Removal of copper by *Pseudomonas putida* strain S4 isolated from copper mines

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A bacterial strain, *Pseudomonas putida* S4, was isolated from smelter drainage of copper mines. The strain exhibited resistance to several heavy metals, like aluminium (Al), zinc (Zn), nickel (Ni) besides copper (Cu). Strain S4 could accumulate Cu from the Cu-supplemented growth medium. In the present study, we have demonstrated the Cu²⁺ removal capacity of this strain from various samples such as mine effluent, low-grade ore and ore-tailings, collected from the mining site. Moreover, ~80% of the accumulated Cu²⁺ could be recovered from the loaded biomass by a simple desorption procedure.

A number of industries, such as, mining, smelting, metal-plating and ore-processing release effluents containing high levels of heavy metals. These waste materials are potent sources of soil and water pollution. High level of these metals in the environment is a cause of concern as their toxicity to microbes is well established. Although many of these metal ions are essential, at elevated levels, they become toxic as they interfere with microbial metabolism. As a result, microbes have adapted various mechanisms of metal resistance, which include efflux, reduced uptake, and sequestration.

The ability of microbes to accumulate metals has attracted the interest of many researchers as this offers a potential alternative to the existing chemical technologies for environmental decontamination and/or recovery of valuable metals. A number of bacterial systems have been identified which can be used for such purposes.

We have earlier reported that a bacterial isolate *Pseudomonas putida* S4 is capable of removing Cu²⁺ from the growth medium. In the present study, the same bacterial strain was applied to the mine-effluent, low-grade ore and ore-tailings to monitor its efficacy in Cu²⁺ removal under more natural conditions. The strain S4 being a natural isolate was expected to possess mechanism/s to manage high levels of Cu²⁺ in order to survive. That the strain is able to accumulate Cu²⁺ could also be demonstrated by the recovery of metal from the loaded biomass by desorption. We thus propose that strain S4 could be employed for environmental bioremediation.

Materials and Methods

Organism—Bacterial strain used in this study was *Pseudomonas putida* S4, isolated from smelter drainage of copper mining site at Khetri Copper Complex (KCC), Rajasthan, India.

Culture media and solutions—A nutritionally defined minimal medium, supplemented with 0.4% gluconate (w/v), as described earlier was used for this study. The pH of the medium was set at 7.0, and the medium was labelled as GMM. Cells were grown in liquid GMM at 37°C, with shaking at 200 rev/min on a Controlled Temperature Shaker Incubator (New Brunswick Inc., USA). The solid medium was prepared by the addition of 1.5% agar-agar to GMM. Cu²⁺ accumulations—Cu²⁺ uptake experiments with ore and ore-tailings were performed in GMM. A pulp density of 5% was used for the experiments. Ore samples were weighed, autoclaved and added to the growth medium, prior to inoculation. Uninoculated controls were also maintained for each set.

Mine effluent was supplemented with gluconate (0.4%, w/v) and inoculated with 10% exponential culture. While ore and ore-tailings sets were incubated at 37°C, 200 rev/min in a shaker incubator for 15 days, effluent sets were kept under similar conditions for 24h only. Samples from these suspensions were withdrawn at different time periods, and analysed.

Sample analysis — Samples collected at different time periods were centrifuged at 5000xg for 10 min at 25°C. Supernatant was collected and analysed for pH, and dissolved fractions of Cu²⁺ and Zn²⁺ were...
estimated by Atomic Absorption Spectrophotometer (Perkin Elmer 3110) at 324.8 nm, and 213.9 nm, respectively. Concentration of the metals in the supernatant was expressed in terms of μg/ml.

For viable counts, appropriate dilutions in 0.85% saline were plated on GMM-agar plates and colonies appearing were counted. All the experiments were performed in triplicate and mean of the three independent sets is represented.

Cell immobilizations—Brick particles (~1 cm³) were washed thoroughly, autoclaved and dried in a hot air oven for 24h at 80°C. Brick particles were soaked in a suspension of stationary phase culture for 4-5h, removed, washed with 0.85% saline several times, and packed in a glass column (33 × 2.5 cm).

Copper removal by the immobilized cells—CuCl₂·2H₂O (1mM) solution was passed through the column at the flow rate of 0.3 ml/min. Column was operated at 25°C. Fractions were analyzed for copper content. An unloaded set of brick particles was always maintained as control.

Recovery of copper from the loaded biomass—1.5mM EDTA (ethylenediaminetetraacetic acid) was used as a desorbing agent. Desorption was carried out at a similar flow rate, as described above. Copper was estimated in each fraction and represented as μg/ml.

Results and Discussion

*Pseudomonas putida* strain S4 is able to grow in presence of 1mM Cu, 2mM Zn, and 1 mM each of Al, Co, and Ni. This strain thus could be characterized as multiple metal resistant strain. In order to demonstrate whether this strain S4 is able to accumulate Cu²⁺ from contaminated samples, we applied this strain to ore and ore-tailings suspension. They contained ~0.85% and 0.65 to 0.1% Cu, respectively.

From the data presented in the Fig.1, it is clear that for the first two days of incubation of ore and ore-tailings, Cu²⁺ was released into the medium. Subsequently, it was taken up by the cells, as indicated by a decline in Cu²⁺ content from second to fourth day. A further increase in the Cu²⁺ amount of the supernatant in case of inoculated ore suspension, during fifth and sixth days of incubation was attributed to the loss of viability. A ten-fold loss of the viability was observed at this stage (data not shown). The addition of fresh inoculum to the ore suspension (Fig.1) resulted in a decrease in the soluble Cu²⁺ content indicating uptake of the same by the fresh biomass. In case of uninoculated controls, amount of Cu²⁺ in the supernatant remained almost constant and release of Cu²⁺ was less as compared to the inoculated sets (Fig.1).

In case of ore-tailings, copper was very limited. Strain S4 could release copper from the ore-tailings also from the third day that became stabilized by the eighth day of incubation, as shown in Fig.1.

The Cu²⁺ removal capability of the strain S4 was assessed by monitoring Cu²⁺ uptake from the mine effluent also. When applied to the mine effluent, strain S4 could remove ~87% of the available Cu²⁺ from the same. Interestingly, the strain S4 could survive and multiply well in the effluent samples simply supplemented with sodium gluconate as a carbon source (data not shown). Addition of carbon-source was required as uptake of Cu²⁺ in the strain S4 is energy-dependent (Saxena and Srivastava, unpublished results).

Cells immobilized on brick particles were efficient in copper removal. Brick particles alone could remove ~72% copper from the 1mM CuCl₂·2H₂O solution. In case of immobilized cells this efficiency was 100% (Fig.2), implying the suitability of this organism for metal removal.

Once metal removal efficiency of strain S4 was established, we looked for the recovery of copper from the loaded cells. When 1.5mM EDTA was passed through the column of immobilized cells, 80% of the adsorbed copper was eluted out (Fig.2).

We also looked at the ability of strain S4 to remove...
Zn$^{2+}$ from the suspensions. The zinc content of uninoculated ore and ore-tailing suspension was $\sim 4.5 \pm 0.93 \mu g \cdot mL^{-1}$. It was observed that strain S4 could remove this metal ion too (data not shown).

The initial pH of the ore and ore-tailing suspension was towards acidic (6.49 and 5.8, respectively) and it shifted towards alkalinity, with the progress of the experiment. The pH of the inoculated sets shifted to 8.6 in case of ore and to 8.0 in case of ore tailings, respectively.

The ore of KCC is chalcopyrite and low-grade ore contains $\sim 0.85\%$ and tailings 0.05 to 0.1% Cu. Traces of other heavy metals, like Zn, Al, Ni and Co are also present in the ore. The multiple metal resistant character of strain S4 could prove advantageous when such a strain is employed for environmental decontamination. The viability loss in the ore samples could be attributed to the presence of trace amounts of metal ions like Ag, As, and Pb to which strain S4 is sensitive. The pH shift towards alkaline range in the inoculated samples can be due to microbial metabolism.

Several bacterial strains have been utilized for removal of Cu from low-grade ore and ore-tailings as bio-recovery of the metals is more cost-effective and less polluting$^{18}$. Bacterial leaching has been successfully applied to recover metals from a wide variety of ores$^{14}$. Biomining and wastewater treatment has become very important as the metals could build-up their concentration and ultimately enter the food chain. These, therefore, serve the purpose of both for environmental protection as well as for metal recovery. Immobilization is an efficient way for recovery of metal, as from the ore suspension it is not possible to recover cells and remove metal$^{19}$. Although the amount of metals in the tailings and effluent was not very high, the potential danger of metal pollution is realized due to their non-biodegradability. Under such a situation it becomes significant to remove even the low concentrations of the contaminated metals lest their concentration builds up to a toxic level. The increase in the Cu$^{2+}$ content in the supernatant of the inoculated sets can be explained as microbe-assisted release of metals, well known in case of P.putida. This organism is able to release Zn from the filter dust, and this function was associated with the production of citric acid as leaching agent$^{19}$. A number of amino acids are also known to solubilize metal from metal sulfides, metal oxide and elementary metals at alkaline pH$^{19}$. A similar phenomenon appears to be occurring with P.putida strain S4. Direct leaching, due to the production of acids, is always associated with very low pH and should have been seen as a drop in the pH as reported in Thiobacillus ferrooxidans$^{15}$.

Multiple metal resistance of the strain S4 makes it suitable for environment bioremediation applications. P.putida strain S4 seems to be more suitable for removal of metals from effluents or industrial waste rather than for bioleaching. Biggest advantage of dealing with this organism is that it is a natural isolate and is able to survive under deleterious environmental conditions. It can thus, respond favourably to bioaccumulation processes.

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