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Remediation Of Mercury Contaminated Soil By Bacillus Sp, And Pseudomonas Sp, Isolated From Rhizosphere Soil

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Abstract: ***Bacillus Sp, and Pseudomonas Sp., is isolated from rhizosphere soil which has the potential to remove mercury from soil. Bacillus Sp, and Pseudomonas Sp, shows the maximum biosorption of Hg(II) ions was obtained at pH – 5 and 6 respectively. The remediation of mercury contaminated soil is studied by addition of nutrient source such as carbon (0.15 – 0.85 g l^{-1}), nitrogen (0.25 – 1.05 g l^{-1}) and phosphate (0.10 – 0.30 g l^{-1}) to the medium. The effect of ionic strength on Bacillus Sp, and Pseudomonas Sp, is optimized by NaCl ranges from 0.10 to 0.30%. The growth profile of mixed cultures in mercury contaminated soil is found to be 1642 cells ml^{-1} after 6 days. The mercury adsorption potential of mixed cultures in the contaminated soil is found to be 60.10% after 6 days at pH – 6 and temperature 35° C. This research indicates that the isolated resistant strains have an important role in adsorption of mercury from the contaminated soil.***

Keywords: ***Mercury, Soil, Bacillus Sp, Pseudomonas Sp, Nutrient sources***

I. INTRODUCTION

India is one among the world's most active mercury industrial centers. Chloroalkali industries are still the major source of mercury release in atmosphere and surface water (Lenka et al., 1992). Mercury pollution in India was contributed by Coal fired plants viz. thermal power plants, steel industries, and cement plants, plastic industry (mercury is used as a catalyst), pulp and paper industry, medical instruments and electrical appliances, certain pharmaceutical and agricultural product (Mody, 2001). Mercury is used in a variety of manufactured products and manufacturing processes (Cooper, 1997) during mining, smelting and ultimate disposal of heavy metal from the industries cause's pollutions and the metals are substantially toxic to plants, animals and microorganisms (Atlas and Bartha, 1993). The major effects of mercury poisoning are neurological and renal disturbances as well as impairment of pulmonary function (Manohar et al., 2002). Therefore the development of technologies for the treatment of soil, sediment and water are of major concern. Conventional methods such as, chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies are generally used for removing metals from aqueous solutions (Grau and Bisang, 1995). These processes may be ineffective or expensive, especially when the heavy metal ions are in solutions containing in the order of 1-100 mg dissolved heavy metal ions L^{-1} (Volesky, 1999a and 1999b). During recent years, the study of microorganisms has contributed important insights into the basic problems an emerging technology that has received more attention in the development of biosorbents with high affinity and specificity (Murugesan and Maheswari, 2007). Microorganisms have evolved resistance mechanisms to deal with heavy metal toxicity, which include volatilization, extracellular precipitation and exclusion, binding to the intracellular sequestration (Roan and Kellogg, 1996). Microbial cell surfaces are usually charged and the passive process involving extracellular accumulation of metals on cell walls has been studied (Sahoo et al., 1992). The mercury resistant bacterial cell accumulated substantial amount of mercury from the medium and volatilized more than 90% of inorganic and organic mercury (Mishra and Nanda, 1997 and Pahan et al., 1996). Many bacterial species such as *Pseudomonas* K – 62

(Tonomura et al., 1968), *Bacillus thuringiensis* (Hassen et al., 1998) and *Pseudomonas fluoresces* BM07 (Noghabi et al., 2007) and fungal species such as *Aspergillus nidulans* (Maheswari and Murugesan, 2009a), *Aspergillus fumigatus* (Maheswari and Murugesan, 2009b), *Rhizopus arrhizus* (Akus et al., 1999), *Phenerochaecte chrysosporium* (Say et al., 2001) and *Aspergillus niger* (Kapoor et al., 1999) have been extensively studied for heavy metal biosorption and the process mechanisms seem to be dependent upon species. In this investigation, the bacterial strain *Bacillus* and *Pseudomonas* Sp, was isolated from rhizosphere soil and its efficiency in removing mercury from the contaminated soil. This study also reports on the effect of carbon, nitrogen, phosphate source and ionic strength on the remediation of mercury from the contaminated soil.

II. MATERIALS AND METHODS

A. ISOLATION AND IDENTIFICATION OF BACTERIAL COLONIES

The soil sample was collected from the rhizosphere region is inoculated into 250 ml of nutrient medium in 500 ml Erlenmeyer flasks. The flask was incubated in a shaking water bath operating at 240 cycles per minute for five days at room temperature. At daily intervals one loop full of enrichment culture from the flask was streaked on nutrient agar plates supplemented with mercury chloride (0.1%) and incubated at 35 °C for 24-48 h. Individual colonies were sub cultured in nutrient agar plates containing same concentration of Hg(II) ions until pure cultures was isolated. The isolated strains was maintained at 4 °C and sub cultured periodically. (Malekzadeh et al., 2002; Lyer et al., 2004). The isolated dominant Hg resistant bacterial cultures were identified based on the identification and characterization of the isolates by Bergey's Manual of Determinative Bacteriology, (1994).

B. OPTIMIZATION OF PH

The effects of the medium pH for Hg(II) ion adsorption rate was investigated at pH of 2, 3, 4, 5, 6, 7 and 8 (which was adjusted with HCl or NaOH at the beginning of the experiment). The growth profile of the resistant isolates was analyzed after the desirable incubation period.

C. MEDIUM OPTIMIZATION

The medium optimization was conducted in a series of experiments by changing one variable at a time, keeping the other factors fixed at a specific set of conditions. Three factors was chosen to obtain higher sorption of mercury from the soil; carbon, nitrogen and phosphate sources. The carbon source (dextrose) was employed at a concentration of 0.15 to 0.85 g l^{-1}, the nitrogen source (yeast extract) at a concentration of 0.25 to 1.05 g $l^{-1}$and phosphate source at 0.10 to 0.30 g l^{-1}in mineral salt medium (KH_2PO_4 – 2.38 g l^{-1}; K_2HPO_4 – 5.65 g l^{-1}; NH_4SO_3 – 2.64 g l^{-1}; $MgSO_4$ – 1 g l^{-1}; $CaCl_2$ – 0.1 g l^{-1}) was studied. The growth profile of the resistant bacterial isolates was analyzed after 24 h.

D. EFFECT OF IONIC STRENGTH ON HEAVY METAL SORPTION FROM SOIL

A series of flasks containing 100 mg l^{-1} of $HgCl_2$ ions at a fixed dosage of the resistant isolates with different NaCl concentration (0.10, 0.15, 0.20, 0.25 and 0.30 g l^{-1}) was incubated at 37ºC. The growth profiles of the resistant isolates were analyzed after 24 h.

E. REMEDIATION OF MERCURY IN THE OPTIMIZED MEDIUM

500g of mercury contaminated soil was placed in the hot air oven (120ºC) for 7 days to kill the indigenous microflora in the soil. After 7 days the dried soil was placed in a metallic tray and it is covered by a polythene sheet to maintain the temperature (35 ºC) for the bioremediation process. *Bacillus* Sp, cultivated in (dextrose – 0.65 g l^{-1}; yeast extract – 0.45 g l^{-1}; K_2HPO_4 – 0.10 g l^{-1}; NaCl – 0.25 g l^{-1}) and *Pseudomonas* Sp., (dextrose – 0.15 g l^{-1}; yeast extract – 0.65 g l^{-1}; K_2HPO_4 – 0.15 g l^{-1}; NaCl – 0.15 g l^{-1}) in the optimized medium. The optimized medium was added to the mercury contaminated soil and it was covered with the polythene sheet.

F. ANALYSIS OF GROWTH PROFILE, PH AND MERCURY CONTENT IN THE CONTAMINATED SOIL

The pH was checked in the contaminated soil up to 7 days periodically. The quantitative estimation of bacterial biomass in soil was analyzed by the method described by Gerdemann and Nicolson, (1963). The soil sample was collected every 24 h up to 7 days was dried and acid digested with 1:1 HCl and the mercury content in the soil was analyzed using AAS (PG – 990).

III. RESULTS AND DISCUSSION

A. IDENTIFICATION OF MERCURY TOLERANCE RESISTANT ISOLATES

In this study the total count of the bacteria in the rhizosphere soil ranged from $132 \pm 0.3 \times 10^{-2}$ to 41×10^{-7}. The dominant mercury resistant strains were identified as *Bacillus* Sp, and *Pseudomonas* Sp., based on the cultural and biochemical characteristics described by Bergey's Manual of Determinative Bacteriology, (1994). The mercury tolerance was tested by growing *Bacillus* Sp., and *Pseudomonas* Sp, in nutrient broth with 0.01 and 0.1% mercury ions. The resistant isolates showed tolerance up to 0.1% of mercury ions. The biochemical basis of resistance noted from the soil strain was demonstrated to be due to enzymatic degradation of organomercurial to Hg^{2+} and the reduction yielded to Hg^{2+} to elemental mercury (Hg^{o}) catalyzed by organomercurial lyase and mercury reductase (Tezuka and Tonomura, 1978). *Pseudomonas* K - 62 a bacterial strain with broad spectrum mercury resistant isolated from phenyl mercury polluted soil has been shown to have approximately 1000 – fold higher resistant phenotype to phenyl mercury than other bacterial

strain such as *Escherichia coli* and *Pseudomonas aeruginosa* (Tonomura et al., 1968). Mercury accumulation strains which was resistant to 400Mm NaCl and retained 95% of its Hg(II) bioccumulating activity in the presence of metal chelators (Chen and Wilson, 1997).

B. GROWTH PROFILE OF THE RESISTANT STRAINS AT DIFFERENT PH

The growth profile of the mercury resistant bacteria isolates at different pH (2 – 8) is shown in Fig – 1. The OD values of mercury resistant *Bacillus* Sp, and *Pseudomonas* Sp., was found to be 0.149 ± 0.03 and 0.915 ± 0.009 at pH 5 and 6 respectively. The amount of adsorbed mercury on dry mass of *Pseudomonas fluoresces* BM07 at pH – 7 were about 3% and 58%. There was an increase in Hg^{2+} adsorption per unit weight of biomass with pH – 7 (Noghabi et al., 2007). In *Phanerochaete chrysosporium* the maximum adsorption of Hg(II) ions was obtained at around pH 7.0 were 65, 58 and 52 mg g^{-1}, respectively and the interaction of the mercury species with the fungal biomass could be primarily with the phosphate groups of the cell walls (Necdet Saglam et al., 1999). During the biosorption of Hg(II) by non-living *P. aeruginosa* PU21 was pH dependent and maximum biosorption was obtained at pH 7.4 (Salih et al., 1998). The pH profile of Hg(II) bioaccumulation by recombinant living *E. coli* remained at the same level within a pH range of 3.0–9.0. Their results demonstrate that in contrast to biosorbents or ion exchange resins, the Hg(II) bioaccumulation system was resistant to pH over a broad (Chen and Wilson, 1997).

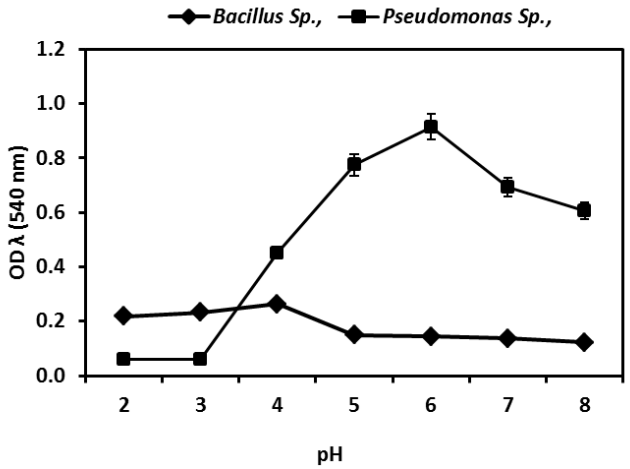

Figure 1: Growth profile of Bacillus and Pseudomonas sp., grown on nutrient broth at different pH at 35º C. Data are means ± standard error (n = 3)

C. GROWTH PROFILE OF THE RESISTANT STRAINS AT DIFFERENT CARBON CONCENTRATION

The effect of different concentration (0.15 – 0.85 g l^{-1}) of carbon source (dextrose) in mineral salt medium along with Hg(II) ions on *Bacillus* Sp., and *Pseudomonas* Sp., is shown in Fig – 2. The OD value for *Bacillus* Sp., increased as the carbon source increased from 0.15 to 0.65 g l^{-1} whereas in *Pseudomonas* Sp., utilized up to 0.15 g $l^{-1}$of dextrose in the mineral salt medium. The mycelium of *Aspergillus nidulans* growth increased as the carbon source with As(III) ions increased from 0.15 to 0.25 g l^{-1}after five days of incubation but decreased at 0.45 g l^{-1} (Maheswari and Murugesan, 2009). *Desulfitobacterium* GBFH couples the oxidation of formate to the reduction of As(V) when formate is supplied as sole carbon source and electron donor. When the isolate was cultured on formate and As(V), the yield of the isolate was lower (OD - 420 ≈ 0.08– 0.01) (Niggermyer et al., 2001). Arsenic (V) reducers, capable of utilizing formate as an electron donor and carbon source for growth, may have a competitive advantage over organisms unable to use this substrate (Ferry and Wolfe, 1976; Gerritse et al., 1999).

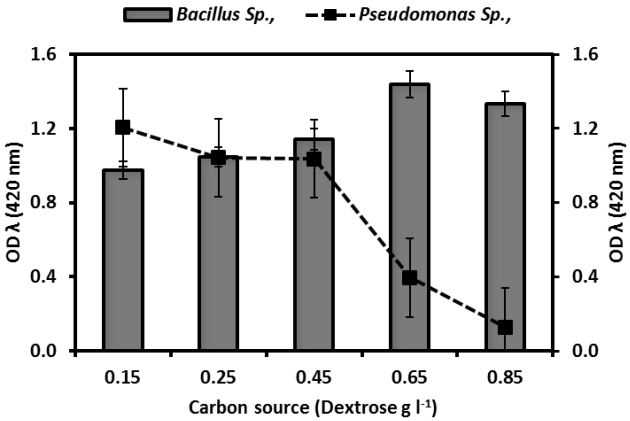

Figure 2: Growth profile of Bacillus and Pseudomonas Sp., grown on mineral salt medium at different carbon concentration for 24 h of 100 mg l^{-1} of mercury ions. Data are means ± standard error (n = 3)

D. GROWTH PROFILE OF THE RESISTANT STRAINS AT DIFFERENT NITROGEN CONCENTRATION

Bacillus Sp, and *Pseudomonas* Sp., uses nitrogen source such as yeast extract (0.25 – 1.05 g l^{-1}) for mercury adsorption is shown in Fig – 3. However in order to obtain high sorption of mercury it is necessary to limit the amount of the micronutrient. The OD value of *Bacillus* sp., at 0.45 g $l^{-1}$of nitrogen source was found to be 1.085 ± 0.04 whereas the OD value for *Pseudomonas* Sp, was 1.095 ± 0.06 at 0.65 g l^{-1}. *Aspergillus nidulans* uses nitrogen source such as yeast extract (0.25–1.05 g l^{-1}) for arsenic sorption. The OD value of the resistant isolate grown on nitrogen source is found to increase from 0.138 to 0.243 g l^{-1} (Maheswari and Murugesan, 2009). Cr(VI)-reducing bacteria may utilize a variety of organic compounds as electron donors for Cr(VI) reduction: the organic compounds are generally limited to natural aliphatics, mainly low molecular weight carbohydrates, amino acids and fatty acids (Cheung and Gu, 2003). The degradation of added organic matter generates a high demand for nitrogen, since the fungi and bacteria that intervene in its transformation have a lower carbon-to-nitrogen ratio than the organic matter they consume. For this reason, organic matter with greater higher nitrogen content is degraded more quickly favoring microbial growth and soil enzymatic activity (Cotrufo et al., 2000).

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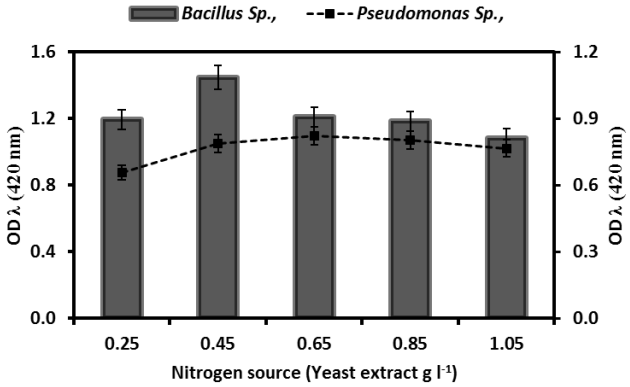

Figure 3: Growth profile of Bacillus and Pseudomonas Sp., grown on mineral salt medium at different nitrogen concentration for 24 h of 100 mg l^{-1} of mercury ions. Data are means ± standard error (n = 3).

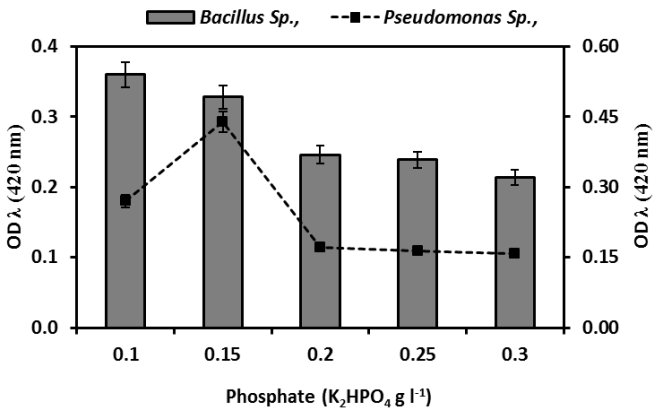

Figure 4: Growth profile of Bacillus and Pseudomonas Sp., grown on mineral salt medium at different phosphate concentration for 24 h of 100 mg l^{-1} of mercury ions. Data are means ± standard error (n = 3)

E. GROWTH PROFILE OF THE RESISTANT STRAINS AT DIFFERENT PHOSPHATE CONCENTRATION

The effect of different concentrations (0.10 – 0.30 g l-1) of phosphate on the mercury resistant bacterial isolates is given in Fig – 4. The OD values of *Bacillus* Sp, and *Pseudomonas* Sp., was found to be 0.540 ± 0.05 at 0.1 g $l^{-1}$and 0.293 ± 0.03 at 0.15 g l^{-1} of K_2HPO_4 (g l^{-1}) respectively after 24 h. The OD value of the two resistant isolates was found to decrease after 0.5 g $l^{-1}$of K_2HPO_4. The mycelium of *Aspergillus nidulans* increased as K_2HPO_4 concentration along with arsenic increased from 0.10 to 0.25 g l^{-1}after five days incubation. The OD value was found to increase up to 0.25 g l^{-1}, and at 0.30 g $l^{-1}$of K_2HPO_4 the OD value decreased (Maheswari and Murugesan, 2009). Rock phosphate altered the stressing effects of metal on *Fusarium oxysporum* with a significant growth improvement at 3 and 6 g l^{-1} rock phosphate, and an increase in amino acids and protein, and a decrease in sugar. Rock phosphate has the highest adsorption affinity for Cd^{2+} (81%) followed by Zn^{2+} (71%) and Mn^{2+} (55%) (Ferry and Wolfe, 1976). The effectiveness of phosphatic clay, a byproduct of the phosphate mining industry, for immobilizing heavy metal (Pb^{2+} , Cd^{2+} and Zn^{2+}) from aqueous solutions. They found that the amounts of metals sorbed onto phosphatic clay decreased in the order Pb^{2+} > Cd^{2+} > Zn^{2+} (Singh et al., 2001). The mechanism of arsenic uptake by the yeast *Candida humicola* was linked to metabolism and the arsenic competed with phosphate in the medium. Soil pH and phosphate addition are the most important factors that control desorption of arsenic (Cox and Alexander, 1973). Rock phosphate significantly reduced Cd^{2+} from soil by about 13 mg kg^{-1} and was effective in decreasing metal uptake by *Pteris vittata*; thus, rock phosphate can be used as an economic amendment for metal-polluted soils (Fayiga and Ma, 2005).

F. EFFECTS OF IONIC STRENGTH ON THE RESISTANT ISOLATES AT DIFFERENT NACL CONCENTRATION

Changes in ionic strength of the medium can cause conformational modification of the functional groups of the bacterial biomasses. It was suspected that these changes would make the reactive functional groups either more or less accessible to mercury ions, there by altering the reduction rate. The reduction rate of Hg(II) ions by *Bacillus* Sp, and *Pseudomonas* Sp, was studied in the ionic strength range 0.10 – 0.30 g l^{-1}NaCl. The increased OD values of *Bacillus* Sp, and *Pseudomonas* Sp, was found to be 0.615 ± 0.02 and 0.770 ± 0.07 at 0.25 and 0.15 g l^{-1} of NaCl respectively (Fig – 5). The reduction of As(III) by *Aspergillus nidulans* was studied in the ionic strength range 0.10– 0.30 g l^{-1}NaCl. The biomass and OD of the resistant isolate increased at 0.15 g l^{-1}NaCl after five days of incubation (Maheswari and Murugesan, 2009). There is not a significant difference in nickel adsorption by zeolite in the presence of NaCl and without NaCl for lower initial concentration 0–200 m^3. The maximum equilibrium capacity is the highest for NaCl dosage 1 kg m^{-3} (195 × g g^{-1}) the lowest for NaCl dosage 1000 kg m^{-3} (100 × 10^{-3} g g^{-1}). The maximum equilibrium capacity for adsorption without NaCl is 156 × 10^{-3} g g^{-1}. The maximum equilibrium capacity is the highest for NaCl dosage 1 kg m^{-3} (195 × g g^{-1}) the lowest for NaCl dosage of 1000 kg m^{-3} (100 × 10^{-3} g g^{-1}). The maximum equilibrium capacity for adsorption without NaCl is 156 × 10^{-3} g g^{-1} (Bakalar et al., 2008). Ammonium sulphate at the concentrations of 0.5 mol l^{-1} and 1 mol l^{-1} was more efficient than other salts for Cr(VI) and Cu(II) removal by dried tea fungal biomass. In the case of Cr(VI) removal the best results were obtained with K_2SO_4 at concentrations of 0.5 mol/l. However, the salt addition (Increasing ionic strength) affected the Cu(II) and Cr(VI) from saline due to the salt effect (Ramovski et al., 2007).

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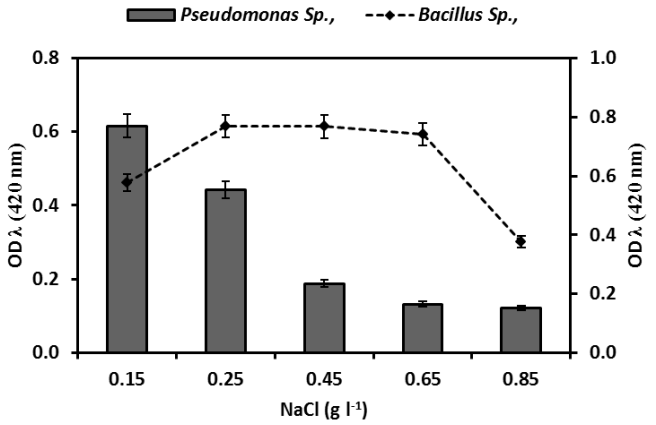

Figure 5: Effect of ionic strength on Bacillus and Pseudomonas Sp., grown on mineral salt medium at different NaCl concentration for 24 h of 100 mg l^{-1} of mercury ions. Data are means ± standard error (n = 3)

G. REMEDIATION OF MERCURY IN SOIL BY MIXED CULTURES

The culture of *Bacillus* Sp, and *Pseudomonas* Sp, in the optimized medium was added to the mercury contaminated soil (500g). The pH of the soil was found to be decreased from 7.34 to 6.54 at the end of 7th day. The cell counts in the soil was increased from 47 to 1654 cells ml^{-1} at the end of 6th day and the cell count decreased after 6 days (Fig – 6). The bacterial biomasses adsorbed mercury from the soil in the presence of dissolved organic matter in the soil. *Aspergillus nidulans* was increased up to 11 days (0.70 g) and the OD values was found to be declined at the end of the 11th day in the optimized media (Maheswari and Murugesan, 2009). At the end of the experiment mercury was significantly lower compared to the beginning. The highest mercury adsorption was found to maximum in the soil at the end of 6th day. It was found that the mercury adsorption in the soil was 60.10% at the end of the incubation period (Fig – 7).

Aspergillus flavus was able to remove 97.50% and 98.73% mercury from shaken and static systems respectively (Kurniati et al., 2014). At a mercury concentration of 10 μg/ml, 89.47% of the mercury was removed by *V. fluvialisa*, whereas *Vibrio parahaemolyticus*, over 40 h of incubation (Jafari et al., 2015). The mercury-removal capacity of *V. fluvialis* was analyzed at four different concentrations (100, 150, 200, and 250 μg/ml) and the efficient bioremediation was observed at a level of 250 μg/ml with the removal of 60% of mercury ions (Kailasam Saranya et al., 2017). The highest arsenic adsorption in the soil by *Aspergillus nidulans* was 84.35% at the end of 11th day (Maheswari and Murugesan, 2009). Soil particle size, organic matter, type and nature of constituent minerals, pH, redox potential and competing ions have all been shown to influence heavy metal adsorption (Chi and Hering, 2000). The maximum uptake by *Spirulina platensis* was found to be 44.56 mg g^{-1} dry weight biomass was obtained at 40 mg L^{-1} of cadmium in aqueous solution (Murugesan et al., 2008).

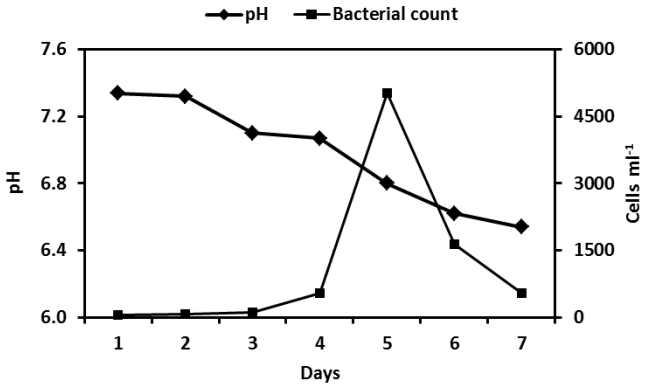

Figure 6: Growth profile and pH of mixed culture (Bacillus and Pseudomonas Sp.,) in mercury contaminated soil

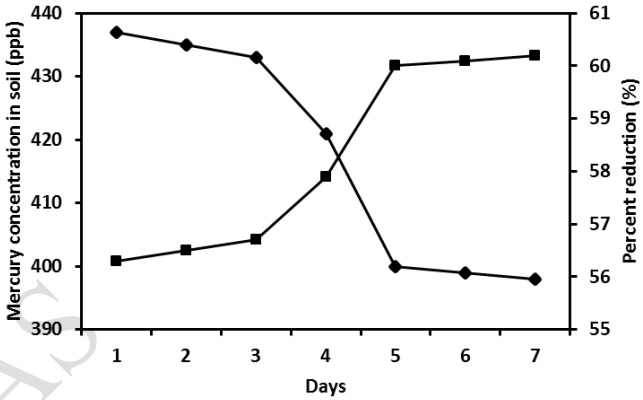

Figure 7: Bioremediation of mercury contaminated soil by mixed culture (Bacillus and Pseudomonas Sp.,) Soil - 1g

IV. CONCLUSION

Results from this study suggest that the *Bacillus* Sp, and *Pseudomonas* Sp, used here is a suitable biosorbent for mercury in soil. Heavy metal removal by adsorbents from soil is strongly influenced by physico-chemical parameters such as ionic strength, pH and the concentration of competing organic and inorganic compounds. The data reported here should be useful for the design and fabrication of an economically viable treatment process for the sorption of mercury from soil.

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