A. phenanthrenivorans*.

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