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De-fluoridation by Mangrove Microbe

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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 isolate could stand up against F- upto 1000 ppm. Shifting the pH from 7 to 6, an increase in de-fluoridation was observed i.e. 46% to 64%. The non-metal absorption efficiency increases on altering pH (pH 7 to 6) i.e. from 200 ppm to 286 ppm Further analysis is based at a range of fluoride conc. 0.5-20 ppm at pH 6 and 7, as the available fluoride in most of the contaminated site is <15 ppm. The bioremediation capability was found in between 40-72%. FTIR analysis substantiated the uptake of the fluoride ions by the biomass. The isolate was molecularly identified in our earlier paper and had received an accession no. MF472596. It appears that this is the first study ever on the bioremediation application towards a non-metal by mangrove microbe.***

Keywords: Halophilic bacteria, Mangrove, De-fluoridation

1. Introduction

Trace elements get potentially toxic within a biological system when they exceed the adequate exposure and safe limit. Fluorides are both organic and inorganic compounds consisting of the non-metallic element fluorine. Fluoride is an essential component for normal mineralization of bones and dental enamel, though being the essential microelement for human health; it has created an endemic health issue (1). Most parts of India the groundwater has been contaminated with fluorine (13-22), which are being used for domestic purposes and consumption of such water has lead to chronic diseases like dental and skeletal fluorosis (5). Fluorine rapidly crosses the cell membrane and is distributed in skeletal and cardiac muscle, liver, skin (2), and erythrocytes (3). It enters through the gastrointestinal tract and remains as hydrofluoric acid inside (4). WHO has established guideline for fluoride not to be more than 1.5 mg/l in drinking water but by an estimation, 200 million people across the world rely on fluoride water crossing this maximum limit barrier (8,12).

Brown and co-worker have proposed several mechanisms proposing the cariostatic effect of fluoride (6). Hamilton and co-worker have proposed a direct fluoride mechanism with in microbes where fluoride inhibits the bacteria to produce acid from carbohydrate (7). This might effect on plaque adhesion and development. None of the mechanisms is exclusive. However fluoride effect on bacterial metabolism varies, as they might prove to be insensitive towards the non-metal or adapt or mutate in its presence. Several microbial strains like *Synechococcus lentus, Micrococcus luteus, Aeromonas hydrophila, Micrococcus varians, Pseudomonas aeruginosa, Escherichia coli, Actinomyces sp., Neisseria sp., Streptococcus matins* been documented in remediation of fluoride (24-26).

Despite of its pronounced toxicity in human, a few microbial research been conducted with this non-metal. 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 fluoride from any toxic site.

2. Materials and Methods

Jal tara water testing kit, Akvo caddisfly (23), was used for defluoridation analysis. UV Spectrophotometer, Agilent Technology, Carry 60, was used for spectrophotometric studies. All media are of Hi-media company and all chemical used are of analytical grade.

Isolate used

Bacillus pumilus (accession no. MF472596) earlier identified in our previous paper was preserved in glycerol stock in -20°C (46). It was revived in Luria Bertani broth by incubating it at 37°C for 48 hr. Fresh inoculums were prepared in nutrient broth which was used further in the study.

Stock Solutions Preparation

A stock solution of fluoride (1000 ppm) was prepared by an inorganic compound fluorocid. 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.

F^- tolerance study of isolates

Various concentrations of the non-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 24 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 F^-concentration to check for the viability (33).

Optimization of growth parameters

Growth characterization

Overnight grown bacterial culture was inoculated in Erlenmeyer flasks; each containing 100ml of nutrient broth supplemented with 1000 ppm of Fluorine solution, incubated at 37°C was used as an inoculums for the analysis of growth pattern at varying fluoride conc. 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.2).

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 3).

Effect of pH on non-metal Absorption

To check the pH effect on biosorption, the biomass of Bacillus pumilus was set incubated at 1000 ppm fluoride concentration with varying pH environment (i.e. 4, 6, 7, 8, 10). 5ml of bacterial suspension from each of the flasks was pipetted out after the incubation period and analyzed at 620 nm (27).

Optimization of F^- uptake by the isolate

Based on the spectrophotometer analysis, the following parameters were chosen for the isolate to be tested under Akvo Cassisfly for F^- reduction.

1) Remediation of non-metal 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 NaF. The media was adjusted to pH 7 and the cultures were incubated at 37°C for 48 h. The incubated cultures were centrifuged at 6500 × g for 20 min, supernatants were used for the determination of the residual non-metal ion contents by using Akvo caddisfly (40,41). Controls without inoculation of the bacteria were prepared to detect the initial non-metal conc.

2) Effect of pH values

As per the spectrophotometer analysis, an optimized pH was identified which the isolate could tolerate for the non-metal and observed for bioremediation. After 48h incubation the incubated cultures were centrifuged at 6500 × g for 20 min. The supernatants were used for the determination of the residual non-metal ion contents by using caddisfly (39,40) keeping controls as standard.

3) Effect of pH values on lower concentration of Fluoride

In nature the F- concentration available for domestic purpose is <15 ppm. Various dilutions ranges (0.5, 1, 2, 4, 6, 10, 12, 15, 18 and 20 ppm) were prepared inoculated with microbe and subjected to pH 6 and 7 at 37°C for a period of 48 h. The supernatants were passed through 0.22 µl syringe filter before analyzing in caddisfly.

Instrumental Analysis of Fluoride Uptake by the Bacteria

The characterization of the biomass was performed to depict the spatial orientation of cells before and after treatment of sodium fluoride. The isolate was grown with and without fluoride solution. Using centrifugation biomass was extracted and sonicated separately. The supernatant was set for analysis under FTIR spectra using Bruker Optik GMBH, Tensor 27 to verify the interaction between the fluoride ions and the functional groups present in the bacterial biomass.

3. Result

A multi-metal tolerance *Bacillus pumilus* was tested for fluoride resistance revealing a 1000 ppm tolerance towards the non-metal. The isolate was subjected for further study. The selection is based on the growth of the organism on the plate containing the same conc. of non-metal, not on spectrophotometer analysis.

Spectrophotometer analysis of *B.pumilus* tolerance towards various F^- concentrations

At 620 nm the isolated was analyzed and found that *B.pumilus* can tolerate up to 1000 ppm of Fluoride (Fig. 1).

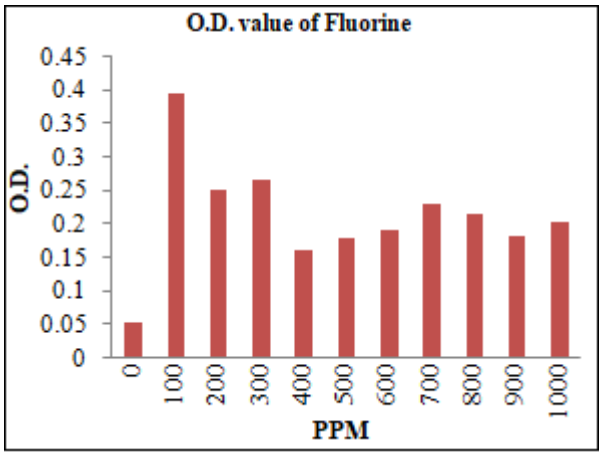

Figure 1: Spectrophotometer analysis of the *B.pumilus* towards Fluoride tolerances

Growth characterization

The growth pattern of *B. pumilus* in the presence and absence of non-metal has been shown in Fig. 2.

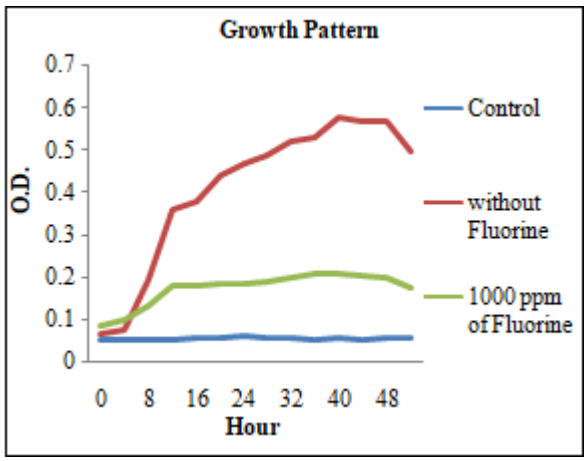

Figure 2: Growth pattern of the isolate

Effect of pH on the isolate

The growth pattern and tolerance towards various pH by *B. pumilus* been shown in Fig. 3.

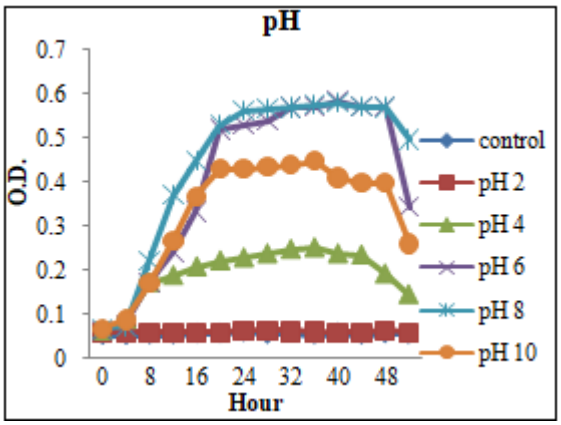

Figure 3: Growth pattern at various pH.

Effect of pH on non-metal tolerance

pH range from 4-7 for the microbe inoculated for 48h is found to be effective in interacting with the non-metal (Fig.4).

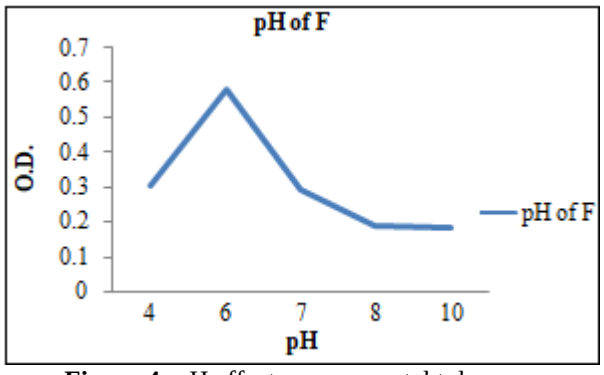

Figure 4: pH effect on non- metal tolerance

Optimization of non-metal uptake by the isolate

Remediation of F^- by the organisms at pH 7

Up to 1000 ppm, the isolate had shown tolerance towards fluoride. Prilimary fluoride detection was done with water testing kit by adding Zirconyl Alizarine. Observation was taken analyzing in fluoride color charts revealing a 40% reduction. As the above method did not give us exact quantity of fluoride within the solution, it was further analyzed in caddisfly using zirconium xylenol orange which reveal a 46% of reduction.

Effect of pH values

Following the spectrophotometer analysis of the isolate for the tolerance towards pH. pH 6 was taken as an optimum pH which was tested for non-metal remediation at the same ppm conc. A reduction of 64% of the non-metal was observed. From the above, we can say that pH plays a key role in remediation.

Effect of pH values on lower concentration of Fluoride

As in nature even in extreme condition, the avail water for domestic purpose do not exceed more than 15 ppm of fluoride conc. So, the isolate remediation capability was tested within a range for 0.5- 20 ppm and a comparision of remediation was done at two different pH (i.e. pH 6 and 7) (Table 1).

Table 1: Remediation study using Claddisfly

PPM	Claddisfly result	
	pH 6	pH 7
0.5	0.2	0.3
1	0.3	0.63
2	0.7	0.9
4	1.32	2
6	1.92	3
10	4	5.3
12	3.36	6
15	8	7.8
18	6.8	10
20	4.4	9

Instrumental Analysis of Fluoride Uptake by the Bacteria

The FTIR spectra of the raw and fluoride-loaded isolate are shown in Fig. 5 and Fig. 6.

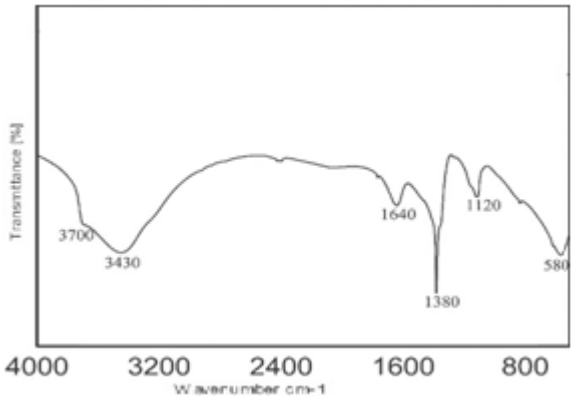

Figure 5: FTIR spectra of raw biomass of *B.pumilus*

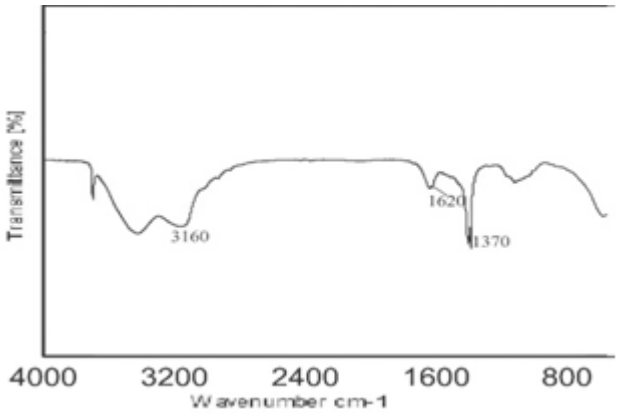

Figure 6: FTIR spectra of fluoride-loaded biomass of *B.pumilus*

4. Discussion

Bacillus pumilus was found tolerating fluoride up to 1000 ppm and subjected for further studies. Detoxifying mechanisms by the microbes in water with high conc. of heavy metals have been explored in our earlier studies (34-36). Incubating the isolated microbe in different conc. of fluoride solution reveals the resistivity of microbe. The gene sequence indicates its close relation with *Bacillus pumilus species* (accession nos. MF472596).

The isolate showed a profound growth pattern in the absence of fluoride. The media without F^- supplement, the isolate

achieved log phase at a much lower time in comparison to the growth in the presence of fluoride. 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 fluoride is more on shifting pH towards acidic i.e. pH 6, 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 binding sites on the cell surface available, as well as metal and non-metal speciation, are affected due to pH variation (41, 42). Ajaz and co-workers reported that pH can greatly influence remediation by microbes (43,44) by influencing the metal / non-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 fluoride remediation under caddisfly as follows:

Remediation of F^- by the organisms at pH 7.
The isolate had shown tolerance towards fluoride and at pH 7 it has shown a 46% reduction when analyzed under caddisfly. The bio-sorption capability by the isolate was measured by disrupting the cells following sonication technique. The bacterial filtered supernatant was analyzed in caddisfly and the absorption value by the isolate was found to be 200 ppm

Effect of pH values
Following the spectrophotometer analysis of the isolate for the tolerance of pH. The isolate at pH 6 shows a reduction of about 64%. The isolate could tolerate F^- only in slight acidic to neutral environment.. From the above, we can say that pH plays a key role in non-metal remediation. Subjecting the isolate for adsorption study it was found that at pH 6 the cells could absorb the non-metal up to 286 ppm. At pH 4 the reduction was slightly less than or equal with pH 7.

Effect of pH values on lower concentration of Fluoride
Following Haq *et.al*., the analyzing procedure the selected isolate *Bacillus pumilus* was prepared by first subjecting it to its highest tolerating fluoride conc. i.e. 1000 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 HNO_3 (45). The data from the caddisfly revealed a non-metal removal of 46% by the isolate, which made clear about the remediation of the non-metal by the isolate. Our results matches with Chouhan et.al., whose isolated microbes *Micrococcus luteus* and *Pseudomonas aeruginosa* showed a reduction of 19% and 22% respectively on incubating for a period of 12 days. A test for 1000ppm was carried out as there are some places like east African rift valley which is situated near an alkaline lake where fluorine level in the water exceeded to 1000 ppm due to combine activity of hypersaline volcanic rocks and geothermal activities. The people nearby this region suffer from skeletal and dental fluorosis.

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 (37). The negatively charged groups on the bacterial cell wall adsorb metal cations, which are then retained by mineral nucleation (38).

Babich and Jalali found the pH value as one of the main factors in the biosorption efficiency and binding to microorganisms (28,29). We have subjected the organisms to set of pH range to determine the fluoride tolerating level. The isolate was found to tolerate fluoride in the range of pH 4-7. At pH 6 the isolate showed luxuriant growth which is taken as an optimized pH and on analyzing the supernatant for fluoride remediation a reduction of 64% was observed. Alkaline condition proved to be negative towards fluoride remediation, our result match with Mukherjee et.al who has used *Staphylococcus leutus* to remediate 85-92% of fluoride form 15ppm solution within 24 h. A decrease in activity was noticed when the pH was shifted towards alkaline (39).

FTIR spectra of raw and fluoride loaded isolate are shown in fig. 5 and 6. A strong band at region of 3700-2900 cm^{-1} represents overlapping peaks of $–NH_2$ and –OH stretching vibration. Peaks at1640 and 1380 cm^{-1} might be due to the existence of N-H bonding in the amino group. C-O stretching of alcohols and carboxylic acids were indicated at band 1120 cm^{-1}. Hence it can be said that the prevalence of hydroxyl, carboxyl amino groups in *B.pumilus* served as attachment sites for F^-. However after fluoride uptake, the peaks of $–NH_2$ shifted from 1640 and 1380 cm-1 to 1620 and 1370 cm^{-1} respectively. This might be indicative of the interaction of the fluoride ions with the bacterium. An additional band at 3160 cm^{-1} only visible in fig. 6 derived from the asymmetrical aliphatic saturated C-H group indicated and intensity reduction of F^-. Compared with standard spectrum, the slight shift in these caused by the superposition with NO_3-peaks.Some weak bonds observed in both adsorbents between 800-520 cm^{-1} indicates the presence of C-C stretching. Thus from the FTIR analysis we can substantiated the fluoride ion has been uptake by the biomass Our results showed similarities with Cherukumilli and co-workers (39, 40).

In general the available fluoride concentration in domestic water in highly contaminated areas is not more than <15 ppm. So, the isolates were exposed to several dilution of fluoride ranging from 0.5-20 ppm at pH 6 and 7. It was observed at the end of incubation period of 48 h that at pH 7 the isolates could remove 40-55% where as at pH 6 the isolate has removed about 60-78% of the non-metal form the solution.

5. Conclusion

The study demonstrates the bioremediation capacity of fluoride by the *Bacillus sp*. exposed at variable pH. The bioremediation efficiency has increased from 40-55% at pH 7 to 60-78% at pH 6. The absorption efficiency increases from 200 ppm to 286 ppm on altering the pH. 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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Author Profile

Divakar Goli has vast experience in Pharmaceutical biotechnology and other fields. He has published over 100 papers, has collaborated with many Pharma industries and coordinating various collaboration projects. Editor of Indian Journal of Pharmaceutical Sciences, the official scientific publication of The Indian Pharmaceutical Association. Invited member of Quality Management and Technical committee of Indian Drugs Manufacturers Association (IDMA) and special invitee to Executive Committee of IDMA-Karnataka Board. Campus Dirctor of Acharya Institutes. Honorary President & Director at Du Laboratories Limited and Iphar Pharmaceuticals Limited.

Sameer Sahoo has experience in Pharmaceutical and Industrial Microbiology. He worked on collection of Mangrove microbial resources, culture condition for scale up, pigmented microbe, Bioremediation of heavy metal and non-metal by bacteria, Microbial fibrinolytic protease; biomass and metabolite enhancement on microbes using ultrasound and Cross microbial Synergistic activity. Co-Author of Pharmaceutical Microbiology book.