International Journal of Science, Environment and Technology, Vol. 9, No 1, 2020, 34 – 47

ISSN 2278-3687 (O)
2277-663X (P)

CHROMIUM BIOREMEDIATION BY A KARNATAKA MANGROVE MICROBE *Bacillus pumilus* MF472596

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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. Only *Bacillus sp.* could stand up against Cr and upto 700 ppm removing nearly 86% of Cr at neutral pH. Chemical analysis was carried out by AAS for rest of the metal ions. Altering the pH to alkaline range the bioremediation efficiency increased to 96%. The metal absorption efficiency increases on altering pH (pH 7 to 8) i.e. from 130 ppm to 210 ppm. Subjecting to a change in pH the metal adsorbed on to the cell surface remains nearly same. The isolate was molecularly identified in our earlier paper and had received an accession no. MF472596. This investigation justifies pH specificity in enhancing bioremediation activity of *Bacillus sp.* towards the metal ion.

Keywords: Halophilic bacteria, Mangrove, Chromium remediation, Adsorption, Absorption.

Introduction

Hexavalent chromium is a human carcinogen and produces a variety of toxic effects (1). The major industrial source of chromium is from chromite ore. Chromium compounds are used for plating, leather tanning, and the manufacture of dyes and pigments, cooking utensils and as wood preservatives. The hexavalent chromium compounds are toxic to the ecosystems, and microbial and plant variants have been found adapting high chromium levels (2). The maximum permissible limit for Cr in water is 0.1 mg/l as per WHO.

Hexavalent chromium is corrosive in nature which can be the cause for chronic ulceration and perforation of the nasal septum. Occupational exposure to chromium can lead to asthma (3). High doses of Cr^{6+} can cause acute renal failure characterized by proteinuria, hematuria, and anuria, but kidney damage from lower-level chronic exposure is equivocal.

Cr^{6+} compounds are more readily absorbed (2–10%) than that of Cr^{3+} compounds (0.5–2%). It easily crosses cell membranes on carriers for sulfate and phosphate. On entering cells, it is reduced intracellularly by ascorbic acid, glutathione, and cysteine, ultimately to Cr^{3+} creating an oxidative stress. It is thought that the toxicity of Cr^{6+} compounds results from damage to

*Received Jan 14, 2020 * Published Feb 2, 2020 * www.ijset.net*

cellular components during this process, generate free radicals and formation of DNA adducts (4).

Various studies have revealed that the microbes must possess a chromium resistant plasmid in order to survive or remediate. A few fungal and bacterial strains such as *Saccharomyces sp., Bacillus sp., Alcaligens sp., Paenibacillus sp., Geobacillus sp., Pseudomonas sp. and Streptomyces sp.* been documented in remediation of chromium.

The present study was carried out with a multi metal tolerant halophilic microbe *Bacillus pumilus* (accession no. MF472596) of Karnataka mangrove region with an objective to use it for remediation of chromium from any toxic site.

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. Molecular analysis was carried out in Trans-Disciplinary University of Health Science and Technology and *Eurofins* genomics India, Bangalore.

Isolate used

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

Stock Solutions Preparation

A stock solution of Chromium (1000 ppm) was prepared from its metal salt i.e. $Na_2Cr_2O_7$. 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.

Metal tolerance study of isolates

Various concentrations of the heavy metal i.e. 100-1000 (mg/L) 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 48 h. The tubes were observed for turbidity which was further analyzed by pipetting out the sample and analyzing under a UV-spectrum. A loopful of the culture was streaked onto the nutrient agar plate containing respective metal

concentration to check for the viability (5).

Optimization of growth parameters:

Salt Tolerance Test

Overnight grown bacterial culture was inoculated in different Erlenmeyer flasks; each containing nutrient broth with different salt concentration (0, 5, 10, 15, 20, 25, 30 and 35 %) for a period of 24 h and streaked on to nutrient agar plates with the same conc. of salts as above. After a period of 24 h, the tubes without visible growth were kept incubated for a period of another 72 h for confirmation (Table 1).

Growth pattern

Overnight grown bacterial culture was inoculated in Erlenmeyer flasks containing 100 ml of nutrient broth supplemented with 700 ppm of metal solutions incubated at 37°C. 5ml of bacterial suspension from the flask was pipetted out after every 4 h and analyzed at 620 nm to monitor the growth pattern (Fig 4).

Effect of pH on the isolate

Bacillus pumilus was set incubated with varying pH environments (i.e.2, 4, 6, 8 and10). 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).

Effect of pH on Metal Absorption

To check the pH effect on bioremediation, the biomass of Bacillus pumilus was set incubated at 700 ppm concentration of chromium 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 (6) (Fig 6).

Optimization of metal uptake by the isolate

Based on the spectrophotometer analysis, the following parameters were chosen for the isolate to be tested under AAS for metal reduction.

1. Remediation of metals by the organisms at pH 7.

One milliliter of the freshly prepared aliquot of the isolate was incubated in 100 ml of nutrient broth media containing the highest tolerating concentration of Cr^{6+} metal ion. The media was adjusted to pH 7 and the cultures were incubated at 37°C for 24 h. The incubated cultures were centrifuged at 6500 × g for 20 min, supernatants were used for the determination of the residual metal ion contents by using AAS (7,8). Controls without inoculation of the bacteria were prepared to detect the initial metal conc.

2. Effect of contact time

Media containing metal solutions adjusted to pH 7 and inoculated with selected isolate was incubated at 37°C for 48 h. The initial and residual conc. of metal within the media was measured as mentioned earlier.

3. Uptake of metal by the organisms at pH 7 (both adsorption and adsorption)

The metal uptake at pH 7 at an optimized temperature and incubation period by the *Bacillus sp*. Following Shetty and Rajkumar method (9) the isolate was cultured on Luria Bertani medium without metal. Cells were harvested by centrifugation at 8000 × g for 10 min., which were then washed twice with de-ionized distilled autoclaved water. The biomass was used for sorption studies.

Biosorption experiments were conducted by keeping the Cr concentration at 700 ppm. At the end of the experiment, the mixture was centrifuged at 8000 × g 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 (10). Metal uptake (mg/g) = V (CI-CF)/w

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

For absorption value the pellet was sonicated at 70 kHz for 15 min at 2 min interval and centrifuged at 10000 × g for 20min. Bacterial free suspensions were ensured by passing the supernatant through a 22µm syringe filter and determined under AAS (7,8).

4. Effect of pH.

As per the spectrophotometer analysis, the optimum pH for remediation by the isolate was analyzed. After 48 h incubation the incubated culture was centrifuged at 6500 × g for 20 min. The supernatants was used for the determination of the residual metal ion content by using AAS (6,7). Control without inoculation of the bacteria was prepared to detect the initial metal conc.

5. Uptake of metal by the organisms at optimized pH (both adsorption and adsorption).

A comparative study was carried out on the uptake of metal at the neutral pH and optimized pH at 37°C for 48 h. Similar method was followed as in step 3 and all analysis was carried out under AAS (6).

Results

Hundred twenty eight halophilic isolates were tested for multi-metal tolerance revealing that chromium is non-tolerable for the rest isolated mangrove microbes. Only *Bacillus pumilus* was found tolerant and selected for further study. The selection is based on the growth of the organism on the plate containing the same conc. of metal, not on spectrophotometer analysis.

Spectrophotometer analysis of the *B.pumilus* on Cr^{6+}.

At 620 nm the isolated was analyzed and found that *B.pumilus* can tolerate up to 700 ppm of the metal (Fig 3).

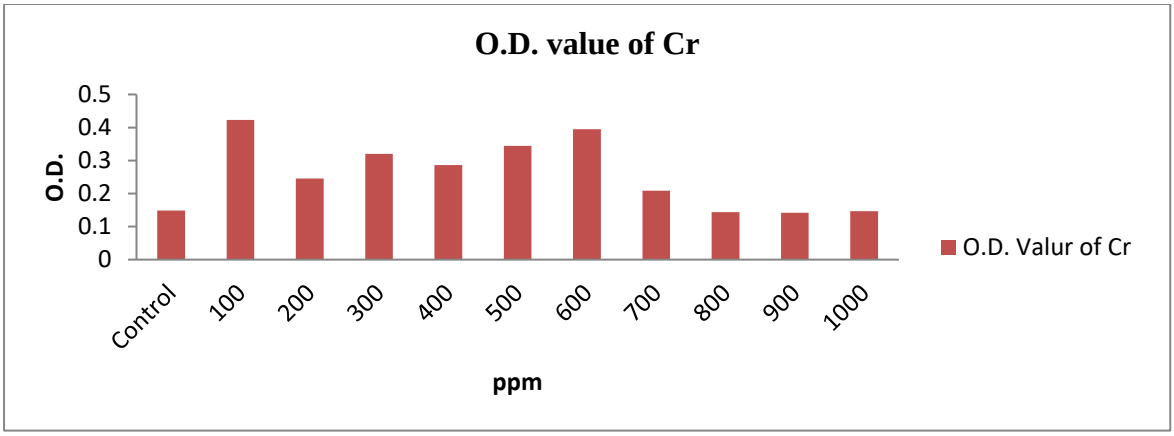

Fig 3. O.D value of isolate in various Cr concentrations.

Growth Parameter.

Salt tolerance test								
Test Isolates	**NaCl Conc. (in %)**							
	0	5	10	15	20	25	30	35
B. pumilus	Nil	+++	+++	+++	+++	++	+	Nil

Table 1: Salt tolerance test.

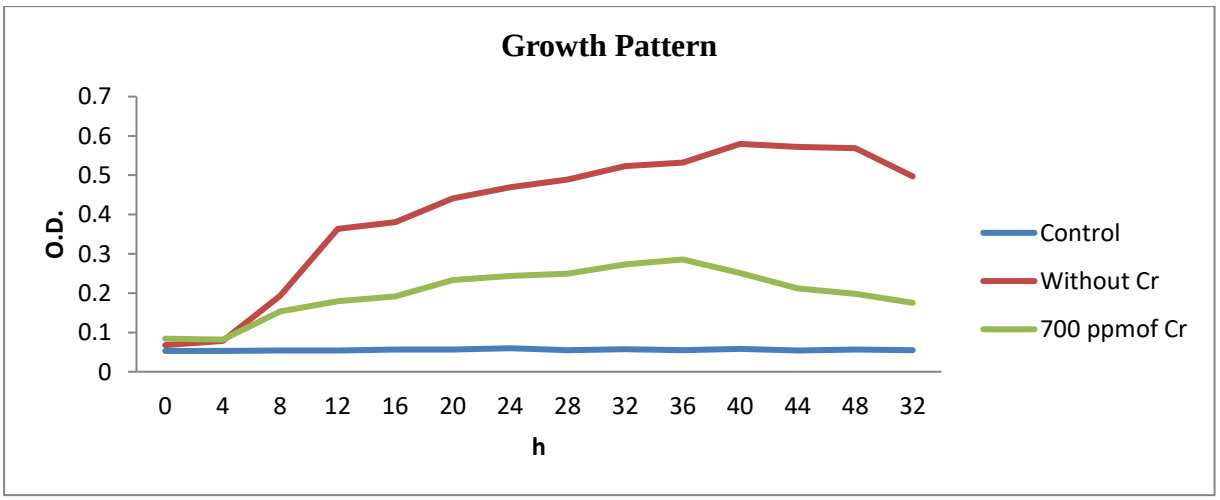

Fig.4 Growth pattern of the isolate.

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

Effect of pH on the isolate

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

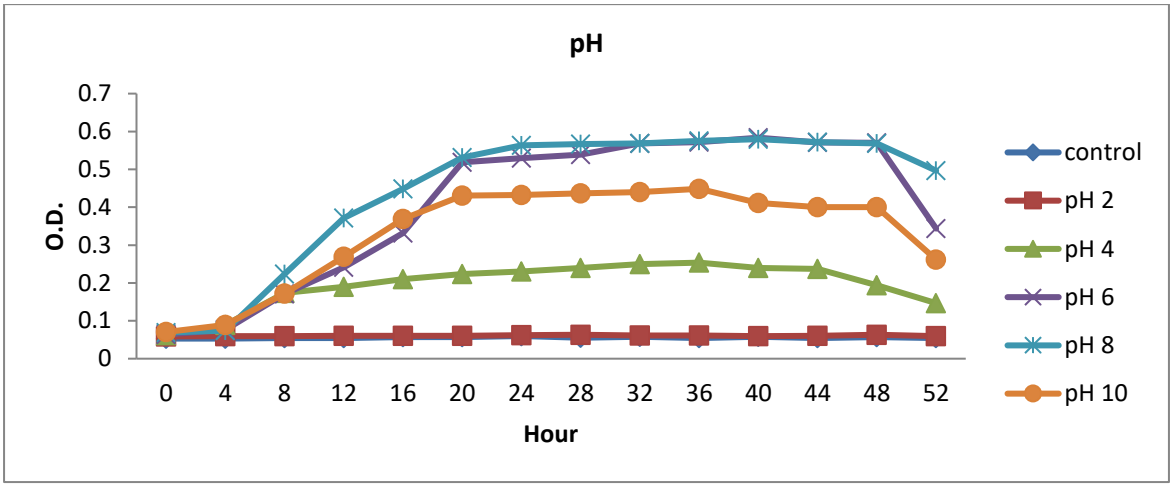

Fig. 5 Growth pattern at various pH.

Effect of pH on metal tolerance.

pH range from 7-10 for the microbe inoculated for 48h is found to be effective in interacting with the metals (Fig.6).

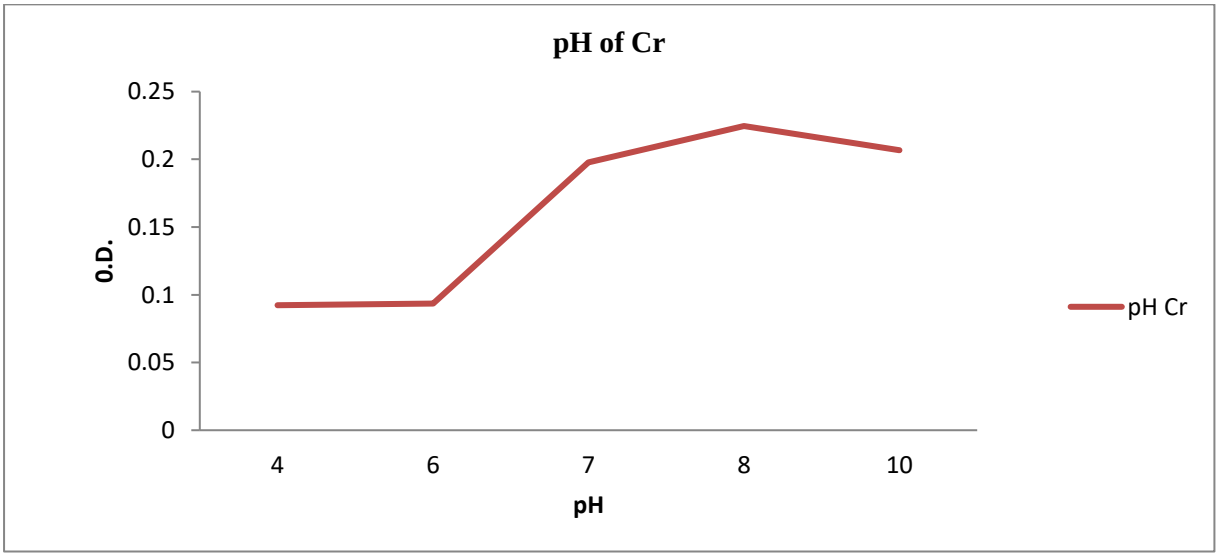

Fig.6 pH effect on metal tolerance.

Optimization of metal uptake by the isolate

1. Remediation of metals by the organisms at pH 7.

Up to 700 ppm, the isolate had shown tolerance towards Chromium. On incubating the isolate with its highest tolerating Cr^{6+} capacity it has shown 86% reduction when analyzed under

AAS.

2. **Effect of contact times.**

At pH 7, the isolate was incubated for a period of 72 h with the highest tolerating conc. of the metal to which the reduction was found to be 87% when analyzed under AAS.

3. **Uptake of metal by the organisms at pH 7 (both adsorption and adsorption)**

The dried pellet was subjected to 700 ppm of Cr. After incubation period the pellet was subjected to both adsorption and absorption studies. The cell pellet was first subjected to eluding buffer followed by sonication, to detect the uptake of the metal by the isolate. Both buffer and supernatant were passed through bacterial filter before AAS analysis. The isolate has adsorbed 550 ppm of Cr metal where as 130 ppm has been absorbed into the cell.

4. **Effect of pH values**

Following the spectrophotometer analysis of the isolate for the tolerance towards pH. pH 8 was taken as an optimum pH which was tested for metal remediation at the same ppm conc. A reduction of 96% of the metal was observed. From the above, we can say that pH plays a key role in metal remediation.

5. **Uptake of metal by the organisms at optimized pH (both adsorption and adsorption).**

The isolate grown in the optimized pH was subjected for metal uptake following the above technique used in step 3. The uptake of metal by the isolate in optimized pH was subjected for comparison with neutral pH. The isolate has adsorbed 570 ppm of Cr metal where as 201 ppm has been absorbed into the cell.

Discussion

Only *Bacillus pumilus* out of 128 isolates was found tolerating chromium, up to 700 ppm which was subjected for further studies. Detoxifying mechanisms by the microbes in water with high conc. of heavy metals have been explored in earlier studies (11,12). Incubating the isolated microbe in different conc. of Cr^{6+} solution reveals the resistivity of microbes. The bacterial strain resists to the heavy metal, shows variation in tolerance at different conc. This variation in the resistant mechanism might be the cause in the varying intolerance towards different conc. of heavy metal. The 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 with metal supplement, the isolate achieved a death phase at a much lower time in comparison to the growth in the absence 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. Below 5% NaCl no growth could be found, luxuriantly growth was observed within a range of 5-15% of NaCl conc. whereas in 20-30% mild growth is seen and 35% onwards no growth has been observed as shown in Table 1. Our study shows similarity with Vreeland *et.al*, isolated *Halomonas elongate* who could grow luxuriantly up to a salt conc. of 32% beyond that, it could not tolerate (41). Optimum pH is shown in fig. 1 which was observed at 620 nm.

The absorbance value towards chromium is more on shifting pH towards alkaline, which is taken as an effective remediation parameter. The evaluation of pH in our work is based on Tehei and Valls conclusion that states the number of cell surface sites available to bind cations, as well as metal speciation, are affected due to pH variation (13,14). Ajaz and co-workers reported that pH can greatly influence heavy metal removal by microbes (15,8) by influencing the metal speciation and solution chemistry as well as surface properties of bacterial cells.

The selected isolate subjected to five different parameters for analyzing the remediation of selected heavy metals under AAS as follows:

1. Remediation of metals by the organisms at pH 7.
2. Effect of contact time
3. Uptake of metal by the organisms at pH 7 (both adsorption and adsorption)
4. Effect of pH.
5. Uptake of metal by the organisms at optimized pH (both adsorption and adsorption).

Following Haq *et.al*., AAS analyzing procedure the selected isolate *Bacillus pumilus* was prepared by first subjecting it to its highest tolerating Cr^{6+}conc. at pH 7 for a period of 48 h. The supernatant was removed at the end of the incubation period by centrifugation method and diluted to 1ppm and acidified with HNO_3 (16).

The data from the AAS revealed a metal removal of 86% 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 dipped in citric acid for elution of metals form the outer membrane to the buffer followed by lysed and acidified with HNO_3 and set for AAS analysis. AAS analysis revealed absorption of about 130 ppm for Cr^{6+} and 550 ppm was found adsorbed to the outer membrane, 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 Hal *et.al*., who reported a 78-86% removal of chromium by *Paenibacillus alvae, B.pumilus, B.Sphaericus and G.sterothermophilus* from the medium. 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 (18,19). A 99% chromium removal by *Pseudomonas sp.* was studied by Roza Maria *et.al.* Lee et al. (1989) indicated that 9% of Cr(VI) was removed in an AS continuous-flow system at which was much lower than the removal (44%) observed by Barth (21) at the same concentration of 15 mg. Lamb and Tollefson (22) reported that up to 35% of Cr(VI) was removed after the addition of 15 mg Cr(VI)/l for a period of 6.5 h in a batch AS reactor.

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 (17,18). The negatively charged groups on the bacterial cell wall adsorb metal cations, which are then retained by mineral nucleation (22). 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 (12). 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 (24,25) in our case.

Babich and Jalali found the pH value as one of the main factors in the biosorption efficiency and binding to microorganisms (26,27). We have set an optimized pH level by the isolate towards the metal. pH 8 is the optimized pH for the isolate to tolerate the metal in which the reduction of metal was found to be 96%. On analyzing the sonicated cells under AAS we found that at pH 8 an absorbance of 570 ppm was found to that of 550 ppm at pH 7. The missing chromium 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 201 ppm adsorption at pH 10 to that of 130 ppm at pH 7. pH variation plays a critical role in the metal remediation from the respective solutions. 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 pH (i.e. pH 8). 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 (28). Rafael *et.al.*, isolated a *Pseudomonas putida* and

found an increase in lead biosorption capacity at pH 6-6.5 (29). Imai and Gloyna (30) demonstrated that 50% of 2 mg Cr(VI)/l added was removed at pH 5 and Cr removal decreased with increasing pH values. Hliher et.al found a complete removal of chromium from the aqueous solution by 5 g/l biomass of *Saccharomyces sp.*at pH 1 in 5 days. On varying the pH (pH 3 and 4), he observed a removal of 62-76% of chromium from the solution (33).

No microbe can treat Chromium pollution unless it can survive in that environment. In order to survive, chromium resistant mechanisms must be present or developed by the microbe. Bopp and coworker states that chromium resistant is a plasmid associated phenomenon (31,32). Due to structural similarity with sulphate, the chromium enters the cells by sulphate transport pathways, but the sulphate uptake is unaltered in presence of chromium ion as studied by Ohtake and Cervantes, identifies a resistant plasmid Chr A gene, which was postulated for outward membrane translocation of chromium anion (34,2). Extracellular reduction of Cr6+ is dominated among bacteria because Cr3+ risking in DNA or protein denaturation. This phenomenon is controlled by detoxifying enzyme as studied by Ramirez et.al (35). As studied by Ackerley YieF dimer, a soluble protein found in cytoplasm may be an acting obligatory chromate reducer (36). The dimer transfer 3 electron to chromate and one to molecular oxygen reducing Cr6+ to Cr3+ in one step unlike Chr R which is another soluble chromate reducer which convert Cr6+ to Cr5+, although Cr5+ is a transient and eventually converted into Cr3+.

Bacteria were found to reduce Cr6+ to Cr3+ at a temperature range of 10-50°C and pH 1-10. The pH and temperature conditions were strongly indicative of the microbial growth which results in Cr6+ reduction. The effect on reduction rate was found following Arrhenius equation. Shen *et.al*., observed that dissolved oxygen is an uncompetitive inhibitor in Cr6+ reduction (37-39). The literature is flooded with discussion of gram-negative bacteria tolerance towards heavy metal stress than of gram-positive ones (40). 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 Cr^{6+} bio-remediation pathway by *B.pumilus*.

Conclusion

The study demonstrates the absorption and adsorption of the chromium metal ion by the *Bacillus sp.* exposed at pH 7 and 8. The bioremediation efficiency has increased from 86% at pH 7 to 96% at pH 8. On studying the bio-sorption efficiency of the bacterium reveals that absorption of the metal ion varies than of pH 7 i.e. 130 ppm to 210 ppm at pH 8. Whereas the

adsorption was 550 ppm and 570 ppm at pH 7 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.

Acknowledgment

The work was funded by RGUHS, grant no. RGU: R&D: Res. Wing 2014-15, dated 13/03/15.-the management of Acharya Institute for the facilities, Dr.P. Mesta, Marine Biologist of IISc field station at Kumta and his team, for their support during fieldwork. Dr. S.P. Balasubramani, Molecular biologist of Trans-Disciplinary University, Bangalore, for helping us in the identification of isolates, the author is greatly indebted to his Mother who was always with him in his efforts. The authors have no conflict of interest to declare.

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