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ISOLATION AND CHARACTERIZATION OF HEAVY METAL RESISTANT BACTERIA FROM INDUSTRIAL EFFLUENT IN JABALPUR CITY, INDIA; CASE STUDY OF IMMOBILIZED BACTERIAL REMEDIATION

Research Article

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ABSTRACT

Due to rapid growth of industrialization and extraction of natural resources heavy metal has been accumulated in soil and water causing toxicity to environment. In this research, waste water from iron- steel industry and plastic industry, Richai (Jabalpur) were analyzed. Total of 76 isolates were obtained from heavy metal contaminant. Sample potent heavy metal (Cu, Co and Zn) degradation was identified as *Klebsiella, Micrococcus, Bacillus, Pseudomonas and Alcaligens*, by morphological and biochemical characteristics. Freely suspended and immobilized bacterial strain was compared for heavy metal degradation at various pH from 2-8 showing highest degradation at 6 and 7. Identified heavy metal resistant bacteria may be useful for bioremediation study of contaminated industrial waste water.

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INTRODUCTION

Accumulation of heavy metal has been arise in soil and water due to the heavy discharge of industrial waste to the environment which is result of growth of industrialization and extraction of natural resources. Since these hazardous heavy metals and toxic chemicals can't be broken down to nontoxic forms, is the major cause that the world is facing today as it not only contaminate the soil, groundwater, sediments, surface water and air but also have long lasting effects on the ecosystem. (Asha and Sandeep, 2013) in their recent study emphasis on the new technologies for the remediation of the natural resources by destructing the pollutants instead of using conventional methods as their is chance of the potential to enter the food chain. It is found scientifically that metals can cause serious health issues if present in body as they disturb our normal functioning, explained by study of (Suranjana and Manas, 2009). But some of this metals when present in low concentration are beneficial like Arsenic copper Iron Nickel etc. High concentration leads to toxicity which are carcinogenic and mutagenic in nature according to study by Salem et al (2000).

There have been a lots of researches carried on heavy metal contamination in the sea from last 10 years due to the major factors like mercury problems and the analytical techniques development. Marine environment are a natural resources of heavy metals (Fe, Cu, Zn, Co, Mn, Mo, Se, Ni, Sn) which are biologically essential. Like metallo proteins or metal-protein complexes they occur in enzymes and respirator as metallo proteins or metal protein complexes they occur in enzymes and respiratory pigments, for example, and may have structural role in polychaete jaws (Bryan and Gibbs, 1980). As a result, studies on metal contamination tells the problem of metal present in natural levels and those which are enriched from anthropogenic sources and may, since metals are considerably toxic, produce unwanted effects.

"Heavy metals" is a massive term, which applies to the group of metals and metalloids with a atomic density higher than 4 g/ cm3, or 5 times or more, higher than water. (Paul and Sinha, 2015) Heavy-metal contamination is not today's problem coming out of industrialization. It developed when humans started working on ores. Since then the use of metals and their effects on the environment have increased, with a major incline during the 19th and 20^{th} centuries U.F€orstner,

G.Wittman, 1983. Usually, most of the heavy metals come to the river from different sources, as a result of natural erosion and weathering or due to anthropogenic, Gupta et al, (2013). Due to the high human activity, natural sources of heavy metals from leaching and weathering of rocks in the environment, are generally of less importance A. Kabata-Pendias, 2001 Dixit et al. Heavy metal are present as a result of precipitation of the carbonates hydroxides and suicides, which settle down and gets into the sedimentation part. The most important anthropogenic sources of heavy metal are various industries and domestic sewage. The heavy metal concentration has been increased in water resources due to continuous discharge of waste effluents from industrial Bao et al, (2014). The various industries like metal industries, paints, pigment, garnishes, pulp and paper, tannery, distillery, rayon, cotton textiles, rubber, thermal power plant, steel plant, galvanization of iron products and mining industries as well as unsystematic use of heavy metal containing pesticides and fertilizer in agricultural fields. These heavy metals have accumulative effect at the low level in drinking water and ground water, Selvapathy et al, (1997).

In this study, the routine of grounding calcium alginate biocarrier was ready by adding of bacterial mass relocate, and to seriously recover heavy metal degradation. Chen et al, (2017). Entrenched immobilization practice, as a technique of immobilization, is widely worn for its easy function, soft and unproblematic to be realized reaction condition, low likelihood of microorganism escape, superior concert at solidity and reuse, high microbial activity and cell capacity, and high immobilization competence which can reach more than 70% compared with other immobilization technologies, such as adsorption, cross-linking, and covalent bonding. In view of these properties, researchers used surrounded immobilization technique to immobilize the microorganisms. Sodium alginatecalcium chloride (calcium alginate) embedding are the most generally used methods of embedding immobilization. However, problems have been encountered in the field of purpose of bioremediation technology due to irrepressible factors, such as the loss of effective strains and substantial reject in the efficiency of remediation, because the ocean is an open and highly mobile system. Immobilized microbial technology is privileged for its high cell concentration, low microbial thrashing, enhanced acceptance of the environment, and high degradation competence of petroleum. Finally, we used the bacteria in laboratory-scale to extravagance heavy metal pollution using immobilized bacteria columns to test the likelihood of metal removal.

MATERIALS AND METHOD

Sampling and pre-treatment

Effluent from Iron- steel industry and plastic industry Richai (industrial area Jabalpur), were obtained from the sedimentation tank of the industry. Before collection water was allowed to settled at room temperature followed by decanting the supernatant sample were collected in pre-sterilized amber bottles pH, temperature, color was observed and B.O.D was locked and sample was kept at 4 degree in ice jacket and was taken to microbiology lab St. Aloysius college For further study sample was processed within 24 hours. All these experiments was performed in triplicates.

Heavy metal determination

Three heavy metals (Cu, Zn, Co) in the waste water effluent were determined Spectrophotometry. Following the standard method of APHA 1981.

Isolation of Heavy Metal-Tolerant Bacteria

Metal tolerant bacterial isolates were collected from different effluent with elevated levels of Cu, Zn, Co (prepared as 10 % stock solutions of CuSO₄,CoCl₂ H₂O, ZnSO₄, salts. El. Bestawy et al, (2012) as described by Oliveira et al, (2001). Tolerant bacteria were isolated from the final stage of the metal enrichment experiment .One-milliliter liquor from each reactor was used to inoculate liquid Luria-Bertani (LB) medium containing the investigated heavy metal (sterilized by filtration) in that reactor. Heavy metal concentrations used during bacterial isolation were 15, 100, 40 and 40 mg/l for the individual Cu, Co, Zn respectively. While a mixture of Cu, Co, and Zn at 2, 10 and 10 mg/l respectively was used for isolation of strains with multiple accumulation ability. After 24 hour incubation at 30°C, 100 rpm of the grown culture was transferred into 10-ml fresh LB medium containing the same metal concentration and left for another 24 h under the same conditions. The highest metal concentration reduced the activated sludge activity by 50 %was amended in Petri dish containing 20 ml sterile LB agar and mixed well. 100 rpm of the grown culture was spread on the surface of the plate and incubated for 24 hour 30°C.

Screening for Heavy metal resistance pattern

Effect of the individual metals on the growth (measured as optical density, OD at 600 nm). An overnight culture of each strain was inoculated in 20-ml LB medium supplemented with definite concentrations of the corresponding heavy metals and incubated for 24 h under the previously mentioned conditions.

Maintenance Medium

Stocks of strains were maintained on standard Luria bertani medium comprising in tryptone (1gm), Yeast extract (0.5gm), NaCl (1gm), bacto agar (1.5), Liu *et al* (1997).

Inoculum Preparation

A loop full of LB slant yeast cells was cultivated in 50 ml liquid LB medium in 250 Erlenmeyer flask at 30 °C on a rotary shaker for 24h at 200 rpm.

Bacterial cells immobilization

Calcium alginate bacterial cells were separated by centrifugation at 10,000 rpm for 10 min. The cells were washed and re-suspended in sterile 50 ml of acetate buffer (pH, 4.0) and 10ml suspension was mixed homogeneously with 90ml volume of aliquot of Na-alginate suspension to contain 2 %(w/v) Na- alginate. Alginate-bio sorbent suspension was added drop wise to 500 ml of 2 %(w/v) CaCl₂ with a pipette. Alginate drops solidified upon contact with CaCl₂, forming beads and thus entrapping bio sorbent particles. The beads were allowed to harden for 30 min and were then washed with sterile physiological saline solution (0.85%, w/v, NaCl) to remove excess calcium ions, Equal number of control beads without cells and immobilized cell beads were used to inoculate 50 ml portions of bio sorption media at 200 rpm at 30⁰C. After 18h, filtration to harvest the beads, centrifugation at $10,000 ext{ x g}$ for the escaped cells and determination of final heavy metals concentrations in the filtrates by atomic absorption were carried out for control beads flasks and beads with entrapped yeast cells flasks .(%) metal removal were calculated; Ksunger *et al* (2003).

Wastewater treatment with immobilized cells

Alginate immobilized cells (≈ 0.25 , 10.12 dry weight /flask) were used to inoculate 50 ml of wastewater in 250 ml Erlenmeyer flasks that adjusted to pH-5.5, respectively, another set of 250 ml Erlenmeyer flask with 50 ml wastewater were treated with 10% (v/v) natural polyelectrolyte and a control wastewater flask kept untreated, incubated at 30°C and 150 rpm for 18 h. and different previous parameters were evaluated.

Preparation of the Heavy metal solutions

The different metal ions were sterilized by filtration through a pore filter of 0.22 um and were added to achieve final concentrations of 15 35, 25 (mg/l) $CoSO_4.8H_2O$; $CuSO_4.5H_2O$, and $ZnSO_4$ respectively in bio sorption media, Liu *et al* (1997). At the end of incubation period, cultures were harvested, centrifuged at 10,000 x g , final metals concentration were determined and metal removal (%) was calculated.

Effect of initial pH on Heavy Metals Removal

Bacterial cell culture in biosorption media at 30° C,200 rpm for 18h adjusted to different pH values (2.5 – 6.5)were harvested, centrifuged ; final metals concentrations in different flasks were measured . (%) metal removal was determined; (Pearce and Sherman, 1999).

Determination of heavy metal concentration in the filtrate

Following metal treatment, culture filtrates were taken at certain intervals, centrifuged at 10,000 x g for 5 min and the clear supernatant liquids was used to determine heavy metals ions concentrations by using spectrophotometer, Varian absorption spectra AA20-NRC. ,Egypt; Ksunger *et al* (2003). Metal removal (%)=Initial metal conc.(mg/l) Residual conc.(mg/l)/Initial metal conc. (mg/l)x100; (Berekaa and Hussein, 2005).

RESULTS

Bacterial strain condition of growth

Out of 76 isolates recovered at different level of metal concentration. 32 were from effluent water and 44 were from effluent effected soil. Toxicity to metal at concentration was checked both on solid and liquid (Figure1). Results of solid medium substantiate liquid media growth profile. Availability of metal compound is higher in liquid than in solid. Thus a particular concentration become more toxic for organism while grown in liquid broth. Their fore tolerance level for a metal was greater in solid media than in liquid. The role of the microbial cell wall in the bio sorption process is to adsorb metal ions in the cell wall itself or pass through the cell membrane into the vacuoles. To balance the stimulatory or inhibitory effects of essential ions and to counteract the toxicity of nonessential metals, all organisms possess homeostatic mechanisms that properly control the cellular accumulation, distribution, and detoxification of metals, bacterial cell provides an ideal system. Gadd (1988) stated that microorganisms can take up nickel

intracellular or the presence of chelating ligands that may be present on the cell surface in trace amount even after washing the biomass thoroughly and before using in bio sorption experiments. Hence, both type of the heavy metal and its concentration affect behavior of bacterial cell bio sorption.

Metal tolerance analysis

Colonies on agar plate with 10 µg/ml of Cu, Co and Zn after incubating for 3-4 days were selected and grown in liquid media with 10-100µg/ml concentration of all above mentioned heavy metal purified bacterium colony was inoculated in liquid medium and metal tolerance concentration was accessed. The bacterial strain with maximum MTC (Metal Tolerance Concentration) was selected for further study (Figure 1). This is may be due to the ratio of protein to hydrocarbon or as (Park and Choi, 2002).stated that after 24 h at 200 rpm at 300C there is no serious metal accumulation of cadmium in the cell which was related to the cell metabolism. Equilibrium time varied according to the bio sorption conditions as it was attained after 30 and 60 min for dead and live cell used for removal of Cu^{2+} , Machado *et al*, (2009); Engle and Kunz, (1995). stated that removal carried out in three steps; rapid binding to the negatively charged groups on the cell wall and passive transport of the metal ion through the cell wall within short time 3-5 min, penetration though cell wall to the cytoplasm and accumulation of the heavy metal in the cytoplasm,. Similarly, (Pearce and Sherman, 1999), Revealed that histidine binds divalent metals, and is routinely exploited by insertion of polyhistidine tracts into proteins, so that the protein can be bound to resins with bound divalent metals ions such as Co^{2+} and Ni².

The bacterial strain with maximum resistance to Cu, Cd and Zn heavy metal was previously identified using morphological, cultural and biochemical characteristics as *Klebsiella* (Isolate1), *Micrococcus* (isolate2), *Pseudomonas* (isolate3), *Bacillus* (isolate4), *Alcaligens* (isolate5). Zinc degradation was found maximum at 4th and 6th day using both freely suspended and immobilized bacterial isolates i.e. 60 and 40% respectively, whereas copper with 70 and 40 % degradation at same day. Slight variation was observed with cobalt which degraded maximum at 6 day showing 65 and 40 % degradation by freely suspended and immobilized bacterial isolate (Figure 2, 3 and 4)



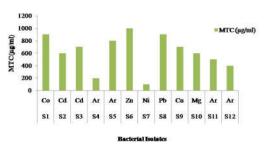


Figure 1 Metal tolerance analysis by various isolated bacteria

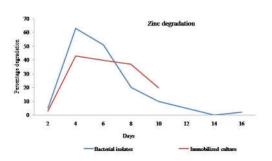


Figure 2 Comparative analysis of freely suspended and immobilized bacteria for Zinc degradation

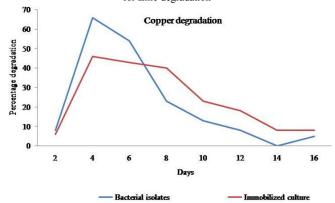


Figure 3 Comparative analysis of freely suspended and immobilized bacteria for copper degradation

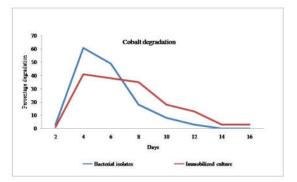


Figure 4 Comparative analysis of freely suspended and immobilized bacteria for Cobalt degradation

Comparative analysis of heavy metal degradation using immobilized bacterial strain

Top three potential heavy metal degrading isolates was further selected for immobilization study. Potent bacterial strain Micrococcus. Pseudomonas. Bacillus Klebsiella. and Alcaligens was immobilized using sodium alginate and calcium chloride. To a certain potential of heavy metal degradation was accessed as function of time. In this experiment, Zinc showed maximum degradation at showing 14.2 % at 7 pH, Copper showed maximum degradation at showing 18.7% % at 7 pH and Cobalt showed maximum degradation at showing 15.6 % at 6 pH (Figure 5). The culture was measured for optical density (600nm). Their study showed relative degradation of heavy metal by immobilized and non-immobilized bacteria. in the same order with alginate because of both yeast cells and the polymeric matrix of alginate beads itself in absorbing the metals, moreover, alkaline earth metal- alginate entrappers matrix is a known procedure, Meena and Raja, (2006) ; Stoll and Duncan, (1997) stated that medium accumulation of heavy metals by microbial biomass with high surface area-to-volume ratio holds great potential for heavy metal removal in both soluble and particular forms, especially when the heavy metal concentrations are low (<50 mg/L) Immobilization offer advantages such as easier separation and reuse of cells, maintenance of higher cell concentrations and stabilization adhesion to supports ; Aloiu *et al*, (2008).

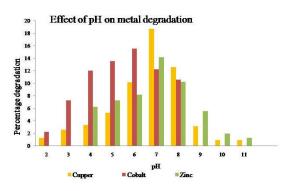


Figure 5 Optimization of pH for Metal degradation by immobilized bacteria

The process by which microorganisms cause changes in metal speciation and mobility are essential components of biogeochemical cycles for metals, as well as all other elements together with carbon, nitrogen, sulfur and phosphorus, with supplementary implications for plant productivity and human health. Ali et al, (2013) approximately all metal-microbe connection shave been examined in the perspective of environmental biotechnology as a means for removal, recovery or of inorganic and organic metal or radionuclide pollutants Ansari et al, (2011); Cassid et al, (1996); Chen et al, (2002). Even though much examine is laboratory based, there have been numerous developments to pilot/ scale, with some processes truly in successful viable operation. It should also be noted that metal removal/ processes conversion are vital though less valued components of customary means of water/sewage treatment as well as reed bed, pond and marsh land technology Chen et al, (2002); Chen et al, (2012); Edema et al, (2001). Molecular and genetic investigation is now furthering our considerate of microbial metal metabolism, including those aspects that have latent in biotechnology Fawole et al, (2001); Aksu and Tezer, (2005). This paper particulars recent advance in accepting of the most important mechanisms of microbial metal transformations within the contexts of bioremediation and environmental biogeochemistry. Mechanisms of microbial solubilization and immobilization of metalloids, radionuclides and linked substances are of apparent probable for bioremediation, with some processes being vital to the function of numerous unbeatenin situ and ex situ processes. Although bio sorption research is rather reserved with slight current progress in an industrial circumstance, work on metal leaching from contaminated matrices, metalloides conversion and bio precipitation is leading to 'field' development, as well as providing elementary systematic insights into metal-microbe relations. As well as the biotechnological on sequence, it should be emphasized that this effort also provides understanding of the biogeochemistry of metalloids cycling in the environment and the central role of microorganisms in

disturbing metal mobility and relocate between different biotic and abiotic locations. The recent study have exposed that the more successful deprivation of cypermethrin at higher concentration could be achieved by immobilized cells of Micrococcus sp. strain CPN 1 than liberally suspend cells. The immobilized microbial system has an benefit of improved rate of degradation, tolerance to higher substrate concentrations and their reuse ability. Hence, the immobilized microbial technology provides a extremely flexible and cost-effective advance that can be used for collapse of pesticide contaminated waste water. Bioremediation, a biodegradation method in which sites unhygienic with xenobiotic are cleaned up by resources of bacterial bio-geochemical processes, quicker in situ, exploits the capability of micro-organisms to weaken the application and/or toxicity of a large integer of pollutants. Interest in processes regarding heavy metal uptake by microorganisms has amplified noticeably in topical years due to the biotechnological potential of micro-organisms in removing and/or recovery of metals. The predictable methods such as man-made ion exchangers are well thought-out as mature technologies. Bio sorption is at rest in its developmental stages, and supplementary improvements in both presentation and expenses can be predictable. Based on the earlier research on the potential of Trichoderma sp. in removing heavy metals, it is completely suggested that Trichoderma sp. should be measured as the agents in bioremediation process.

Solubilisation of heavy metal at different pH by immobilized bacteria

Inocula of heavy metal- resistant immobilized bacterial isolates were prepared using 16 hr logarithmic phase cells. Concentration of heavy metal was _µg/ml to be treated by immobilized bacteria with pH as function. pH from 2-8 was adjusted and effect of various pH was accessed. Percentage of cupper removal was maximum at pH 7, for cadmium maximum at pH 6 and for zinc maximum at pH 7 (fig 5). On increasing pH, the bio sorption capacity increased as there is an increase in ligands with negative charges which results in increased binding of cations because the ligands on the cell are closely associated with the hydronium ions. With increased pH, the hydronium ions are gradually dissociated and the positively charged metal ions are associated with the free binding sites; Ahlya et al, (2003). Little metal removal was observed at pH 2.5 is an indication of competition of excess of protons for the same binding sites on the cell wall. The iron, cadmium and copper uptake increased with increasing pH, to the maximum near pH 5.5 then decreased at 6.5 pH. At pH values higher than 6.5, metal ions precipitated, so it is not conducted, due to the high concentration of OH- ions in the adsorption medium to avoid possible hydroxide precipitation and bio sorption Ahlya et al, (2003).

Indicated the importance of pH in the bio absorptive process

It affects the solution chemistry of the metals, and the activity of the functional groups in the biomass. The more rapid increase in metal removal in accordance with pH may be presumed by larger degree of deportation of the cell wall at higher pH because the hydrolysis degree of heavy metal in the solution at a given pH was nearly constant regardless the microbial species.

CONCLUSION

The work presented in this paper shows that isolation from indigenous sources like effluent soil and water can be an effective design to remediate heavy metal contaminated water using immobilization technique. Present study revealed that more effective degradation of Cu, Cd and Zn at higher concentration could be achieved by immobilized cells of Micrococcus, Pseudomonas, and Klebsiella, Bacillus Alcaligens then freely suspended cells. This method could be helpful in developing useful system for evaluation of natural potential of environment samples. This study further could be used for developing global bioremediation methodology. As immobilized microbial technology provides highly versatile and cost effective approach for degradation of heavy metal in waste water.

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