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Isolation, characterization and identification of fluoride tolerant bacterium *Escherichia sps.* strain FRB-1

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Abstract

The toxic impacts of high doses of fluoride have been witnessed on microbial community in form of reduction of number and diversity, which in-turn influence the soil fertility. Such ecological systems generally harbor microbes with varying mechanisms of fluoride tolerance. The present study focuses on the isolation, characterization and identification of fluoride tolerant bacterial isolate FRB1 isolated from soil of Bharatpur district, Rajasthan, India. The soil was characterized for fluoride content and other physicochemical characteristics as pH, electrical conductivity, organic carbon and organic matter, chloride, water holding capacity and available phosphorus. The growth reduction study on FRB 1 with increasing fluoride stress was used to determine the minimum inhibitory concentration (3000ppm). The stain FRB-1 as identified by 16SrDNA sequencing followed by alignment of the amplified sequence with nrdatabase of Genbank through BLASTn analysis showed 97% homology with *Escherichia sps.* The bacterium was further characterized for staining *and* biochemical characteristics. The high fluoride stress tolerance assures its survival as well as preponderance in stressed soil systems indicating their possible application in natural or artificial remediation of toxicant exposed degraded soil systems.

Keywords: Fluoride tolerance, soil fertility, aquifers, *Escherichia*, ground water

Introduction

Fluoride is one of the most abundantly present toxicant in natural waters. The increased doses of fluoride in ground water has been witnessed around the globe. Due to the shortage of clean surface water the deep underground waters are harnessed to provide sufficient water for various purposes. The aquifers in fluoride belt contain dissolved fluoride which brings excessive fluoride on surface soil and water (Chowdhury *et al.*, 2019)**.**

The anti-microbial potential of fluoride pose a serious toxic effect on natural soil microbiota (Van Loveren, 2001). This decline rapidly subjects the soil to degradation making it unsuitable for agriculture in due course of time (Chatterjee *et al.*, 2020). Such soil systems can be restored by microorganisms which can tolerate toxic stress and provide rapid mineralization of soil organic matter and other contaminants (Mukherjee, Sahu and Halder, 2017; Pal *et al.*, 2022). Thus the fluoride content is considered an important factor in shaping of the microbial community (Zhang *et al.*, 2019).

The restoration of such degraded soil systems involve an interplay of soil microbial metabolism with mineralization or cycling of contaminants. The soil with poor microflora does not show a progressive improvement of soil quality indicators due to poor mineralization of complex organic components and restricted biogeochemical cycling of other elements. The restoration of such soil samples is possible with the growth and preponderance of bacteria which can survive in high concentrations of toxicants.

The fluoride endemic areas provide long term exposure of toxicant concentration generally induces the development of tolerance mechanisms (Chellaiah *et al.,*2021; Pal *et al.*, 2022). Therefore, in the present study the isolation of fluoride resistant bacterium was performed from fluoride stressed sites of Bharatpur

District Rajasthan, India. The groundwater contamination of fluoride in Bharatpur district has been witnessed in the hydrogeological report of Bharatpur district (2013) where seven out of nine blocks were reported to contain > 3mg/L (high concentration) and two block with 1.5-3mg/L (moderately high) fluoride in ground water.

Material and Methods

Soil sample collection

Soil samples were collected from different areas in Keoladeo, Ghana National Park, Bharatpur in sterile polypropylene zip lock bags. These samples were stored in refrigerator 4˚ C before analysis and subjected to bacterial isolation within 24 hours.

Soil Characterization

The soil samples were characterized for pH, Electrical conductivity, organic carbon, organic matter, available phosphorus, chloride content, water holding capacity, calcium (Ca^{2+}), magnesium (Mg^{2+}), available phosphorus and fluoride content using standard protocols (Maiti, 2004, APHA 2005)

Isolation of Fluoride tolerant bacteria

Soil dilutions (10^5-10^{10}) were inoculated on sterile nutrient agar plates (Himedia) spiked with 500ppm of sodium fluoride (NaF; Himedia) and incubated for 1-3 days at 35˚ C in incubator (Remi). The pure colonies of 500ppm tolerant bacteria were isolated by repeated quadrant streaking on nutrient agar plates.

Determination of Minimum Inhibitory concentration (MIC)

The pure bacterial isolate was subjected to increasing doses of NaF (500-3000ppm) in Nutrient broth (pH-7.4) at 35˚ C with shaking (100rpm) in shaker incubator (Remi) for a period of 24-96h. The growth at each dose was measured as optical density after each 24h of incubation using UV-Vis spectrophotometer (Systronics) to estimate the MIC dose which can inhibit the bacterial growth completely.

Identification of Fluoride tolerant bacteria-

The pure DNA of strain FRB-1was obtained by phenol-chloroform method and subjected to 16S rDNA partial sequencing (outsourced-Bioaxis DNA Research Centre Pvt.Ltd, Andhra Pradesh, India). The genomic DNA 16S rDNA sequence was PCR amplified using universal primers(forward primer-5’ AGA GTT TGA TCC TGG CTC AG 3';reverse primer-5' ACG GCT ACC TTG TTA CGA CTT 3') (Chellaiah *et al.,* 2021,Maniatis*et al.,* 1989, Sacchi*et al.*, 2002).

The PCR product was purified as single band on agarose gel and used for DNA sequencing. BLASTn analysis of 16S rDNA sequence with nrdatabase of NCBI genbank provided the similarity match (top 10 matches are shown in table 2) with Distance matrix tree.

The sequencing result of the strain was supported by biochemical characterization from Bergey’s Manual of Determinative Bacteriology (Holt *et al.,* 1993) using the microbiology laboratory manual Cappuccino and Sherman, 2002.

Results and Discussion

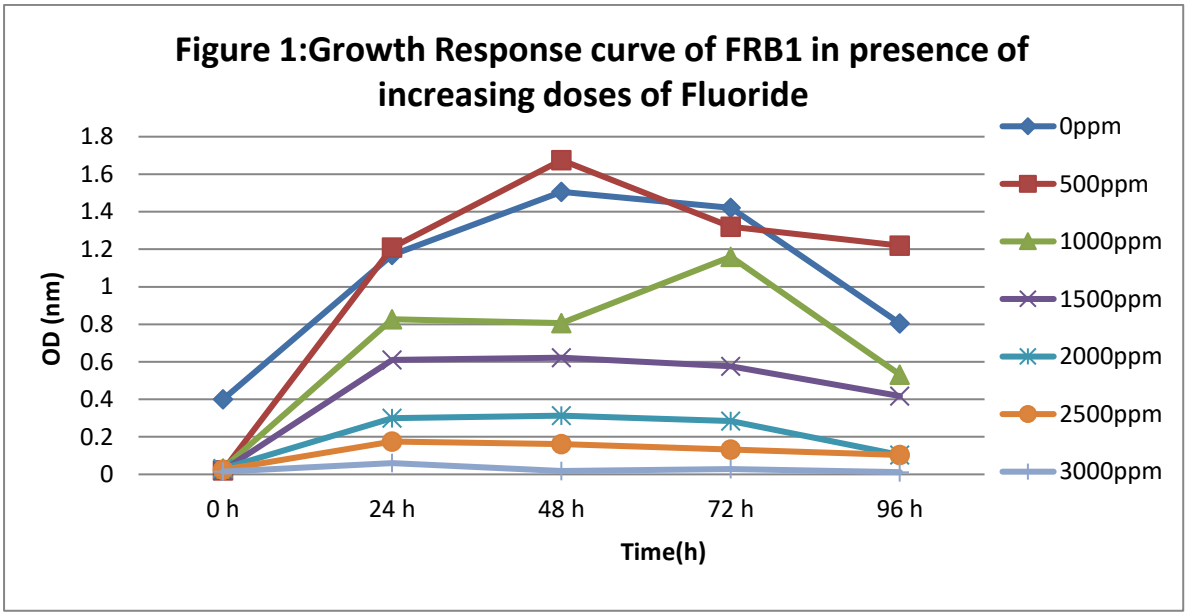
Soil Sample characterization

The physicochemical characteristics of soil sample are summarized in table 1.

Table no-1.Physicochemical characteristics of FRB-1 isolation source			
Parameters	**Result**	**Parameters**	**Results**
pH	7.38-7.55	Ca^{2+} Present in the Soil (ppm of soil)	406.81 ± 9.8
EC (mS)	1.2-2.58	Mg^{2+} Present in the Soil (ppm of soil)	254.11 ± 8.33
Organic Carbon (%)	0.721 ± 0.018	Water holding capacity	65%
Organic Matter (%)	1.24± 0.033	Fluoride Content (mg/g of soil)	43.8
Chloride (mg/l)	55.17±2.4	Available phosphorus (kg/hact)	25.646±2.44

Isolation of Fluoride tolerant bacteria and determination of MIC

The bacterium FRB -1 formed white, opaque, round colonies with smooth margin. This isolate was able to grow in presence of 2500ppm of sodium fluoride (Figure 1). In the four-day growth measurement study (optical density) an initial increase in growth followed by a plateau period and decline fluoride tolerant bacterium was observed at all doses (500 ppm to 3000ppm). As no growth was observed at 3000 ppm, this dose was considered as MIC.



Identification and characterization of bacterium FRB -1

The 16 S rDNA sequence alignment of FRB-1 with nrdatabase of NCBI indicates closest similarity with genus *Escherichia.* The top 10 similarity sequence alignment are shown in table 3. The distance matrix tree which provides an idea about the evolutionary relationship of the isolate with genetically close organisms is shown in figure 2. The staining and biochemical tests of bacterium FRB-1(table 2) support the similarity with the genus *Escherichia* with minor variation. Thus, on the basis of nucleotide homology and biochemical characteristics the bacterial isolate FRB-1 was designated as *Escherichia sps.* strain FRB-1.

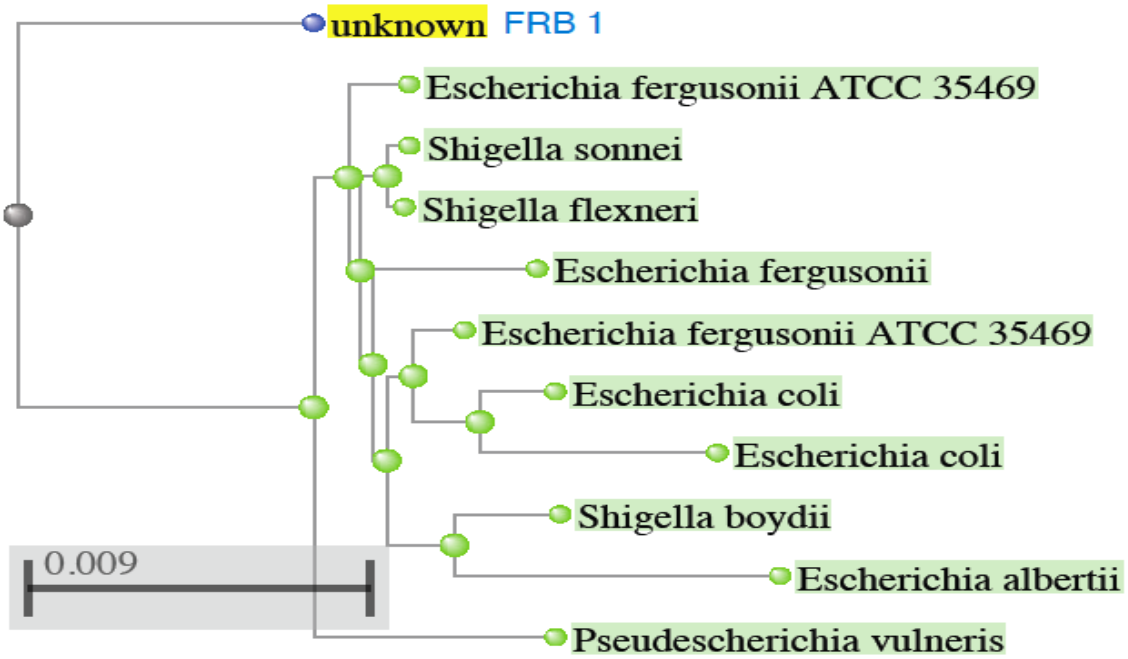
Table 2: Staining and biochemical characteristics of bacterial isolate FRB-1			
Staining		**Enzyme activity test**	
Gram staining	-ve	Starch hydrolosis	-ve
Acid fast staining	-ve	Casein hydrolysis	-ve

Endospore staining	-ve	Gelatin Liquifaction	-ve
Shape	Rod shaped	Nitrate reduction	+ve
IMVic test		Catalase activity	-ve
Indole production test	+ve	**Carbohydrate Utilization test**	
Methyl red test	-ve	Sucrose	-ve
Voges-Proskauer	+ve	Lactose	-ve
Citrate Utilization	-ve	Dextrose	-ve

Table 3. Top ten 16 S rDNA sequence alignment matches for bacterial isolate FRB-1 .

Description of 16 S rDNA partial sequence	Scientific name	Max.Score	Percent Identity	Accession No.
Escherichia fergusonii ATCC 35469	*Escherichia fergusonii ATCC 35469.*	1696	97.96	NR_027549
Shigella flexneri strain ATCC 29903	*Shigella flexneri*	1690	97.86%	NR_026331.1
Shigella sonnei strain CECT 4887	*Shigella sonnei*	1690	97.86%	NR_104826.1
Escherichia fergusonii strain NBRC 102419	*Escherichia fergusonii*	1685	97.65%	NR_114079.1
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, complete sequence	*Escherichia fergusonii*	1679	97.65%	NR_074902.1
Escherichia coli strain NBRC 102203	*Escherichia coli*	1677	97.55%	NR_114042.1
Shigella boydii strain P288	*Shigella boydii*	1674	97.55%	NR_104901.1
Pseudescherichiavulneris strain ATCC 33821	*Pseudescherichiavulneris*	1652	97.04%	NR_119109.1
Escherichia coli strain U 5/41	*Escherichia coli*	1652	97.04%	NR_024570.1
Escherichia albertii strain Albert 19982	*Escherichia albertii*	1646	097.04%	NR_025569.1

Figure 2:-Distance matrix tree of the bacterial isolate FRB-1 drawn using 16S rDNA sequences.



DISCUSSION

The major causes of fluoride induced bacterial growth inhibition have been summarized in literature (Marquis, 1995). The prime mechanisms is the weak acid effect due to inhibition of membrane F-ATPase activity which play role in the proton transport. Secondly, it can disturb the metabolic activity by inhibition of various enzymes like catalase, urease, enolase and heme based proxidases etc. (Marquis, 1995; Marquis, Clock and Mota-Meira, 2003).

However, many bacterial strains have developed resistance to high doses of fluoride in order to thrive in fluoride affected or endemic regions. The bacteria have developed various mechanisms of fluoride tolerance of which provides an insight on the molecular resistance capacity. The membrane proteins play an important role in the evasion of fluoride toxicity by either preventing its entry or by speedy export from the cell. Another, way of reduced toxic effect can be through the induction enzymes which can mitigate the fluoride toxicity. The role of chloride channel permease or Eric F protein overexpression have been observed on in fluoride resistant *S mutans* strain (Murata and Hanada, 2016). These are antiporter channels which exchange fluoride (F-) for protons(H+) and reduce cellular fluoride availability.

Some workers suggest that the long term exposure to fluoride can induce fluoride resistance by means of mutations or selection of mutant forms in fluoride rich environment. This can be due to riboswitches which are activated by presence of fluoride and act by changing or inducing genes expression responsible for fluoride resistance like the fluoride antiporters, activate expression of genes which are inhibited by fluoride (Baker *et al.*, 2012; Liu *et al.*, 2017). The possibility of involvement multiple loci on fluoride resistance in *S. mutans has been reported* (Liao *et al.*, 2018). In this study the fluoride resistant mutants were compared with the wild type for shared single nucleotide polymorphism (mutp, glpfp and glpf) which was suggested to provide resistance. The mutation in mutp (which lies in promoter region of fluoride antiporter) showed overexpression of fluoride antiporters .

Fluoride resistance has also been observed in bacteria of genus *Escherichia* has been reported by many worker (Stockbridge *et al.*, 2012; Chellaiah *et al.,* 2021) with variable dose tolerance. The presence of crcB which encodes for fluoride antiporters has been observed using the gene specific primers (Chellaiah *et al.*, 2021). A subclass of CLC anion transporter CLC^F has been studied to export fluoride in *E.coli* (Stockbridge *et al.*, 2012).

Although, considerable research in the field of fluoride research has been done but the exact mechanism of fluoride resistance in any bacterial isolate cannot be completely predicted. The study of fluoride resistance in bacteria provides us with valuable information about the genetic adaptability in microbes under stress along with an opportunity to develop bioremediation strategies using such bacteria.

CONCLUSION

It can be concluded from the study that fluoride exposure in environmental regimes can promote the development of fluoride tolerance mechanisms in bacteria. Such bacteria can play an essential role in bioremediation of degraded land systems by un-inhibited growth and involvement in biogeochemical cycling of organic and inorganic compounds.

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