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Bioremediation of Lead by a Halophilic Bacteria *Bacillus pumilus* Isolated from the Mangrove Regions of Karnataka

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Abstract: ***One bacterial isolate Bacillus pumilus out of hundred and twenty-eight screened for tolerance against Pb, Cd, Ba, Cr, Fe, Cu, and F was found to be resistant against all. Hence it was selected up for detail study. The Bacillus sp. could stand up to 900 ppm of Pb and remove nearly 96% of Pb at neutral pH. Subjecting to a change in pH nearly 45% of metal found to be adsorbed on to the cell surface. Rupturing the cell for absorption studies reveals absorption of 412 ppm and 462 ppm at pH 6 and 8 respectively as compared to 296 ppm at neutral pH. The isolate was molecularly identified in our earlier paper and had received an accession no. MF472596. This investigation justifies pH specificity in enhancing biosorption activity of Bacillus sp. towards the metal ion.***

Keywords: Halophilic bacteria, Mangrove, Lead remediation, Adsorption, Absorption

1. Introduction

Pb, Cd, and Hg are toxic to the human body even at their lowest concentration. They cannot be degraded like that of organic materials; rather they persist in environment complicating their remediation, where they are introduced to the food web by the biological systems (1-3). A typical turn over time of Lead is about 220-5000 yrs (5) its historical use as a fuel, additive or pesticide has increased its concentration has upto1000 fold in the environment over the past three centuries (4) and listed it as a top toxic substance. Accumulation of Lead in a humans causes severe deleterious effects such as neurodegenerative impairment, renal failure, reproductive damage and cancer (6-10) for which a concentration of <10µl/L as a safe permissible level in drinking water by WHO is recommended (2).

This Pb mobility may continuously affect both microbial populations and higher organisms, making re-colonization in a contaminated area particularly difficult. Several investigations have shown lead interference with microbial growth, morphology, and biochemical activities, as the metal toxicity damage their DNA, protein, and lipids even by replacing the essential ions within the enzymes (12-14), a declining biomass and diversity have been noted within a lead-contaminated region (15-19).

Microbes have evolved resistant (20,21) mechanisms towards the toxic effect of the metal by extracellular precipitation, exclusion, volatilization, bio-methylation cell surface binding and intracellular sequestration (22,23) and enhanced siderophore production. Several cyanobacterial and bacterial strains like *Synechococcus, Anabouena, Oscillatoria brevis, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas fluorescence, Bacillus megaterium, Salmonella choleraesuis*, and *Proteus penneri* been documented in remediation of Pb (24-27).

The present study is a detail study on Pb remediation carried out with *Bacillus pumilus* (accession no. MF472596) from Karnataka mangrove region, to use it for remediation from any toxic site.

2. Materials and Methods

Atomic Absorption Spectrophotometer, SHIMADZU, AA600, was used for bioremediation analysis. UV Spectrophotometer, Agilent Technology, Carry 60, was used for spectrophotometric studies. Sonicator Probe, Life core, ENUP-500, used for sonicating the bacterial cells. All media are of Hi-media company and all chemical used are of analytical grade.

2.1 Isolate used

Bacillus pumilus **(**accession no. MF472596**)** earlier identified in our previous paper was preserved in glycerol stock in -20°C (72). It was revived in Luria Bertani broth by incubating it at 37°C for 48 hr (28,29). Fresh inoculums were prepared in nutrient broth which was used further in the study.

2.2 Stock Solution Preparation

A stock solution of Lead (1000 ppm) was prepared from its metal salt i.e. $Pb(NO_3)_2$. The glassware used for this purpose were leached in 2N HNO_3 and rinsed several times with distilled water before use to avoid any metal contamination. Two liters of stock solution was prepared in distilled water and slightly acidified with HNO_3 (10 to 20 ml of 2% HNO_3) to prevent precipitation, and was sterilized at 121°C for 15 min.

2.3 Metal tolerance study of the isolate

Various concentrations of lead (i.e. 100-1000 ppm) were prepared in a final volume of 10 ml in Hi-media nutrient broth, to which 1 ml of 24 hr old isolated bacterial cultures

were inoculated at 37°C for 24 h. The tubes were observed for turbidity which was further analyzed by pipetting out 5ml of the sample and analyzing under a UV-spectrum. A loopful of the cultures was streaked onto the nutrient agar plate containing respective metal concentration to check for the viability (65).

2.4 Optimization of growth parameters

2.4.1 Growth characterization

Overnight grown bacterial culture in Luria Bertani medium with 5% salt conc. was used as an inoculum for the analysis of growth pattern. It was inoculated in different Erlenmeyer flasks; each containing 100ml of nutrient broth supplemented with 900ppm of Lead solution incubated at37°C. 5ml of bacterial suspension from each of the flasks was pipetted out after every 4 h and analyzed at 620 nm to monitor the growth pattern fig.4.

2.4.2 Effect of pH on the isolate

Bacillus pumilus was set incubated with varying pH environments (i.e. 2, 4, 6, 8 and 10). 5ml of bacterial suspension was pipetted out after every 4 h and analyzed at 620 nm to monitor the growth pattern and tolerance (fig 5).

2.4.3 Effect of pH on Metal Absorption

To check the pH effect on bio sorption, the biomass of Bacillus pumilus was set incubated at 900 ppm lead conc. with varying pH environments (i.e. 4, 6, 7, 8 and 10). 5ml of bacterial suspension from each of the flasks was pipetted out after the incubation period and analyzed at 620 nm (35) fig.6.

2.5 Optimization of metal uptake by the isolate

2.5.1 Determining the metal remediation by the resistant isolate at pH 7

One milliliter of the aliquot of the isolate was incubated in 100 ml nutrient broth media containing highest tolerating conc. (900ppm) of $Pb(NO_3)_2$ metal ion. The media was adjusted at pH 7 and incubated with the culture at 37°C for 24h. The incubated culture was centrifuged at 5000 × g for 20 min. The supernatants were used for determination of the residual metal ion contents by using AAS. Control culture without inoculation of the bacteria was prepared to detect the initial metal conc.

2.5.2 Absorption of metal by the organisms at pH 7(following cell disruption method)

The absorption of metal in neutral pH at an optimized temperature and incubation period. The cultures were centrifuged at 6500 × g for 20 min. The supernatant was discarded and the pellets were washed with de-ionized water several times. The pellets were first sonicated followed by centrifugation at 10000 xg for 20min. The supernatants were passed through bacterial filters and determined under AAS for metal uptake (30).

2.5.3 Pb adsorption by the resistant bacteria at pH 6 and pH 8

Determining the remediation data of lead by AAS analysis, it was subjected for adsorption studies. The spectrophotometer analysis revealed two tolerating range of pH the metal solution. Following Shetty and Rajkumar method (68) the isolate was cultured on Luria Bertani medium without metal. Cells were harvested by centrifugation at 8000 xg for 10 min., which were then washed twice with de-ionized autoclaved distilled water. The biomass was used for sorption studies. Biosorption experiments were conducted at two different pH (6 and 8) keeping the Pb concentration at 900 ppm. At the end of the experiment, the mixture was centrifuged at 8000 xg for 10 min separating pellet from the supernatant.

The metal-laden pellet was suspended into 5mL of the eluant solution (citric acid (0.1M)) to which metal is slowly is elude out. Remaining concentration of metal in the supernatant along with the eluant was analyzed in AAS by keeping a blank of the metal solution in parallel to avoid confusion between bio-sorption and possible metal precipitation. The metal uptake in mg/g dry wt. was calculated as per Volesky and May-Phillips (56). Metal uptake (mg/g) = V (CI-CF)/w

CI- intial metal conc. (ppm),CF- final metal conc. (ppm), V-volume of reaction(L), w-total biomass (g).

2.5.4 Absorption of metal by the organisms at pH 6 and pH 8. (following cell disruption method)

After eluding the metal from the outer surface of the pellet the biomass was disrupted using sonication technique followed by centrifugation at 10000 xg for 10min. The supernatants were passed through 0.22µm syringe filters before determining under AAS for metal intake (37, 38).

3. Results

Hundred and twenty eight halophilic isolates were tested for multi metal and a non metal tolerance. Bacillus pumilus was found to be potent. It stands up to 900 ppm of lead which was subjected further. The selection is based on the growth of the organism on the plate containing the same conc. of metal, not on spectrophotometer analysis.

3.1 Optimization of growth parameters

3.1.1 Spectro analysis of the *B.pumilus* on lead

At 620 nm the isolated was analyzed and found that *B.pumilus* can tolerate up to 900 ppm of Lead (Fig 3).

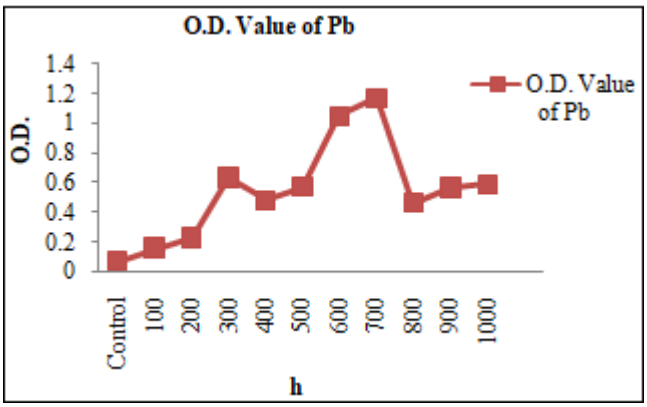

Figure 3: O.D value of isolate in various Pb concentrations

3.1.2 pH and incubation tolerance test

The growth pattern of *B. pumilus* in the presence and absence of Pb has been shown in Fig. 4.

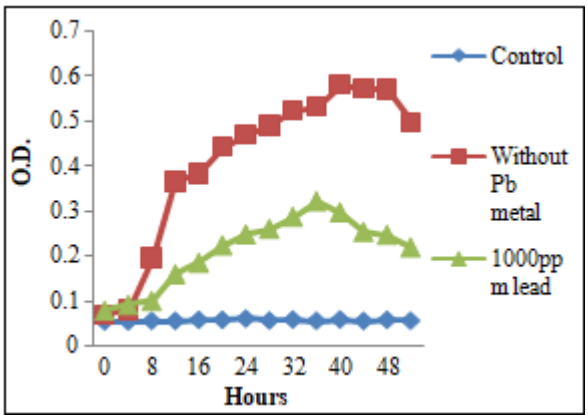

Figure 4: Growth pattern of the isolate.

3.1.3 Effect of pH on the isolate

The growth pattern and tolerance towards various pH by *B. pumilus* been shown in fig. 5.

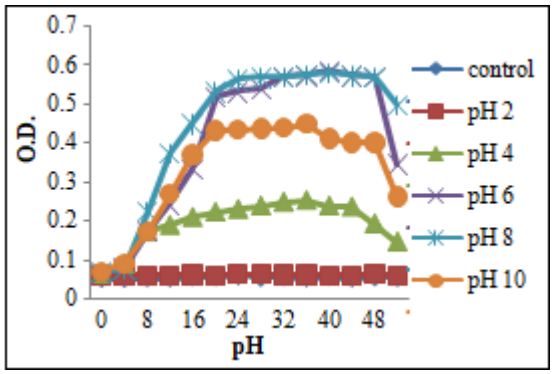

Figure 5: Growth pattern and pH tolerance of *B.pumilus*.

3.1.4 Effect of pH on metal absorbency

From the absorbency value, the isolate tolerance towards lead is suitable at pH 6 and 8 (Fig.5).

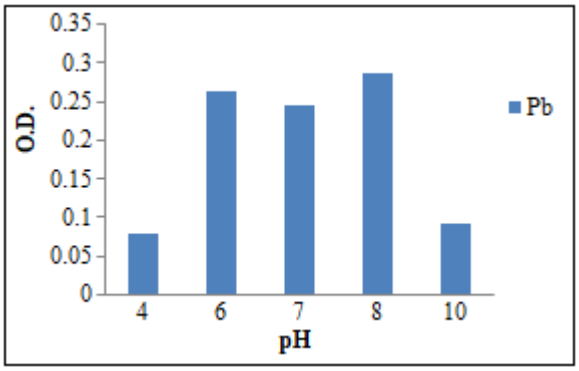

Figure 6: pH and incubation period effect on metal absorbency

3.2 Optimization of metal uptake by the isolate

3.2.1 Remediation of Metal by the isolate (pH 7)

Up to 900 ppm, the isolate had shown tolerance towards Lead. On incubating the isolate with its highest tolerating Pb capacity it has shown a 96.8% reduction when analyzed under AAS. To measure the bio-sorption capability by the isolate the cells were disrupted following sonication technique. The bacterial filtered supernatant was analyzed in AAS and the absorption value by the isolate was found to be 296.18 ppm

3.2.2 Determination of resistant bacteria on Pb adsorption at pH 6 and pH 8

Following the spectrophotometer analysis of the isolate for the tolerance of pH (highest and lowest is considered), it was tested for metal remediation as well as bio-sorption test at the same ppm conc. for Pb. pH 6 is the lowest range for the isolate to tolerate Pb in which the reduction of the metal range was found to be 99.3%. The isolate could tolerate Pb up to pH 8 showing a reduction up to 99.4%, as compared to pH 7 where the reduction was 96.8%. From the above, we can say that pH plays a key role in metal remediation. Subjecting the isolate for adsorption study it was found that at pH 6 the cells could absorb the metal up to 446.8ppm and at pH 8 the cells could do the same up to 447.4 ppm.

3.3.3 Absorption of metal by the organisms at pH 6 and pH 8. (following cell disruption method)

The bacterial pellet was disrupted by sonication technique after adsorption study. The intake of metal by the bacterium in varying pH is, at pH 6 the bacterium could absorb Pb metal to 412.15 ppm whereas in pH 8 and the uptake of metal in this range was found to be 442.55 ppm.

4. Discussion

Bacillus pumilus was found tolerating lead up to 900 ppm and subjected for further studies. Detoxifying mechanisms by the microbes in water with high conc. of heavy metals have been explored in earlier studies (39-41). Incubating the isolated microbe in different conc. of Pb solution reveals the resistivity of microbes. The bacterial strain resists to the heavy metal, shows variation in tolerance even among the isolates found in the same region and the phenomenon of this variation in the resistant mechanism is the cause in the varying intolerance towards different conc. of heavy metal. The BLAST hits of KBORMPorg obtained from 16s rRNA gene sequence indicate its close relation with *Bacillus pumilus species* (accession nos. MF472596).

The isolate showed a profound growth pattern in the absence of metal. The media without metal supplement, the isolate achieved log phase at a much lower time in comparison to the growth in the presence of metal. The growth of the isolate can be seen up to 36-40 hr after which it is found to be in standard phase till 48th hr before touching the decline phase. The highest absorbance value towards lead is more on shifting pH towards alkaline and acidic i.e. pH 8 and pH 6, which are taken as an effective remediation parameter. The evaluation of pH in our work is based on Tehei and Valls conclusion who states the number of cell surface sites available to bind cations, as well as metal speciation, are affected due to pH variation (42, 43). Ajaz and co-workers reported that pH can greatly influence heavy metal removal by microbes (44-51) by influencing the metal speciation and solution chemistry as well as surface properties of bacterial cells.

The selected potent isolate was subjected to three different

parameters for analyzing the Pb remediation under AAS as follows;

1) Remediation of metal by the organisms at pH 7.
2) Determination of resistant bacteria on Pb adsorption at pH 6 and pH 8.
3) Absorption of metal by the organisms at pH6 and pH8 following cell disruption method.

Following Haq *et.al*., AAS analyzing procedure the selected isolate *Bacillus pumilus* was prepared by first subjecting it to its highest tolerating Pb conc. i.e. 900 ppm at pH 7 for 48 hr. The supernatant was removed at the end of the incubation period by centrifugation method and diluted to 1ppm and acidified with HNO3 (52).

The data from the AAS revealed a metal removal of 96% by the isolate, which made clear about the biosorption of the metals by the isolate. The culture pellet was thus collected and rinsed thrice with PBS and lysed by applying sonication with amplitude of 100 for a period of 20 mins with 45 sec interval after every 3-4 mins and acidified with HNO3 and set for AAS analysis. AAS analysis for the sonicated cell for Pb was 296.18 ppm, which clearly confirmed us that not only the isolate has the capability to tolerate the metal and remove them from their respective metal solution but also has the capability of up taking them successfully. The above results corroborate with the work of C.H.Kang *et.al.,* who reported a removal of Pb up to 68% and 54% by *E.cloacae* K-46, KJ-47 from the medium within 72 h (70). Bezverbnaya and Odokuma studied resistant to the heavy metals toxicity by *Bacillus sp.* and *Aeromonas sp.* concluding that the persistence of these isolates in the presence of the respective heavy metals may be as a result of the possession of heavy metal resistant plasmids (53,54). Castillo-Zacarías and co-workers who isolated phenol-resistant bacteria in Monterrey, México from industrial polluted effluents found a Cd^{2+} removal rate of 23 to 78% by *E. Cloacae*, 23 to 64% by *P.aeruginosa* and 24 to 64% by *K. pneumonia* (55). Hanjung Gua and co-worker isolated *Bacillus L14* from *Solanium nigrum* reported a removal of 80.4% of Pb within 24 hrs. 99% Pb removal by members of *Enterobacteriaceae* family isolated from Vembanond lake was studied by M.Sowmya and co-workers (71). Similar results are obtained on *Vibrio harvei* as studied by Abd-Elnaby *et.al*.(56).

Metal ion to the cell surface binding may be due to covalent bonding, electrostatic interaction, Van-der Waals forces, extracellular precipitation, redox interaction or combination among the processes (57). The negatively charged groups on the bacterial cell wall adsorb metal cations, which are then retained by mineral nucleation (58).

Surface activity and kinetic energy of the solute became more efficient in sorption activity with the rise in temperature, which promote the active uptake or attachment of the metals to the cell surface, respectively (59). The heavy metal removal by *B.pumilus* was found to be decreased by increasing temperature above 40°C, these results disagree with the results obtained by Mameri and co-workers (60-62) in our case.

Babich and Jalali found the pH value as one of the main factors in the biosorption efficiency and binding to microorganisms (63,65). We have set a highest and lowest pH tolerating level by the isolate towards the metal. pH 6 is the lowest range for the isolate to tolerate the metal in which the reduction of metal was found to be 99.3%. pH 8 is the highest in which the reduction of metal was found to be 99.4%. On analyzing the sonicated cells under AAS we found that at pH 6 an absorbance of 412.15 ppm was found to that of 442.55 ppm at pH 8. The missing Pb metal was searched for in the adsorption mechanisms by the cell by calculating as per Volesky and May-Phillips the study reveals a data of 446.8 ppm adsorption at pH 6 to that of 447.4 ppm at pH 8. pH variation plays a critical role in the metal remediation from the respective solutions (31). An increase in remediation percentage was noticed in all the cases. The uptake of the metal by the isolate has increased when subjected to a shift towards an alkaline or acidic pH (i.e. pH 6 and 8). Rafael *et.al.,* isolated a *Pseudomonas putida* and found an increase in lead biosorption capacity at pH 6-6.5. Lopez *et.al.* studied multi-metal resistant Pseudomonas Fluorescens 4F39 stating that the affinity series for the bacterial accumulation of metal cations increased on pH variation (36).

A bacterium follows several mechanisms in order to prevent itself from heavy metal toxicity. This can be carried out by accumulating inside them in the form that is non-toxic or expel the metal to outside after converting their chemical composition or blocks the metal entry into the cell.

The present study reveals efficiency of the bacteria in removing more than 95% of lead from the aqueous solution. Further study has revealed that 50% of the removed metal is found on the surface and inside the cell mass. Studying the analysis a probable hypothesis can be drawn out that the remediation occurs by following the metal and cell wall interaction or bio-mineralization. In cell wall the metal must have binded with teichoic and techuronic acid in the peptidoglycan layer which have hold some of the metals on the surface. Whereas the second process is common with halophilic microbes in remediation. The inorganic products synthesised by the microbe reacts with the organic compound forming a organic-inorganic hybrid which leads to a non-toxic product to dwell inside the body.

There is another probability of the organisms to be siderophore. Where the organisms has released the iron to outside due to deficiency of iron in the environment, and the heavy metal rush to take the place of iron.

All the above process are mediated by physical parameters i.e. change in pH and temperature. Our results collided to the mechanisms results, as a slight pH deviation has lead to an enormous absorption and adsorption of metal by the isolate.

The literature is flooded with discussion of gram-negative bacteria tolerance towards heavy metal stress than of gram-positive ones (31). So, this gives us a chance to explore more of gram-positive bacteria in this mangrove vegetation and further studies will be based on finding Pb bio-remediation pathway by *B.pumilus*.

5. Conclusion

The study demonstrates the absorption and adsorption of the lead metal ion by the Bacillus sp. exposed at variable pH. The bioremediation efficiency has increased from 96.8% at pH 7 to >99% at pH 6 and 8. On studying the bio-sorption efficiency of the bacterium reveals that absorption of the metal ion greatly varies than of pH 7 i.e. 296.18 ppm to 412.15 ppm and 462.55ppm at pH 6 and 8 respectively. Whereas the adsorption was 446.86 ppm and 441.42 ppm at pH 6 and 8 respectively was found. Thus, pH plays a key role and stresses the importance of bacteria in an eco-friendly method in mitigating environmental pollution. The mangrove environment can be used to isolate many other microbes, which can be effective in bioremediation potential.

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