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REMOVAL OF COPPER VIA BIOREACTOR BY SOIL ISOLATE PSEUDOMONAS STUTZERI

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ABSTRACT

Prevalence of heavy metals in effluent is a major cause of environmental damages. The most prevalent ones include barium, cadmium, chromium, copper, iron, lead, manganese, nickel and zinc.

As bacterial strain CMG463 identified as Pseudomonas stutzeri removed highest concentration of copper it was also exploited for the removal of copper by developing bioreactor/biofilters and copper level in the out flow of bioreactor determined the removal efficiency. CMG463 cells were immobilized by developing biofilm on sponge; it was also examined by scanning electron microscopy. The biofilter demonstrated efficient removal of copper i.e. 90% from solution containing copper. CMG463 showing highest removal of copper could be exploited for remediation of sites contaminated with copper.

Keywords: Copper, biofilter, heavy metals, bioremediations

INTRODUCTION

Heavy metals are pervasive contaminants of great concern as they are non degradable and thus persistent. These metals are used in various industries from which effluents are consequently discharged into the environment (Zvinowanda et al., 2009). Introduction of metals in various forms into the environment can produce numerous modifications of microbial communities and affect their activities. In order to survive in heavy-metal polluted environments, many micro-organisms have developed means of resistance to toxic metal ions.

Copper is used as a biocide in agriculture fields and also used as a growth promoting nutrient in animals (Jacela et al., 2010). The discharge of copper into the environment through both natural sources and anthropogenic sources. The total flux of copper to the atmosphere is approximately 75,000metric tons/year of which 5000-13,000 tons are deposited into the ocean through both wet and dry deposition (Nriagu, 1979), approximately 75% of the atmospheric emissions are from anthropogenic sources. The most important natural discharge of copper to the atmosphere is windblown dust. Other sources of solid copper wastes include fertilizer production and municipal and industrial sewage.

Copper containing minerals of both technological and natural origins have been identified in the suspended particles of the surface and deep waters of the North Atlantic and pacific oceans (Jedwab, 1979).

Soluble copper levels in uncontaminated freshwater usually range from 0.5-1.0μgL^{-1} increasing to $\geq$ 2μgL^{-1} in urban areas. Variable concentrations of copper are also found in various aquatic plants and animal varies with respect to its industrial discharges.

Bioremediation is the restoration of environments through living (biological) systems rather than mechanical or toxic means and it is environment friendly. These processes are applied to clean the effluents, contaminated ground water and soil. For the development of this technology microorganism especially bacteria are of great importance. They have the ability to reduce the toxicity of metals by several mechanisms such as bio-sorption, biotransformation, bioaccumulation/uptake and bio-precipitations. This ability of bacteria can be harnessed in biotechnological applications for the removal/control of excess metal in various environments such as industrial and other wastes.

The ability to withstand the presence of high concentrations of a metal salt intracellularly called bioaccumulations. It plays an important role in the detoxification of hazardous heavy metals. The uptake of metal ions onto the cell surfaces and their subsequent translocation into the cell are well known natural processes but are highly specific (Hughes and Poole 1989). It is usually related to their ionic potential determined as a ratio of the charge and ionic radius. Element of low ionic potential easily form soluble cat-ions such as Na^{+} and Ca^{+}, whereas elements of high ionic potential P^{5-} and N^{5-} form soluble anions. Microbial cells can intracellularly and or extracellularly accumulate both metabolically essential and non-essential metals such as chromium, cadmium, copper, nickel, lead, iron, germanium, silver and zinc. Several species of bacteria have been reported for the accumulation/uptake of metal such as *Citrobacter* species accumulated cadmium and uranium (Macaskie and Dean, 1984; 1987; Macaskie *et al*., 1988), *Pseudomonas syringae* accumulated copper (Cooksey and Azad, 1992), *Pseudomonas stutzeri* accumulated Germanium (Dyke et al., 1990) etc.

Packed bed reactor, in this type of reactor cell immobilized in the form of beads, disc, chips, pellets etc. or biofilm form on solid support materials (such as rock, stone, crushed granite, plastic usually made up of polyvinyl chloride (PVC), polypropylene, polyethylene) can be packed into column such reactor system is very easy to use and ideal for remote rural sites. The solution or wastewater to be treated is passed through a settled bed held in a column and discharged continuously at the other end. The out flow can be monitored to determine the efficiency of reactors. Hollo et al (1979) has reported removal of heavy metal through packed bed reactor. The removal was a simultaneous during denitrification of an industrial wastewater by *Pseudomonas aeruginosa* biofilm on PVC chips. In this study the bacterial strain previously identified as *Pseudomonas stutzeri* (Badar et al., 2000) was used for bioremediation of copper.

MATERIALS AND METHODS

Bacterial strain

A bacterial strain CMG463 was selected from CMG stock culture after screening the resistance and accumulation of copper (Badar et al 2000).

Selection of support materials for the Development of Biofilm

Several support material were tested for the development of bacterial biofilm such as wood shavings, charcoal grids and sponge pieces. The main criteria was the ability to provide surfaces for attachment, high porosity and economically feasible. All these material were tested in order to select the best material, which was used to develop the bioreactor for the removal of copper.

Development of biofilm in batch culture

The support materials were washed three times with distilled water and air dried and weighed 0.5gms into 25ml nutrient broth in 100ml flask and sterilized by autoclaving. 1ml of

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overnight grown culture was inoculated in nutrient broth having support materials and incubated at 37°C for a week in a shaker incubator and each day samples were observed visually and finally biofilm was observed under scanning electron microscope.

Development of biofilm in chemostat culture

The support material which showed good biofilm in batch culture was selected for continuous culture for obtaining maximum biofilm biomass on support material required for the development of bioreactors. In continuous culture the biofilm was developed in 1L glass vessel. The temperature, pH and growth or optical density at 600nm was monitored periodically. The medium was supplied until the visible film of bacterial culture was observed.

Bioreactors/biofilters for the removal of copper

The bioreactor or biofilter consisted of pyrex glass column of 20 cm × Ø5.08 cm filled with support material (foam pieces of approx. 1.5 cm^2) coated with bacterial biofilm fitted with rubber stoppers on both ends. The bed volume was 250ml and the pyrex glass tubes of 2 mm inner diameter were fitted into the rubber stoppers to serve as out flow ports. Silicon tubing was used for conducting the effluent. A single multi channel peristaltic pump was used to challenge the effluent/synthetic wastewater containing copper (100 µM) into the bioreactors at the rate of 33ml/h. The control column was identical in every respect except that it did not contain biofilm. The outflow from the bioreactors was collected periodically and quantified removal of copper by spectrophotometric based copper assay as described by Sommers and Garroway (1957).

Electron Microscopy

Scanning electron microscopy (SEM)

The biofilm biomass was observed under scanning electron microscope. The biofilm coated support material were fixed in 2.5% glutaraldehyde for 1hr and then dehydrated in 50%, 70%, 90%, 100%, 100%, 100%, 100% ethanol 30mins each. The dehydrated samples were dried at critical point in polar critical point dryer and mounted on metal stubs and sputter coated with gold. The samples were observed under scanning electron microscope (2300 Hitachi).

Energy Dispersive X-ray Analysis (EDAX)

For energy dispersive X-ray analysis (EDAX) thicker sections (200-300 nm) of specimen were cut and examined by scanning transmission electron microscopy (JEOL JEM-100CXII) using a LINK ISIS X-ray analyser to determine elemental distribution in the cells.

RESULTS

Bacterial Strain

A bacterial strain CMG463 identified as *Pseudomonas stutzeri* was selected in this study have the ability to accumulate copper inside the cell. Due to this property the strain was selected for the development of biofilters or bioreactor for the removal of copper.

Selection of Support Material

Three different support materials were selected for developing biofilm. These were sponge pieces, wood shaving and charcoal grits of approximately 1.5 cm^2, 1×0.5 cm^2 and 0.01× 0.01cm^2 in size respectively (Figure1 A, B, and C).

A B C

Figure 1. Support materials for the development of bacterial biofilm. A, Wood shavings (pertal); B, Sponge pieces and C, coke grits

Development of Biofilm in Batch Culture

In preliminary examination visible attachment of biomass of CMG463 was observed on sponge only where as scanty or negligible biofilm was seen on charcoal grits and wood shavings. The support materials, sponge and charcoal grid were also observed under scanning electron microscope (SEM). SEM revealed the presence of bacterial film only on sponge pieces whereas no biofilm was observed on charcoal grits. The biofilm on sponge also showed the large amount of extracellular matrixes forming the network of fibres, which anchor the biofilm with the support material and the cells (Figure 2A and B). Therefore sponge was selected for developing biofilm in bulk that is chemostat culture for the development of lab- scale bioreactor.

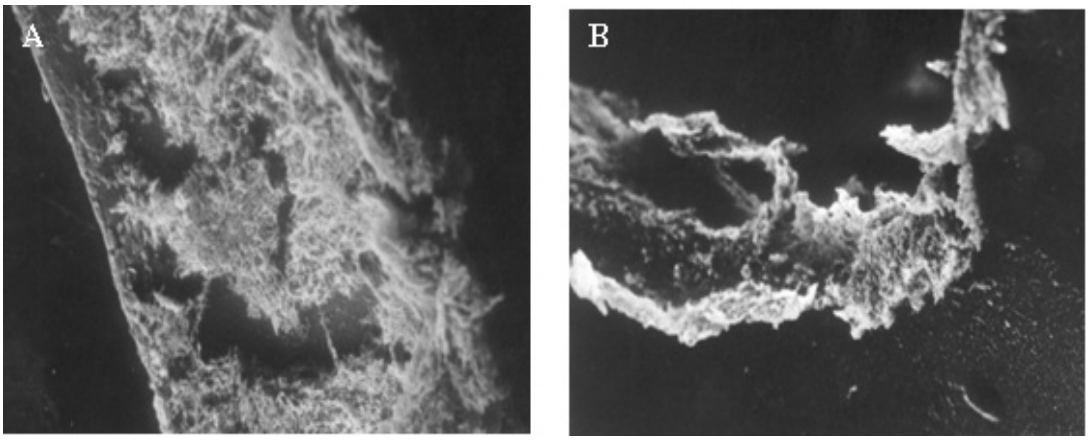

Figure 2. Scanning Electron Micrograph (SEM) of CMG463 Biofilm. A, showing biofilm on edge of sponge; B showing biofilm inside the sponge

Development of Biofilm In Chemostat Culture

The maximum growth was obtained at 1.6 $O.D_{600}$, the steady state of growth is equivalent to 0.92 μ_{max} in batch culture. In a continuous system the growth medium was diluted with the rate of 0.72ml/min. Consequently the rate of cell division in the biofilm at slow elution rates is regulated by the rate of fresh medium flow. In chemostat culture the μ_{max} is 0.144 and the chemostat was switched to continuous medium after 119hrs whereas after 200hrs detectable bacterial biofilm were seen on sponge pieces afterward chemostat was turned off and sponge

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pieces were harvested which was subsequently used for the biosorption of copper through bioreactor. The sponge pieces were also examined under scanning electron microscope showed presence of multi-layer biofilm. The excessive biofilm was observed at the edges of the sponge whereas the pores remain un-coagulated which allowed the passage of fresh medium and air (2 A and B).

Removal of Copper from Lab-Scale Bioreactor

In mini column bioreactors having capacity of 20ml, was preliminary used to remove copper. ~90% copper was removed and continued for 48hrs afterwards the activity of removal was decreased to 50%.

In 250 ml glass column bioreactor more than 90% copper was removed from 6 bed volumes of wastewater containing copper (100µM) and the efficiency of bioreactor for the removal of copper remained of about 48 hrs (Figure 3).

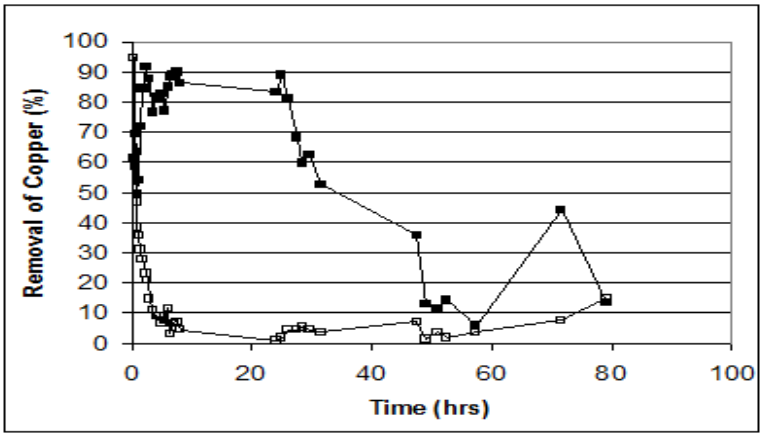

Figure 3. Removal of Copper through mini scale Bioreactor.Filled square represent removal of copper by biofilm coated sponge pieces where as hollow square represent control

EDAX of biofilm coated sponge

Microanalysis of biofilm coated sponge pieces for the elemental detection revealed the presence of copper along with Phosphorus, calcium, potassium and iron (Figure 5). These metals possibly came from growth medium (Figure 4).

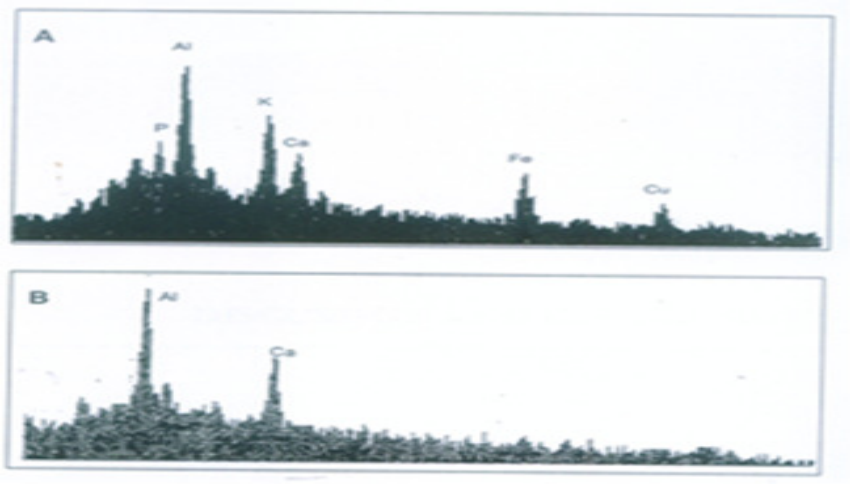

Figure 4. Energy Dispersive X-ray analysis of sponge. A, sponge coated with biofilm of CMG463 was harvested after the treatment of copper containing solution showing visible peaks of Cu, Fe, Ca, K, P, and Al. B, analysis of sponge pieces without biofilm (control experiment)

DISCUSSION

As CMG463 highest accumulation or removal of copper , it was suggested for the selection of biofilm formation for the remediation of copper. Several supporting materials such as sponge pieces, cokegrits and wood shavings were examined for the development of biofilm. Commercially available inexpensive sponge pieces were preferred because it replenished the requirements for ideal support material. The preliminary examination i.e., scanning electron microscopy also revealed the presence of greater biofilm on sponge pieces. The biofilm of CMG463 was developed on sponge in bulk in chemostat. Biofilms were harvested after one week from chemostat apparatus. This ability of biofilm formation or association with the solid support might be due to the excessive production of polysaccharides that has been reported earlier by Raihan et al. (1992) and Ahmed et al. (1997). It is a general experience that bacteria adhering onto a surface frequently secrete an exo-polysaccharides matrix in order to cement themselves the scanning electron micrograph of CMG463 biofilm on sponge also revealed the presence of network like matrixes of exo-polysaccharides. The bioreactors, containing biofilm coated sponge pieces were packed in a glass column and challenged with copper (100µM) solution with the flow rate of 33ml/hr. It was found that the efficiency of copper removal was 90%. In contrast with control column containing sponge pieces devoid of any film also showed removal of copper but it gripped only for few hours because of the adsorption whereas after saturation it showed no removal. Qureshi et al., 2001 have reported that the biofilm of Pseudomonas aeruginosa removed 85% of copper from the wastewater through bioreactor containing biofilm on PVC rings. Further the EDAX analysis of sponge that was harvested from the bioreactor after treatment of copper containing solution revealed the presence of copper peak along with the Fe and P. The presences of these metals were from the growth medium representing that CMG463 also have the ability to co-accumulate these metals. Similarly the accumulation of various metals i.e. cadmium, Uranium, lanthanum and thorium has been reported (Yong and Macaskie, 1998; Macaskie and Dean 1987, 1989).

CONCLUSIONS

The indigenous bacterial strain CMG463 is the best candidate, which has the ability to remove copper efficiently from the solution. It has been found that this technology can be scale up, which subsequently provides a solution for the environmental bioremediations.

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