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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF FLUORIDE RESISTANT BACTERIA: POSSIBLE ROLE IN BIOREMEDIATION

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Microorganisms found in industrial effluents and near the sites of the contamination can be used to indicate pollution and detoxify the contaminated water resources. Emergence of xenobiotic resistant bacteria among them might be potential application in bioremediation. The objective of this study was to isolate and characterize fluoride resistant bacteria from soil and water samples of different regions of India. Five isolates were recovered from different samples which were found to be fluoride resistant. Two of them effectively reduced the fluoride from their media. Through the current study it can be predicted that fluoride pollution results in selective pressure that leads to the development of fluoride resistant among bacterial populations, probably through the mechanism which involved high affinity anion binding compounds called ionophores. Resistant microbes may play a bioremediative role by transforming and concentrating these anions so that they are less available and less dangerous.

Every trace element is potentially toxic when safe and adequate exposure is exceeded. Fluoride (F) ion is protoplasmic poison and a very small amount of this element can be tolerated by any living cell and known to cause several biochemical alterations [1]. In many parts of the world, toxic effects of fluoride are a major public health problem resulting mainly from long-term consumption of water with high F levels [2]. Fluoride concentration in drinking water up to 1 ppm is safe for human body but above this limit is considered deleterious to health [3]. It is found to induce oxidative stress and DNA damage, leading to apoptosis and cell cycle changes in rat oral mucosal cells and hepatocytes [4]. It has been established that therapeutic exposure to fluoride may result in their covalent binding to DNA, which may lead to DNA damage and could be an initial event in the process of chemical carcinogenesis [5]. It is a chemically active ionized element which can affect oxygen metabolism and induce the production of $O_2^{-\bullet}$ free radicals [1, 6].

The problem of fluoride concentration in ground water resources has become one of the most important toxicological and geo-environmental issues in India [7, 8]. Treating fluoride-containing wastewater has been an important issue following the development of the various industries. Various physical and chemical techniques are employed for the decontamination of water. In this context, several methods have been proposed to remove F from aqueous solution. Unlike organic pollutants, the toxicity of fluoride ion is inherent in its atomic structure, and it cannot be further transmuted or mineralized to a totally innocuous form. However, its oxidation state, solubility and association with other inorganic and organic molecules can vary; microbes as well as higher organisms may play a bioremidiative role by transforming and concentrating these anions so that they are less available and less dangerous.

Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic elements. Among the various adaptation mechanisms, metal sorption, mineralization, uptake and accumulation, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form, and efflux of xenobiotics from the cell has been reported [9–13]. These mechanisms are sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another [14]. The detoxifying ability of these resistant microorganisms can be manipulated for bioremediation of toxic elements in wastewater. Microbial species, such as *Pseudomonas aeroginosa*, *Klebsiella pneumoniae, Proteus mirabilis* and *Staphylococcus*, have been shown to be relatively efficient in bioaccumulation of uranium, copper, lead, and other metal ions from polluted effluents [15–17]. The bioremediation of xenobiotics using microorganisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its potential application in industry. Many plants and bacteria secrete high-affinity anion-binding compounds called ionophores. The ionophores bind specific chemical forms of anions [18, 19]. This anion-ionophore complex is

then absorbed back into the organism for utilization. Such organisms may provide the opportunity to make fluoride less available and less dangerous.

The aim of the study was to isolate, identify and characterize fluoride resistant bacteria that may play a role in bioremediation of excessive fluorides from environments.

MATERIALS AND METHODS

Sample collection. Environmental samples of water and soil were collected from different sites of Gwalior and Morena regions of Madhya Pradesh, India. Fifteen water samples (2 well water, 7 tap water and 6 drinking water supply) from in and around the Defence Research and Development Establishment (DRDE), Gwalior and 10 (5 well water and 5 drinking water supply) from different regions of Gwalior and Morena, were collected in 50 ml in pre-sterilized containers. 5 soil samples (3 from the grounds of Railway station, Morena and 2 from grounds of DRDE, Gwalior); 5 g each were collected in pre-sterilized containers.

Isolation and detection of bacteria from samples. The water samples of well/tap/drinking water were allowed to stay at room temperature (RT) for 2 h for the debris to settle down. 0.5 ml supernatant of each of these samples inoculated into 5 ml brain heart infusion (BHI) broth and incubated at 37°C for overnight. 1 g of soil samples in quantity each was inoculated in 10 ml of sterile 0.05 M phosphate buffer saline (PBS), pH 7.4 and incubated at RT for 2 h for the debris to settle down. The rest of the procedure was the same as given for water samples.

Adaptation of cultures on media with sodium fluoride. The samples showing turbidity in BHI broth tubes were cultured (100 μl each) on BHI agar media containing different concentrations of sodium fluoride (from 5 mM to 200 mM) and incubated at 37°C overnight. Different colonies were picked up from agar media with 100 mM sodium fluoride and inoculated each in 5 ml BHI broth with 100 mM sodium fluoride. After overnight incubation at 37°C these broth cultures were further sub-cultured on BHI agar plates with the same concentrations of fluoride and incubated at 37°C for overnight. After 3 to 4 consecutive subcultures on media with 100 mM sodium fluoride cultures were subjected to Gram's staining, biochemical characterization, protein profiling by SDS-PAGE and identification of their bacterial origin by 16s rDNA PCR.

Identification of bacterial isolates. Biochemical characterization of bacteria was carried out according to Bergey's manual [20]. Following tests were performed for characterization of isolates.

Methyl red test. To check the ability of bacteria to perform mixed-acid fermentation we used methyl red test. The test organisms were inoculated in the culture tubes containing sterile glucose (5 g)- phosphate (5 g)-peptone (7 g) mixture prepared in 1.0 l of distilled water. Control tube contained sterile glucose-phosphate-peptone mixture without bacteria. Tubes were inoculated with 100 μl of overnight culture in 5 ml of broth and cultivated at 37°C for overnight. After that 5 drops of methyl red solution were added in each tube and appearance of bright red colour was observed.

Voges–Proskauer (V-P test). V-P test used to detect acetone in a bacterial broth culture. The test organisms were inoculated in the culture tubes containing sterile glucose-phosphate-peptone mixture as described above. Control tube contained sterile mixture without bacteria. Both inoculated and control tubes were incubated at 37°C for overnight. After the incubation was over, 12 drops of V-P reagent and 2–3 drops of reagent V-P 2 were added in each tube. The tubes were agitated gently for 30 s for aeration. The tubes were then kept for 30 min at room temperature. The development of crimson to pink colour in the medium was observed.

Catalase test. Catalase test was performed following the method of Evans and Kloos [21]. One drop of hydrogen peroxide (H_2O_2) solution was placed on the glass slide. Single colony of overnight grown culture was picked up from BHI agar medium and mixed with H_2O_2 drop. Formation of bubbles indicated the presence of catalase.

Oxidase test. Oxidase reagent (1% N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride) was prepared. Filter paper was moistened with this reagent. Overnight grown organisms were smeared over the paper with the help of a glass rod or plastic loop or platinum wire. Change in the colour was observed within 10 s. Formation of purple colour shows the presence of oxidase.

Indol test. The test organisms were inoculated in the culture tubes containing sterile 1% peptone. Control tube contained sterile peptone without bacteria. Both inoculated and control tubes were incubated at 37°C for overnight. After that, 1 ml of Kovac's reagent containing isoamyl alcohol (75 ml), *p*-dimethylaminobenzaldehyde (5 g) and concentrated hydrochloric acid (25 ml) was added gently and after 10–15 min red colored ring in the surface alcohol layer of the broth was appeared.

Nitrate reduction test. In order to determine if bacteria can reduce nitrate, the test organism is inoculated into nitrate reduction broth, an undefined medium that contains large amounts of nitrate. Both inoculated and control tubes were incubated at 37°C for 18–24 h. After incubation reagent A (0.8 g sulfanillic acid and 100 ml 30% acetic acid) and reagent B (500 mg N,N-dimethyl-1-naphthylamine and 100 ml 30%

acetic acid) each of 0.5 ml were added. Then 10 μl of zinc (1.0%) was added and results were recorded. Positive test develops as red color after the addition of sulfanilic acid and N,N-dimethyl-1-naphthylamine which disappears on addition of zinc. Negative test means that no color appers after the addition of the same reagents which turns red on addition of zinc.

Triple sugar iron agar test (TSI). To test the ability of bacteria to ferment sugars and to produce hydrogen sulfide we used TSI test. TSI agar medium (HI Media, India) was used to prepare the slants for the test. The test culture was streaked on it and incubated at 37°C for 24 h. Black color developed along the streak line or throughout the medium indicated H_2S production.

Yellow color appeared in the butt shown acid production due to glucose fermentation. Red /pink color of the butt shown no acid production. Yellow color developed in the slant indicated acid production due to lactose or sucrose fermentation of broth. Pink color of the slant indicated that fermentation of lactose or sucrose did not occur.

Protein profiling. SDS-PAGE was carried out for the protein profiling of all the isolates according to the procedure of Laemmli [22]. Overnight-grown broth cultures of all the isolates (1 ml each) were sedimented by centrifugation at 3000 g for 30 min and suspended in 1.0 ml of sterile distilled water. 1.0 ml of 2× sample buffer was added and boiled for 10 min in a water bath for preparation of protein samples. 10–15 μl of the samples along with standard molecular weight markers were loaded on 10% SDS-PAGE. Electrophoresis was carried out initially at 10 mA till the samples crossed stacking gel and then the current was increased to 15 mA till the dye front reached the bottom of the 10% gel. The gel was removed from the plate and stained using Coomassie brilliant blue R-250 (Sigma, USA) overnight and detained using the destaining solution (acetic acid- methanol- water in the ratio of 10 : 30 : 60) with 2–3 changes till the stained bands were clearly visible.

Amplification of 16Sr DNA. *Chromosomal DNA preparation.* The chromosomal DNA was prepared from 5 ml 24 h- grown cultures of every isolate according to the standard procedure of Maniatis et al. [23]. Briefly, each culture was harvested by centrifugation at 3000 g for 20 min and the pellet was suspended in 1ml of solution I [25 mM Tris Cl buffer (pH 8.0), containing 10 mM EDTA and 50 mM glucose] followed by 2.0 ml of solution II (0.2 M NaOH, 1% SDS) at RT for 5 min. Then equal volume of phenol–chloroform (2 : 1) was added, vortexed and centrifuged at 18000 *g* for 15 min to collect the aqueous layer. After that equal volume of chloroform–isoamyl alcohol (24 : 1) was added twice to remove the proteins. DNA from this aqueous layer was precipitated by $1/10^{th}$ volume of sodium acetate and 2 volumes of absolute alcohol at –20°C overnight or at –70°C for 3 h followed by centrifugation at 18000 *g* for 30 min. The pellet of DNA was suspended in 70% ethanol, air dried and dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1mM EDTA. The purity of the DNA was checked in gel electrophoresis using 0.8 percent agarose and stored at –20°C until use.

Primers and PCR protocol. Primers for universal regions in 16S rRNA gene for confirmation of bacterial identity were obtained from M/S Genetix (New Delhi). The two universal oligonucleotide primers used to amplify the 16S rRNA samples were as follows: forward primer, 5'-CGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3'; and reverse primer, 5'-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3'; annealing temperature was 50°C. An initial denaturation at 95°C for 3 min was followed by denaturation at 95°C for 40 s, annealing at 50°C for 1 min, extension at 72°C for 2 min. This was followed by a final extension at 72°C for 7 min. Product formation was confirmed by 1% (w/v) agarose gel electrophoresis

Measurement of fluoride content in the medium. Isolates were grown in liquid BHI media as described above. Fluoride content was estimated at the day 0, 2, 4, 6, 8, 10 and 12. Cells were harvested by centrifugation at 2500g for 10 min at 4°C. After centrifugation, supernatants were collected and fluoride content was measured by using Orion ion analyzer (Orion, EA, USA). Since only isolates 1 and 4 were showed extra protein bands in protein profiling only these isolates were used for estimation of fluoride uptake whereas isolate no. 5 was used as a control.

RESULTS

Isolation of fluoride resistant bacteria. BHI broth inoculated water samples (well/tap/drinking) and soil samples of Gwalior and Morena regions showed bacterial turbidity after overnight incubation at 37°C. Following subculture in BHI broth/agar plates supplemented with different concentrations of NaF ranging from 5 mM to 200 mM, the growth was observed only with well water or soil samples. Three isolates were recovered from well water sources, 2 from Gwalior region and one from Morena region while 2 isolates were recovered from soil samples collected from Morena region. No growth was observed with tap/drinking water samples. After 3–4 consecutive subcultures in BHI broth/agar plates supplemented with 5 mM to 200 mM NaF these 5 isolates (isolate 1 and 2 from well water, Gwalior, isolate 3 from well water, Morena, and isolate no. 4 and 5 from soil samples of Morena region) could show their growth till 100 mM concentration of

Table 1. Biochemical testing of fluoride resistant isolates

Isolate no.	Methyl red	Voges-Proskauer test	Oxidase	Catalase	Indol	Nitrate reduction test	Triple sugar iron test*	Organism identified
1	–	+	+	+	–	–	K/K	*Micrococcus luteus*
2	–	+	+	+	+	–	K/A	*Aeromonas hydrophila*
3	–	+	+	+	–	+	K/A	*Micrococcus varians*
4	–	+	+	+	–	–	K/K	*Pseudomonas aerugenosa*
5	+	–	–	+	+	–	A/A	*Escherichia coli*

* A/A – Glucose, sucrose, lactose fermentation. K/A – Glucose fermentation, no H_2S production. K/K – No acid, no H_2S production.

NaF after which a decline in the growth of these isolates was observed.

Gram staining and biochemical characterization. Out of the 2 well water isolates of Gwalior region one was found to be gram-positive and the other was found to be gram-negative bacillus. The well water isolate of Morena region was found to be gram-positive and the soil isolates of Morena region were found to be gram-negative cocco bacilli (data not shown). Table 1 depicts results of biochemical testing of all the fluoride resistant isolates. Isolates no. 1, 2, 3, 4 and 5 were identified as *Micrococcus luteus, Aeromonas hydrophila, Micrococcus varians, Pseudomonas aeruginosa* and *Escherichia coli* respectively. Fig. 1 shows the colony of bacterial strains grown on BHI agar in the presence of 100 mM fluoride. Growing colonies are identified as *Micrococcus luteus*, *Micrococcus varians* and *Pseudomonas aerogenosa*.

Protein Profile. To study the protein profile, all the fluoride resistant isolates were grown in BHI broth with or without 100 mM fluoride, overnight at 37°C. The proteins were resolved on to 10% SDS-PAGE. Approximately 25–30 bands could be observed with all the isolates after staining. Isolate no. 1 grown in BHI broth with 100 mM fluoride showed the expression of protein at 30 kDa regions in comparison to its growth in BHI broth without fluoride. No significant difference was observed with the protein profiles of isolates no. 2 and 3 when grown in BHI broth with or without fluoride (Fig. 2). Isolate no.4 revealed the expression of three extra protein bands with molecular weights of 20, 22 and 25 kDa in presence of fluoride (Fig. 3).

PCR identification of universal region of 16srDNA in fluoride resistant bacteria. DNA was extracted from all the fluoride resistant bacterial isolates. Optimum concentrations of $MgCl_2$ and annealing temperatures were worked out on to these DNA for standardization of PCR protocol. Following standardization, optimum amplification of 1.5 kb fragment in DNA samples of all the isolates was obtained with 1.5 mM $MgCl_2$ and annealing temperature of 50°C for 30 cycles. Presence of DNA can be confirmed by the elec-

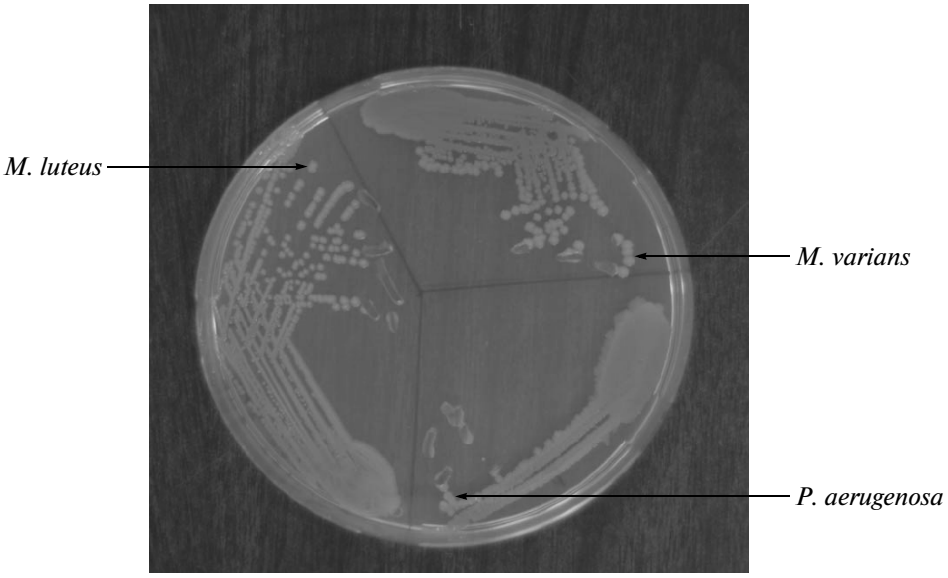

Fig. 1. Growth of fluoride resistant isolates on BHI agar plate with 100 mM NaF.

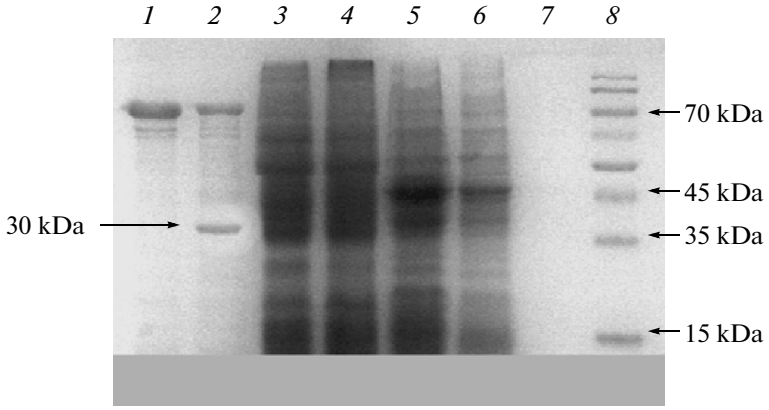

Fig. 2. SDS-PAGE of bacterial isolates grown on the medium with or without fluoride. *1* – isolates no. 1, medium without fluoride; *2* – isolate no. 1, medium with fluoride; *3* – isolate no. 2, medium without fluoride; *4* – isolate no. 2, medium with fluoride; *5* – isolate no. 3, medium without fluoride; *6* – isolate no. 3, medium with fluoride; *7* – control without protein; *8* – molecular weight markers.

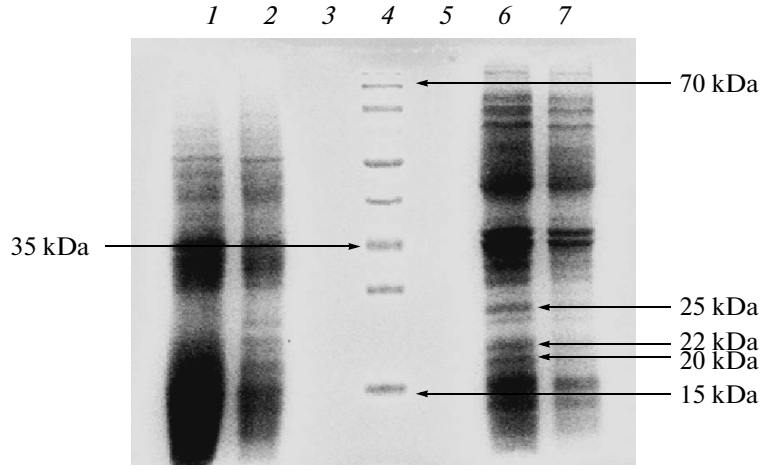

Fig. 3. SDS-PAGE of bacterial isolates grown on the medium with or without fluoride. *1* – isolate no. 5, medium with fluoride; *2* – isolate no. 5, medium without fluoride; *3* – control without protein; *4* – molecular weight markers; *5* – control without protein; *6* – isolate no. 4, medium with fluoride; *7* – isolate no. 4, medium without fluoride.

trophoresis of the PCR product on an agarose gel containing 4 μl of ethidium bromide (2 mg/ml of distilled water). PCR identification for universal regions in 16S rRNA gene of bacterial isolates confirms the bacterial identity of isolates (Fig. 4).

Fluoride reduction by isolates. Since protein profiling of isolates 1 and 4 revealed different patterns when grown with and without fluoride, these two isolates were estimated further for the percentage reduction in the fluoride content. Percentage reduction was determined for 7 alternate days i.e. at the day 0, 2, 4, 6, 8, 10 and 12. As indicated in Table 2 the percentage reduction after day 12 through isolate no.1 and 4 were found to be 19.8% and 22.1% respectively whereas iso-

Table 2. Reduction of fluoride by isolates no. 1, 4 and 5

Isolates no.	Reduction in fluoride content (%) after days (0–12)						
	0	2	4	6	8	10	12
1	0.0	0.4	3.4	5.1	10.4	12.8	19.8
4	0.0	1.8	6.1	7.8	13.2	18.3	22.1
5	0.0	0.0	0.5	0.8	1.1	1.4	1.4

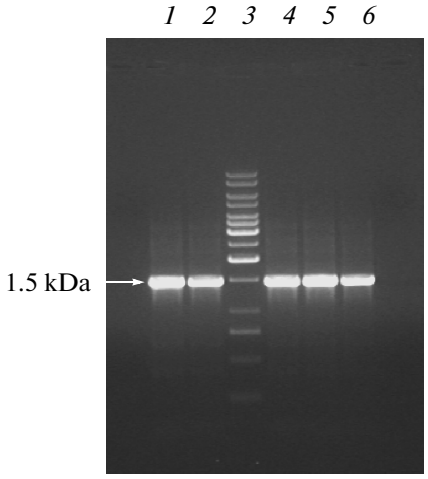

Fig. 4. PCR-amplication of 1.5 kb fragment of 16S rDNA from different bacterial isolates. *1* – isolate no. 1; *2* – isolate no. 2; *3* – 1 kb DNA ladder; *4* – isolate no. 3; *5* – isolate no. 4; *6* – isolate no. 5.

late 5 did not show any difference in the percentage reduction of the fluoride content.

DISCUSSION

Xenobiotics exert their toxic effects on microorganisms through various mechanisms. Tolerant bacteria could survive in these habitats and could be isolated and selected for their potential application in the bioremediation of contaminated sites [24, 25]. In the present study, 5 bacterial isolates which were found to be fluoride resistant were isolated from water and soil samples. The isolates then further identified and characterized by biochemical properties. All the isolates were found to be gram-negative. They were tested for their capability to accumulate fluoride and found to significantly reduce the fluoride content from the media.

Fluoride compounds have been used for many years as additives in toothpaste, mouthwash, and drinking water in order to reduce the incidence of caries. Once entered into the human system fluoride readily gets absorbed through gastrointestinal tract or lungs, based on the route of exposure. Excess to fluoride exposure leads to a disease known as fluorosis. The incidences of fluorosis being recorded at regular intervals in our country imply that the problem of endemic fluorosis is highly pertinent in India and other developing countries. Abnormal levels of fluoride in drinking water and prevalence of hydrofluorosis have been reported in several regions of India [26, 27].

Several methods have been adapted for defluoridation of water. The removal of excessive fluorides from water is usually been accomplished by means of activated clay [28], magnisite [29] and activated carbon from coconut shell impregnated with aluminum ions [30]. However, these existing technologies have the problem of leachates that might alter the water quality, making it unsuitable for drinking process. These methods could not find any practical application because of high capital and operating cost and complexity of operating procedure. Moreover conventional methods consume high amounts of energy and large quantities of chemical reagents. Bioremediation of toxic xenobiotics has advantageous over other techniques as it is cheap, non destructive and contamination remains localized [31]. It is well recognized that microorganisms have a high affinity for metals and non metals by variety of mechanisms [32, 33]. Since microorganisms have high surface area to volume ratio because of their small size therefore they provide a large contact area that can interact with elements in the surrounding environment [34]. These bacteria are capable of accumulation of xenobiotics and exist in soil and near the site of contamination have adapting mechanisms to the contaminant and such adaptation might allow strains to achieve dominance in a localized habitat. Researches show that several plaque populations like *Actinomyces, Neisseria spp*. are capable of initial growth in fluoride [35] Strains of *Streptococcus matins* is capable of *in vitro* growth at levels up to 1.0 mg F/ml. Bacterial cell have the unique property of concentrating fluoride within their cells [36, 37].

Fluoride is more likely to exist with the combination with proton (HF), which can readily cross cell membrane and acts similarly to a proton conductor. Keeping this in mind, microorganisms with the ability to tolerate and reduce fluoride can be employed for the detoxification of environments contaminated with fluoride.

Hence, concerted efforts should be made to develop bioremediation method using microorganisms. Microbial remediation of fluoride may prove to be effective and need attention as it requires no chemicals and microbes are native to the contaminated water. Moreover there is no study undergoing with bioremediation of fluoride contaminant. The present study was undertaken to isolate, identify and characterize microorganisms from environmental samples of water and soil that can to grow in presence of fluoride. Five bacterial strains, 3 from well water supplies and 2 from soil samples collected from Gwalior or Morena regions of India were recovered. These isolates were grown in BHI broth/agar media with or without different concentrations of sodium fluoride ranging from 5–200 mM. Stable resistance to 100 mM fluoride has been demonstrated by all these isolates after three to four consecutive subcultures.

Presumptive identification of these isolates was done by Gram staining and biochemical tests (Table 1). Isolates no.1 and 2 obtained from well water source of Gwalior region were identified as *Micrococcus luteus* and *Aeromonas hydrophyla* respectively. The isolates no. 3 and 4 obtained from well water source of Morena region were identified, as *Micrococcus varians* and *Pseudomonas aerugenosa* respectively. The isolate no. 5 obtained from soil sample collected from Morena region was identified as *E. coli*. 16srDNA PCR employing universal primers further confirmed the simi-

larity of these isolates. The DNA extracted from all these isolates could amplify 1.5 kb universal region of prokaryotic 16S rRNA gene (Fig. 4). A molecular approach based on 16S rDNA is useful in detecting bacterial community structure changes, because these genes are conserved and present in all bacteria [38]. To check out the pattern of protein expression in the present study, the isolates recovered from well water and soil samples of both Gwalior and Morena regions were grown on media supplemented with 100 mM fluoride and proteins were resolved on 10% SDS-PAGE. Staining revealed the appearance of extra protein band at 30 kDa regions in isolate no. 1 grown in media with fluoride in comparison to its growth in media without fluoride. Similarly the isolate no. 4 revealed the expression of three extra protein bands with molecular weights of 20, 22 and 25 kDa when grown in presence of fluoride as compared to its growth in media without fluoride (Fig. 3). In these isolates extra protein bands expressed in response to fluoride may act as ionophores. Many plants and bacteria secrete high-affinity anion-binding compounds called ionophores that can bind specific chemical forms of anions from their environment [18, 19]. One of the important finding of the present study was that both the isolates recovered were able to reduce the fluoride concentration up to a significant level. Reduction was found to be up to 19.8% and 22.1% after 12 days through isolate no. 1 and 4 respectively. Isolate 5 which was used as control did not show any significant change in the fluoride reduction. These results support the hypothesis that the isolates expressing ionophores within the cytosol are found to be efficient in removing fluoride from the media.

The results conclusively suggest that these bacteria actively reduce fluoride contamination that may provide opportunity to develop a new bioremediation technique for extracting excess of fluoride in a simple way. These organisms may play a bio-remedial role by transforming and concentrating the fluoride anions from the environments so that they are less available and less toxic. However, the present study deals in the restricted domain of identifying the fluoride concentrating microbes and not on the development of bioremedial system for the purpose. Thus we further recommend that after fluoride accumulation, removal of these cultures must be an integral part of bio-remedial process for reducing fluoride burden of water-bodies.

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