Isolation, Identification and Characterization of Fluoride Resistant Bacteria: Possible Role in Bioremediation

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Abstract — 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.

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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\(_2^\cdot\) 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 bioremediative 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 aeruginosa*, *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
secretes 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 grounds of DRDE, Gwalior and 2 from grounds of Railway 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 (H₂O₂) solution was placed on the glass slide. Single colony of overnight grown culture was picked up from BHI agar medium and mixed with H₂O₂ 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-naphthyamine 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