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Isolation, identification and molecular characterization of fluoride tolerating bacteria

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Abstract
In India, according to WHO, fluoride content of drinking water in most of the cities is above the permitted level (1.5mg/L) and this high content of fluoride can lead to deformation of teeth and bones. Therefore, there is a need to reduce excessive fluoride from drinking water. The objective of this work was to study fluoride degradation by bacteria isolated from the ground water samples of 05 areas namely Nalgonda, Krishna, Karimnagar, Nacharam and Hitech City. Enrichment and isolation was carried out for all the samples in Brunner's media, BHI and LB media. The optimal *pH* and temperature of the selected bacterial isolates were found to be 7.0 and 37 ^{0}C respectively. Based on biochemical tests and morphological characters, selected bacterial strains were identified as *Pseudomonas* spp. *Pseudomonas* isolates showed fluoride tolerance of up to 100ppm. Isolation of plasmid DNA was carried out by Mini Prep method and it was run on agarose gel electrophoresis. The effect of bacterial isolates (showing presence of plasmid DNA) on fluoride content was checked by inoculating the bacteria in water samples and estimating the initial and residual fluoride content by colorimetric method. It was observed that the water sample treated with *Pseudomonas* has shown decreased levels of fluoride suggesting their ability to degrade and utilize fluoride for their growth. Presence of plasmid DNA suggests probable role in fluoride tolerance which can be further elaborated by plasmid curing, transformation etc. in future studies. Antibiotic sensitivity of the isolates was checked against different antibiotics from which they showed resistance to Ampicillin and Penicillin and sensitivity to Streptomycin. Thus such findings may be useful in designing a novel bacterial strain that can be used to return the altered environment to its original condition.

Keywords: Fluoride, *Pseudomonas,* plasmid, bioremediation, antibiotics

Introduction
Fluorine is the lightest member of the halogen group and is one of the most reactive of all chemical elements. It is not, therefore, found as fluorine in the environment. It is the most electronegative of all the elements which means that it has a strong tendency to acquire a negative charge, and in solution forms F^- ions. [13]. It accounts for about 0.3 g/kg of the Earth's crust and exists in the form of fluorides in a number of minerals, of which fluorspar, cryolite and fluorapatite are the most common. Fluoridation is the addition of fluoride compounds into drinking water, to adjust concentrations to levels between 0.8 and 1.0 mg/L for the beneficial effect of tooth decay prevention. Studies have shown that children drinking fluoridated water can expect to have up to 35% less tooth decay than those drinking non-fluoridated water. According to WHO, the permissible limit of fluoride in drinking water in India is 1.5 mg/L *i.e*. (1.5ppm) [3].
Most of the fluoride found in groundwater is naturally occurring from the breakdown of rocks and soils or weathering and deposition of atmospheric volcanic particles. The main sources of local pollution with fluorine compounds such as HF, SiF4 or H2SiF6 are industrial plants processing apatite and phosphorite, or using cryolite, white clay or fluosite.
Fluoride can also come from:

- Runoff and infiltration of chemical fertilizers in agricultural areas
- Septic and sewage treatment system discharges in communities with fluoridated water supplies
- Liquid waste from industrial sources

A daily intake of around 10-20 mg/day for adults and as low as 3-8 mg/day for children has been found to be harmful. Using these limits,the rough water safety limits of 1 mg/L to 1.5mg/L have been arrived at in the context of India [11].

In regions where natural water contains elevated levels of fluorine (higher than 1.5mg/L), people often develop spotted teeth. Intake of an excessive amount of fluorine (e.g. with water containing more than 5mg/L) can lead to deformation of extremities. Many epidemiological studies of possible adverse effects of the long-term ingestion of fluoride via drinking water have indicated that fluoride primarily produces effects on skeletal tissues (bones and teeth). When drinking water contains 3-6 mg/L fluoride, skeletal fluorosis occurs and over 10m/L Crippling skeletal fluorosis develops. Skeletal fluorosis leads to impairment, disability and subsequently makes the affected subject handicap [4].

Of the 85 million tons of fluoride deposits on the earth's crust, 12 million are found in India [22]. Endemic fluorosis resulting from high fluoride concentration in groundwater is a public health problem in India [14]. The available data suggests that 15 States in India are endemic for fluorosis (fluoride level in drinking water >1.5 mg/L), and about 62 million people in India suffer from dental, skeletal and non-skeletal fluorosis. Out of these; 6 million are children below the age of 14 years [21]. Groundwater is considered as the major source of drinking water in most places on earth.

India was one of the worst fluorosis affected countries, with large number of people suffering. This is because a large number of Indians rely on groundwater for drinking purposes and water at many places is rich in fluoride [12]. The main source of fluoride in groundwater is the rocks which are rich in fluoride. Most of the people affected by high fluoride concentration in groundwater live in the tropical countries where the per capita consumption of water is more because of the prevailing climate [6]. Some regions in north western and southern India are heavily affected with fluorosis [17, 2]. Similarly, the rocks in southern India are rich with fluoride which forms the major reason for fluoride contamination in groundwater [6], and the granites in the district of Nalgonda, Andhra Pradesh contain much higher fluoride than the world average fluoride concentration of 810 mg/kg.

Telangana is second in the list of states associated with drinking water problems as a new Union health ministry data shows close to 1,174 hamlets across the state being affected by fluorosis [15]. The worst hit is Nalgonda district where the fluoride levels are as high as 7mg/L. The fluoride levels in these districts range from 2 to 7 mg/L. People suffer from various skeletal deformities like genu varum, genu valgum, antero posterior bowing of tibia, kyphosis, exostosis etc, and muscular tenderness, neck rigidity, stiffness of joints and mental retardation [1].

A remedy developed by the Government called -The Nalgonda technique showed a major drawback that daily operations require a trained and conscientious operator. In this Technique two chemicals, alum (aluminium sulphate or kalium aluminium sulphate) and lime (calcium oxide) are added to and rapidly mixed with the fluoride contaminated water [9]. The major cause for concern with the lime and alum technology is that if the dose of alum is not adhered to, there is a possibility of excess aluminium contaminating the water. The maximum concentration of aluminium permitted is 0.03 mg to 0.2 mg/L of water according to BIS, as an excess is suspected to cause Alzheimer's disease [16]. The use of bacteria in reduction of fluoride content is preferred since several soil bacteria have the ability to degrade sodium fluoride and also bioremediation does not leave toxic byproducts. In central Australia, seven fluoro acetate degrading bacteria genera including *Acinetobacter, Arthrobacter, Aureobacterium, Bacillus, Pseudomonas, Weeksella, and Streptomyces* were isolated. [20, 22]. Other areas in Telangana affected with high fluoride levels are: Karimnagar, Adilabad, Rangareddy, Warangal, Mahbubnagar and Medak.

Microorganisms are a part of the biosphere that has received little attention in research so far, when it comes to fluoride pollution. Fluoride is very immobile in soil, which can be beneficial for groundwater resources but have a very opposite effect for the microbial community. The aim of this present study was to isolate, identify and characterize fluoride tolerating bacteria that may play a role in bioremediation [18].

Materials and Method

Water Sample

The water samples used for the isolation of fluoride tolerating bacteria were collected from five places- Nalgonda, Krishna, Karimnagar, Nacharam and Hitech city.

Reagents

Standard sodium fluoride solution (2ppm), Alizarin solution (reagent A) and Zirconium oxychloride solution (reagent B) used for Scott Sanchis method were freshly prepared.

Media

For enrichment, shake flask culture technique was used. All the enrichment and isolation media were autoclaved at 121 °C/15psi./20 minutes. For all the five samples enrichment was carried out for three generations using three media *i.e.* Brunner's media, [10] Brain Heart Infusion and Luria Bertani broth for 7 days at 120rpm in the orbital shaker. All the above flasks were incubated at 37 °C at 120rpm (1st enrichment). From every flask, 5ml was re-inoculated to the flask with same medium composition aseptically and further incubated at 37 °C shaker condition of 120rpm (2nd enrichment). Then from every flask a loopful of culture was streaked on sterile Brain heart infusion agar plate and the plates were incubated at 37 °C for 24hrs to get isolated colonies of bacteria. The bacterial colonies were selected based on the differences in colony morphology. The well isolated colonies were grown on sterile Nutrient agar slants as pure cultures and maintained at 10 °C as stock. The strains were sub-cultured once in every 25 days and the purity was checked periodically. The colony characters were identified based on the colony morphology and staining characters. The different characters studied for each bacterial isolate included- size, shape, color, margin, elevation, opacity, consistency, gram character and motility.

Identification of bacterial isolates

The bacterial isolates were subjected to morphological, cultural and biochemical studies which included Gram staining, Motility by Hanging Drop technique. Standard Biochemical tests included Indole, Methyl red, Voges Proskauer and Citrate Test, TSI slant, Catalase and Oxidase tests.

Water Analysis tests

Most Probable Number

The number of coliform bacteria present in the water samples was determined by performing MPN test. Total coliforms can be detected and enumerated in this multiple-

tube fermentation technique. Double strength and single strength Mac Conkey's broth tubes were inoculated with measured amounts of the water sample and results were checked using standard Mac Crady's chart.

Biological Oxygen Demand:
BOD measures the amount of oxygen required or consumed for the microbiological decomposition (oxidation) of organic material in water. High BOD content indicates more pollution. All the five water samples were tested for their BOD levels by performing Wrinkler's method.

Chemical estimation of fluoride
Scott Sanchis method
This method works on the principle of color reduction technique. F^-ions are made to react with Zirconyl-alizarin red S complex which forms more stable, colorless fluorozirconate (ZrF^-_5) anion. In this process Zr ions are displaced from the red dye Zirconyl alizarin complex, whereby its color intensity decreases. The lowering of intensity can be directly related to the concentration of F^- ions. Zr has a greater affinity for F^- than for the dye. Alkalinity Al^-, Cl^-, Fe, Po^-_4, SO^-, may interfere with color development period. This method is directly applicable to samples in the fluoride range 0.05-1.4mg/L.
Standards were prepared in the range 0.2-2.0 mg/L. After adding reagent A and B and incubation for 1 hour, OD was taken at 525nm [8].

Determination of Minimum Inhibitory Concentration
Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation. The MIC was determined by preparing 10-100ppm of standard Sodium Fluoride solution. The tubes were inoculated with the isolates and they were incubated for 24hrs at 37 °C. Positive and negative control tubes were made for interpretation of the results.

Isolation of plasmid DNA and Agarose Gel Electrophoresis
The commonly used method of plasmid DNA isolation is Miniprep method (Alkaline Lysis Method) which involves 3 steps as growth, harvest and lysis of bacteria and isolation of plasmid DNA [5].
Pure cultures of all 14 isolates were grown overnight in 25 ml of Sterile Luria-Bertani broth (Hi-Media) and cell pellet was harvested by centrifugation at 6000rpm/10minutes/160 C. The cells were suspended in 100 µL of ice cold Solution-I (50mM glucose,25mM Tris-Cl,10mM EDTA, pH 8.0); 200 µL of Solution–II (0.2 N NaOH,1%SDS) and150 µL of Solution-III (8M Potassium Acetate). After proper mixing the tubes were kept on ice for 5 minutes ensuring complete suspension of the cell pellet. Equal volume of PCI (Phenol+ Chloroform+ Isoamyl alcohol in the ratio 25:24:1) was added, mixed and centrifuged at 10,000rpm/10minutes/160 C. After centrifugation, the aqueous phase (that contains plasmid DNA) was separated from organic phenol phase. Double volume of ice cold absolute alcohol and 1/10th of the total volume of 3M sodium acetate was added in this aqueous phase and kept at R.T. for 15minutes. For the precipitation of plasmid DNA, the tubes were kept at 40 C for 48 hours. After precipitation step, the tubes were centrifuged at 12,000rpm/20 minutes/160 C. The supernatant was discarded and 500 µL of 70% ethanol was added, mixed properly and centrifuged at 12,000rpm/10minutes/R.T. The supernatant containing ethanol was discarded and the pellet was dried at R.T. to allow complete evaporation of ethanol residue from the pellet, the pellet was further suspended in 20 µL of TE buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris, pH 8.0) and was further analyzed by agarose gel electrophoresis or stored at -20 °C until ready for use.

Fluoride degradation by the Isolates
All five water samples were inoculated with isolates H11 and H12 (showing presence of plasmid DNA) for 4 days in orbital shaker at 37 °C for 120rpm. Estimation of fluoride content before and after incubation was carried out by Scott Sanchis method and the concentration was determined according to the standard fluoride estimation graph. OD was measured at 525nm.

Antibiotic sensitivity Test by Disc Diffusion method
Three bacterial isolates were tested for their sensitivity to different antibiotics by means of Kirby –Bauer Disc diffusion method. The following antibiotics were used: Penicillin (P), Ampicillin (A), Kanamycin (K) and Streptomycin (S).

Results and Discussion
Identification and characterization of fluoride tolerating bacteria
A total of 14 isolates were found, of which 8 isolates were found to be *Pseudomonas spp*. Identification of fluoride tolerating bacteria was carried out by studying morphological, cultural and biochemical characteristics. Results of 02 bacterial isolates used for final fluoride degradation are mentioned in Table 1, and 2.

Most Probable Number
MPN was carried out for all the five samples and the number of coliforms was observed. The MPN index was calculated (Table 3).

Biological Oxygen Demand
BOD was calculated for all the five samples to detect the microbial load in the drinking water samples. (Table 4) All of the samples showed considerably high BOD values, especially Krishna showed maximum levels.

Scott Sanchi's method
Standards of sodium fluoride were prepared in the range of 0.2-2mg/L by following the standard procedure. The results were plotted on a standard graph. (Figure-1)

Isolation of plasmid DNA and Agarose Gel Electrophoresis
Two isolates, H11 and H12, amongst 14 isolates, showed the presence of plasmid DNA on performing Mini Prep method of plasmid DNA extraction. (Figure-2)

Fluoride degradation by the isolates
For studying the effect of bacteria on fluoride degradation, 02 bacterial isolates- H11 and H12(showing presence of plasmid DNA) were inoculated in all five water samples and incubated in orbital shaker at 37 °C for 120rpm/4days. It was seen that H11 showed fluoride degradation in the range of 51-57% whereas H12 degraded fluoride 40-84% from different water samples used. (Table 5 and 6).

Antibiotic sensitivity/Minimum Inhibitory Concentration
Antibiotic sensitivity test was carried out by disc diffusion method. The zone of inhibition was measured in millimeter and the resistance and sensitivity was determined for the selected 02 isolates, H11 and H12, against 4 antibiotics, Penicillin (P), Ampicillin (A), Streptomycin (S), Kanamycin (K). Both the isolates were found to be sensitive to Streptomycin and Kanamycin and resistant to Penicillin and Ampicillin (Table-7) The MIC was determined to check the level of tolerance of the bacterial isolates to the fluoride. It was observed that both *Pseudomonas* isolates could tolerate concentration of fluoride up to 100ppm.

Table 1: Biochemical results of the bacterial isolates

Isolates	Gram Nature	I	MR	VP	Citrate	Urease	Catalse	Oxidase
H11	*Gram negative coccobacilli*	-	-	+	+	-	+	+
H12	*Gram negative coccobacilli*	-	-	+	+	-	+	+

Key: + -positive result; - negative result; I- Indole, VP- Voges Proskauer, MR-Methyl red

Table 2: Triple Sugar Iron result

Isolates	Triple Sugar Iron			
	Butt	Slant	H_2S	Gas
H11	Red	Red	-	-
H12	Red	Red	-	-

Table 3: Most Probable Number (MPN)

Samples	10ml	1ml	0.1ml	Combination of positives	MPNindex for100ml
I	5/5	0/5	1/5	5-0-1	23
II	5/5	3/5	0/5	5-3-0	80
III	4/5	5/5	3/5	4-4-0	34
IV	1/5	0/5	0/5	1-0-0	2
V	5/5	3/5	0/5	5-3-0	80

Table 4: Biological Oxygen Demand

Samples	BOD(mg/L)
I	8.8
II	24
III	3.6
IV	5.4
V	3.4

Table 5: Fluoride degradation values for Isolate H11

Sample No.	Before Inoculation	Conc. of fluoride from std. Graph	After Inoculation	Conc. of fluoride from std. Graph	% degradation
1	0.34	1.7ppm	0.16	0.8ppm	52.95%
2	0.35	1.75ppm	0.16	0.8ppm	54.29%
3	0.32	1.6ppm	0.15	0.75ppm	53.16%
4	0.33	1.65ppm	0.16	0.8ppm	51.52%
5	0.33	1. 65ppm	0.14	0.7ppm	57.58%

Table 6: Fluoride degradation values for Isolate H12

Sample No.	Before Inoculation	Conc. of fluoride from std. Graph	After Inoculation	Conc. of fluoride from std. Graph	% degradation
1	0.34	1.7ppm	0.18	0.9ppm	47.06%
2	0.35	1.75ppm	0.18	0.9ppm	48.58%
3	0.32	1.6ppm	0.19	0.95ppm	40.63%
4	0.33	1.65ppm	0.05	0.25ppm	84.85%
5	0.33	1. 65ppm	0.15	0.75ppm	54.55%

Table 7: Zones of inhibition (mm) observed on performing Antibiotic Sensitivity Test.

Isolates	Antibiotic (30mcg)	Interpretation
H11	K	S
	S	S
	A	R
	P	R
H12	K	S
	S	S
	A	R
	P	R

Key: K-Kanamycin, P-Penicillin, S-Streptomycin, A-Ampicillin.; R-Resistant, I-Intermediate, S- Sensitive

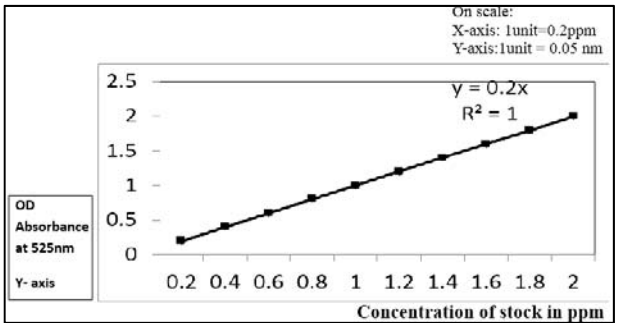
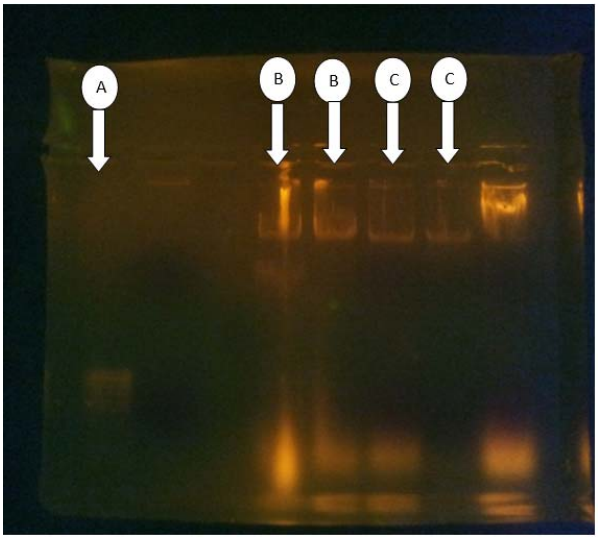


Fig 1: Estimation of Standard Fluoride content by Scott Sanchi's method



Key: Lane A- Ladder, Lane B-Isolate H11, Lane C-H12

Fig 2: Isolation of plasmid DNA and Agarose Gel Electrophoresis

Discussion

In the current study it was observed that a total of 14 bacterial isolates were determined, of which 8 isolates were *Pseudomonas*. 02 isolates (H11 and H12) were able to tolerate high concentration of fluoride levels. Water analysis results by MPN and BOD also showed a significant microbial load contamination. The bacterial isolates also showed the presence of plasmid DNA which could be one of the factors for the high fluoride tolerance levels in drinking water. Chemical estimation using Scott Sanchi's method was carried for estimation of fluoride content of water sample before and after bacterial inoculation. The isolates showed antibiotic resistance to Penicillin and Ampicillin. Thus the results of current study provide a basic tool for biological removal of fluoride from drinking water which can be further exploited at larger scale bioremediation to solve the problems associated with fluoride contamination of water.

Conclusion

According to WHO, the permissible limits for fluoride in drinking water is 1.5mg/L. The fluoride levels in the drinking water samples especially from Krishna district, (Pamuru mandal) are as high as 8.6mg/L [19]. The presence of high fluoride content in the water can be attributed to the prolonged dry spell in that district. High fluoride content in Nalgonda ranged from 0.1 to 8.8 mg/L. Weathering of rocks and evaporation of groundwater are responsible for high fluoride concentration in groundwater of this area apart from anthropogenic activities including irrigation which accelerates weathering of rocks [7]. This study was therefore carried out to remove excess fluoride in drinking water samples with the use of microbes.

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