ISOLATION AND CHARACTERIZATION OF FLUORIDE RESISTANT BACTERIAL STRAINS FROM FLUORIDE ENDEMIC AREAS OF WEST BENGAL, INDIA: ASSESSMENT OF THEIR FLUORIDE ABSORPTION EFFICIENCY

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ABSTRACT: In the present investigation, two highly fluoride ion (F) tolerant bacterial strains, *Bacillus cereus* FT1 (GenBank Acc. KP729612) and *Bacillus marisflavi* FT2 (GenBank Acc. KP729613) were isolated from soil samples collected from F endemic areas of Birbhum district (Rampurhat block II) of West Bengal, India. The F tolerance limit and absorption efficiency exhibited by these two bacterial isolates were monitored for 72 hr at 24 hr intervals. At lower F concentrations (10–100 ppm), the F absorption efficiency of the bacterial strains was not affected. However, at a higher F concentration (730 ppm) the absorption efficiency was significantly increased compared to the control strain (*B. licheniformis* ONF2). The concentration of F in the culture medium of *B. cereus* FT1 and *B. marisflavi* FT2 was reduced from 730 ppm to 570 ppm and 730 ppm to 580 ppm at 72 hr, respectively. To monitor F toxicity, growth curves were prepared at two different concentrations, 1500 ppm and 3000 ppm of NaF. In both cases, the lag phases in the growth curves were extended. However, the bacterial growth was not completely inhibited. The F tolerance efficiency exhibited by these two bacterial isolates was again confirmed by cell morphology study using a scanning electron microscope. To the best of our knowledge, this is the first report on F absorption by the bacterial strains, *B. cereus and B. marisflavi*.

Keywords: Absorption efficiency; Bacterial isolates; Fluoride tolerance; Scanning electron microscopy.

INTRODUCTION

Fluorine is one of the most abundant elements on earth and acts as a major environmental toxicant originating from both natural and industrial sources.[1] The level of the fluoride ion (F) in surface water is gradually increasing due to rapid industrialization and contamination with pesticides like cryolite and sulfuryl fluoride.[2] The long term consumption of F containing water exerts various adverse effects on health including skeletal and dental fluorosis in both man[3-6] and domestic animals.[7-11] The fluoride ion acts as a protoplasmic poison and a very small amount of this anion can alter several biochemical processes inside living cells.[12] From recent research, F is found to induce oxidative damage in cells by producing reactive oxygen species (ROS) and modulating intracellular redox homeostasis.[13,14] F can also induce genotoxicity and bind with DNA leading to DNA damage which could be the initial event of chemical carcinogenesis.[15-17] In addition, F can modulate expression of many genes related to the phase I and phase II detoxification systems at the transcriptional level.[18,19] Thus, the increasing concentration of F in groundwater resources is now becoming an important toxicological and geo-environmental concern.[20]

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People in a few villages (Noapara, Atla, Junidpur, etc.) in West Bengal (India) consume water with a high F content and fluorosis remains as a major health hazard in these villages.[21] In recent years, a few microorganisms have been reported to be an important bioremediation tool against xenobiotics. Microbes are known to have a variety of mechanisms to tolerate and utilize toxic elements like lead, cadmium, nickel, etc.[22,23] They can adapt to toxic environments through different ways like mineralization, metal sorption, enzymatic oxidation or reduction, and the efflux of xenobiotics from the cells.[24] However, reports regarding the bio-absorption of F are scanty. Only a single literature is available reporting F resistant *B. flexus*.[25] The detoxifying mechanism exhibited by these microorganisms might be a future tool for developing engineered bacteria for the reduction of F levels. Therefore, the present investigation was undertaken with the objectives of (i) screening, isolation, and characterization of F tolerant bacterial strains; (ii) evaluating the absorption efficiency of F by these selected bacterial strains; and (iii) studying the effect of F on bacterial growth kinetics and cellular morphology using scanning electron microscopy.

MATERIALS AND METHODS

Sampling and isolation of bacteria: Soil and drinking water (from hand-pumps) samples were collected in triplicate from six different sites in three villages, namely Atla, Nowapara, and Junidpur, located in Rampurhat block II (24º10’34”N and 87º52’56”E), Birbhum district, West Bengal, India. In order to isolate the F tolerant bacteria, 1 g of soil was dissolved in 10 mL of sterile distilled water and serial dilutions were made according to the standard method.[26] The isolation of the F tolerant bacteria was done in tryptone soya agar (TSA) plates containing 300 ppm of sodium fluoride (NaF). Six morphologically different bacterial colonies were obtained and pure cultures made by the repeated streaking method. On the basis of the F tolerance level, two bacterial strains named FT1 and FT2 were selected for further studies. The minimum inhibitory concentration (MIC) for these two selected bacterial strains was found with TSA plates prepared with different concentrations of NaF (1500 ppm–5000 ppm).

Fluoride estimation in water samples: The collected water samples were subjected to F concentration measurement using a Thermo Scientific ORION STAR A214 ISE meter following the manufacturer’s protocol. In brief, 1 mL of TISAB III reagent was added to 10 mL of water sample, mixed carefully, and measured using a F selective electrode. The ion analyzer electrode was calibrated using standard F solutions (1, 10, and 100 ppm) prepared from a 100 ppm F standard solution (Orion 940907).

Characterization of the selected bacterial isolates: In order to determine the colony morphology, these two selected bacterial strains, FT1 and FT2, were separately cultured on TSA plates and incubated for 24 hr at 37ºC. The colour, margin, elevation, and surface morphology were checked under a scanning electron microscope. Gram staining was carried out according to the standard method. The biochemical characterization of these selected bacterial strains was done using biochemical characterization kits (HiCarbo kit, HiMedia).

16S rRNA sequence analysis: The proper identification of the selected bacterial strains was done by 16S rRNA sequence analysis[26] with minor modifications. In brief, genomic DNA was extracted following the SDS-lysozyme method. Both the quantity and quality of the extracted DNA were checked in a nano-spectrophotometer (Eppendorf Biospectrometer). The amplification of the 16S rRNA gene was carried out in standard conditions in a thermal cycler (GeneAmp 9700, ABI). The PCR products were bi-directionally sequenced using forward AGAGTTTGATCMTGGCTCAG (B27 F) and reverse GGTTACCTTGTTACGACTT (1492 R) primers, respectively. The sequenced data obtained were edited, aligned, and submitted to the NCBI GenBank for accession numbers. The phylogenetic tree of the selected bacterial strains was prepared using Mega 6 software. The guanine and cytosine content (G+C) was calculated and plotted using an online calculator program (http://www.endmemo.com/bio/gc.php).

F absorption by the selected bacterial strains: In order to check the F absorption, the selected bacterial strains FT1 and FT2 were cultured in tryptone soya broth (TSB) medium containing 1500 ppm NaF and incubated for 3 days at 37ºC. The F concentration was estimated at 24, 48, and 72 hr using the Thermo Scientific ORION STAR A214 ISE meter following the same protocol as described above. The initial F concentration of the culture medium was estimated before inoculating the bacterial strains. *B. licheniformis* ONF2 (Acc. No. JX912557) was used as a control.

Effect of NaF on bacterial growth kinetics: The bacterial strains FT1 and FT2 were cultured separately in 100 mL of tryptone soya broth (TSB) medium for 16 hr. 100 μL of seed culture from each container was taken and mixed in freshly prepared 100 mL of TSB medium containing 1500 and 3000 ppm of NaF. This was followed by incubation at 37ºC under a continuous shaking mode (100 rpm). In order to check the growth kinetics, 1 mL of culture from each container was taken in a sterilized condition and the optical density (OD) was measured at 600 nm using a spectrophotometer for 12 hr at 1 hr intervals. A culture broth without NaF was used as a positive control. The growth kinetics of *B. licheniformis* ONF2 (Acc. No. JX912557) at 1500 ppm of NaF were used as negative control.

Effect of NaF on bacterial cell morphology: The bacterial strains FT1 and FT2 were cultured in TSB medium for 10 hr in the presence of 0 (control) and 1500 ppm of NaF. 10 μL of bacterial culture was taken on a cover slip and a thin layer made, followed by air drying and heat fixation. Dehydration of the sample was done with increasing concentrations of alcohol (30, 50, 70, 90, and 100%; 30 min each). The cover slip was then mounted in a stub, coated with gold particles, and observed under a scanning electron microscope (Model Hitachi S530).

Statistical analysis: The calculation of the standard error, one way ANOVA, and a DMRT analysis for significance testing were performed using Microsoft Excel 2010.

RESULTS

Very high levels of F were found in the drinking water samples collected from the three villages (Atla, Junidpur, and Nowapara) including a level of 16 ppm at two sampling sites in Nowapara (Table 1).

Table 1. Fluoride level in drinking water collected from three villages of Birbhum, West Bengal, India

Sampling sites	Concentration of fluoride (ppm)					
	Sample number 1	Sample number 2	Sample number 3	Sample number 4	Sample number 5	Sample number 6
Atla	8	7	10	8	9	7
Nowapara	12	16	14	13	16	14
Junidpur	12	10	14	9	8	11

Six bacterial strains were isolated from the F contaminated soil to check the F tolerance ability. Two strains namely FT1 and FT2 showed the highest F tolerance (1500 ppm), and thus were selected for further studies (Table 2).

Table 2. Screening of F tolerant bacterial strains (+ = growth, – = no growth)

Bacterial strains	Sodium fluoride (NaF) concentrations (ppm)				
	300	600	900	1200	1500
FT1	+	+	+	+	+
FT2	+	+	+	+	+
FT3	+	+	–	–	–
FT4	+	+	–	–	–
FT5	+	+	+	–	–
FT6	+	+	+	+	–

The minimum inhibitory concentrations (MIC) of these two bacterial isolates, FT1 and FT2, were measured to be 6000 and 3400 ppm NaF, respectively. Primarily, these two bacterial strains were identified by their morphological and biochemical properties as listed in Table 3. The colony morphology of FT1 was round, convex, semitransparent, off-white in colour, smooth surfaced, and with

irregular margins, whereas, FT2 was irregular, convex, transparent, and yellow in colour. Both the isolates were Gram positive rods. It was observed that both FT1 and FT2 were able to efficiently utilize inulin, glycerol, rhamose, and esculin. FT2 could also utilize maltose, fructose, dextrose, galactose, trehalose, sucrose, arabitol, and melezitose, whereas, it was negative for α-methyl-d-mannoside, lactose, xylose, malonate, raffinose, melibiose, manose, l-arabinose, sodium gluconate, inositol, α-methyl-d-glucoside, sorbitol, mannitol, adonitol, dulcitol, erythritol, cellobiose, xylitol, and sorbose.

Table 3. Morphological and biochemical characteristics of the selected strains (+ = positive reaction, – = negative reaction)

Characteristics		Bacterial strains	
		FT1	FT2
Gram staining property		+	+
Colony morphology	surface	smooth	smooth
	margin	irregular	irregular
	elevation	convex	convex
	colour	off-white	yellow
	shape	round	irregular
	opacity	semi-transparent	transparent
Biochemical characterization	inulin, glycerol, rhammose, esculin hydrolysis	+	+
	citrate, salicin	+	–
	maltose, fructose, dextrose, galactose, trehalose, sucrose, arabitol, melezitose	–	+
	α-methyl-d-mannoside, lactose, xylose, malonate, raffinose, melibiose, manose, l – arabinose, sodium gluconate, inositol, α-methyl-d-glucoside, sorbitol, mannitol, adonitol, dulcitol, erythritol, cellobiose, xylitol, sorbose	–	–

The guanine and cytosine (G+C) contents and their respective graphs are presented in Table 4 and Figure 1, respectively.

Table 4. Guanine (G) + cytosine (C) content of the selected bacterial strains

Bacterial strains	G+C content (%)	A+T content (%)
Bacillus cereus FT1	53.29	46.71
Bacillus marisflavi FT2	54.53	45.47

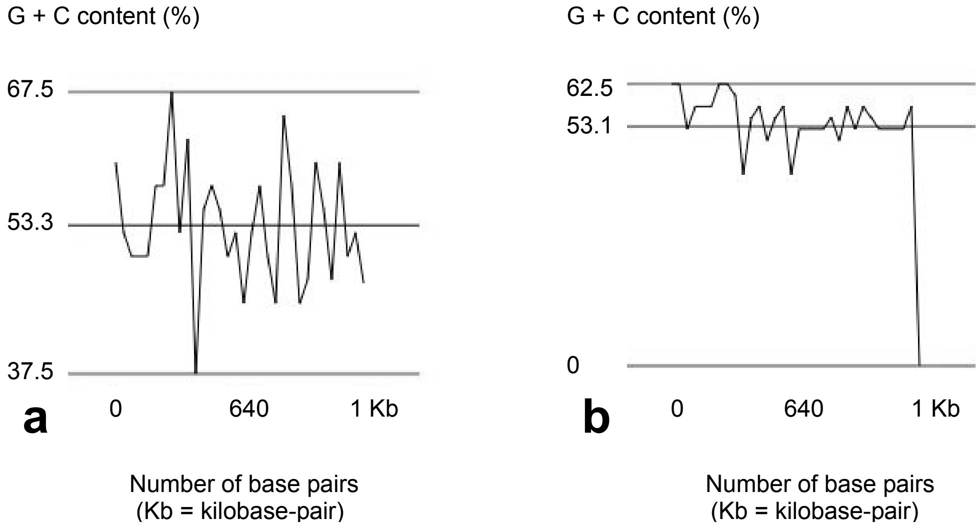


Figure 1. Guanine (G) + cytosine (C) content graphs of two bacterial strains. a: *B. cereus* FT1 and b: *B marisflavi* FT2.

Finally, these bacterial strains were identified up to species level by 16S rDNA sequence analysis (Figure 2).

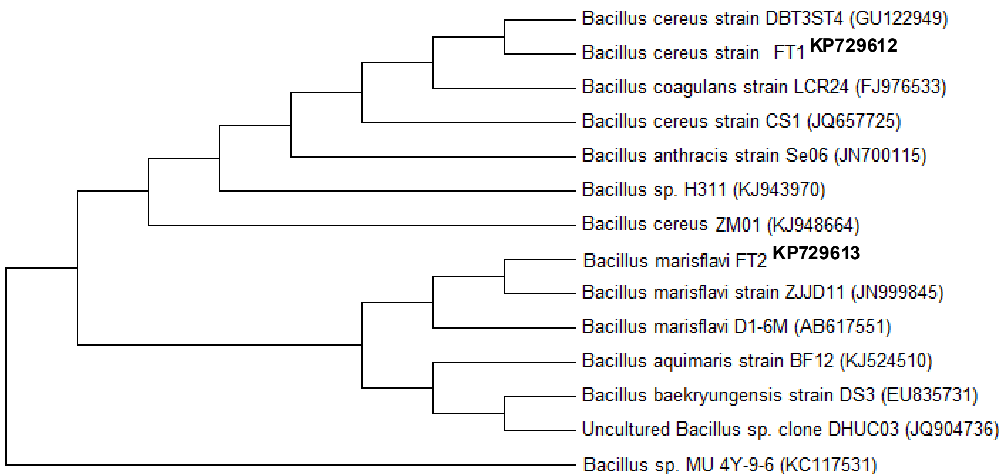


Figure 2. The phylogenetic relationship of the two bacterial isolates with their close homologs taken from the NCBI database. The phylogenetic tree of these two bacterial strains was constructed using Mega 6.0.

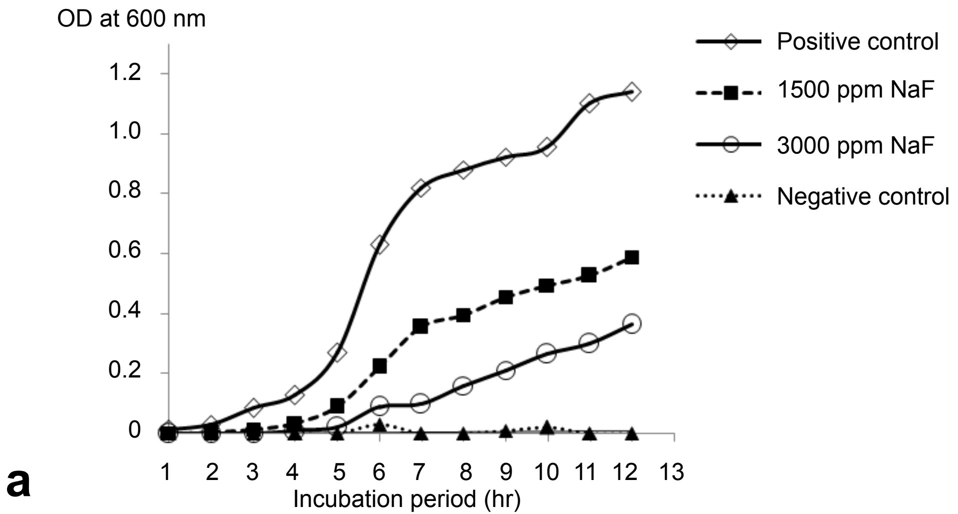
The bacterial isolates FT1 and FT2 were identified as *B. cereus* (GenBank Acc. KP729612) and *B. marisflavi* (GenBank Acc. KP729613), respectively. the F absorption exhibited by these two bacterial, strains *B. cereus* and *B. marisflavi,* is shown in Table 5. The F concentration in the medium decreased gradually at 24, 48, and 72 hr. The concentration of F in the culture medium of *B. cereus* and *B. marisflavi* reduced from 730 ppm to 570 ppm and 730 ppm to 580 ppm at 72 hr, respectively, while no such change was detected with the control strain.

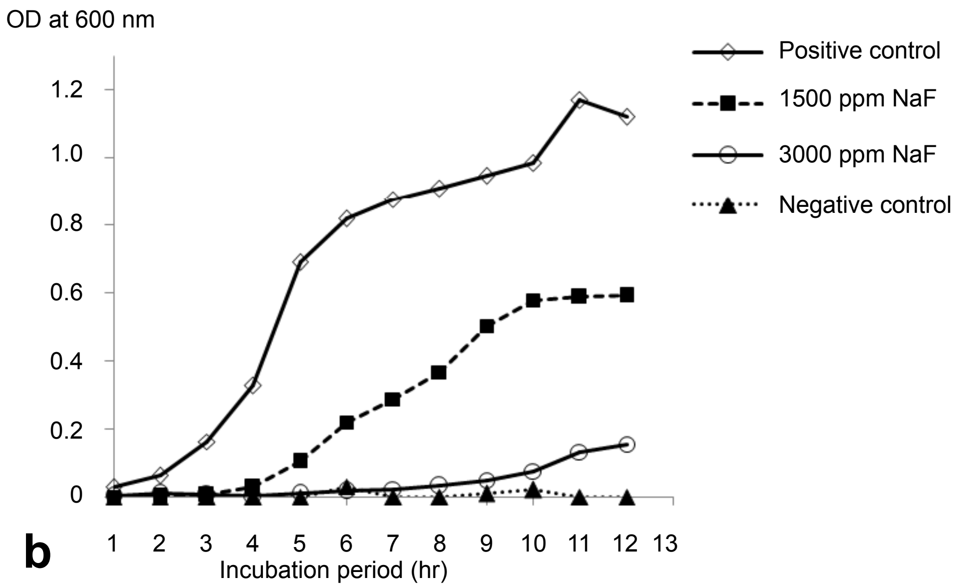
Table 5. F absorption efficiency exhibited by bacterial isolates. Data are presented as mean±SEM, n=3.

Bacterial strains	Concentrations of fluoride (ppm)			
	Initial	Day 1	Day 2	Day 3
Bacillus cereus FT1	730±9.6[a]	680±9.25[a]	610±7.26[b]	570±7.79[b]
Bacillus marisflavi FT2	730±9.6[a]	690±8.94[a]	590±7.12[b]	580±7.53[b]
Bacillus licheniformis ONF2 taken as control	730±9.6[a]	728±9.4[a]	720±9.7[a]	722± 8.9[a]

[a,b]Different lower case letters indicate the presence of a significant difference, $p<0.05$, whereas the same lower case letters denote no significant difference.

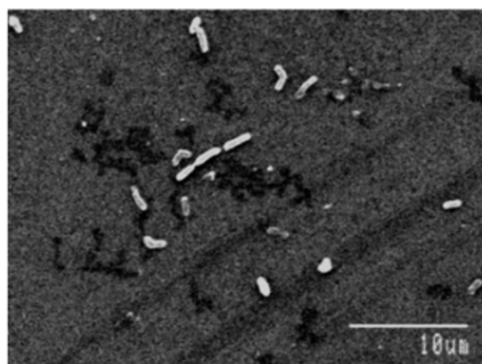
The effect of F on bacterial growth at 1500 ppm and 3000 ppm of NaF is represented in Figure 3. *B. cereus* showed an extended lag phase in the presence of NaF compared to the control. A higher concentration of NaF (3000 ppm) was found to be highly toxic in the case of *B. marisflavi*.



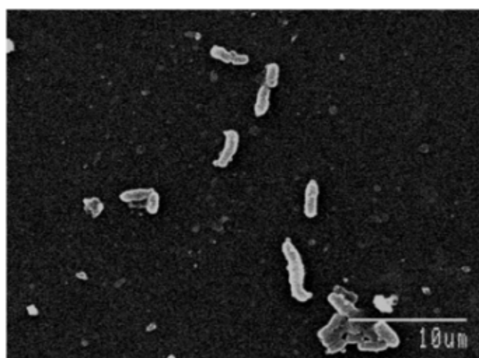


Figures 3a and 3b. The bacterial growth curve in the presence of 1500 and 3000 ppm of NaF. a: *B. cereus*, b: *B. marisflavi*. A bacterial culture without NaF was used as a positive control. The growth kinetics of *B. licheniformis* in the presence of 1500 ppm of NaF was used as a negative control.

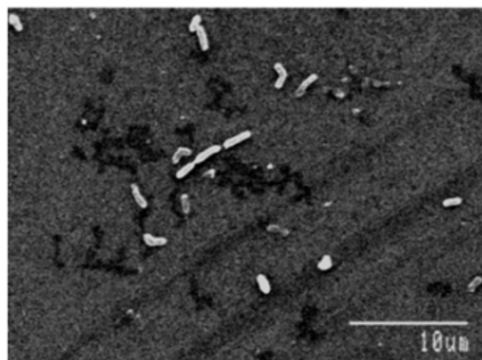
In order to examine the toxic effect of F, scanning electron microscopy was performed. Interestingly, 1500 ppm of NaF failed to impose any adverse effect on bacterial morphology which might be related their tolerance efficiency (Figure 4).



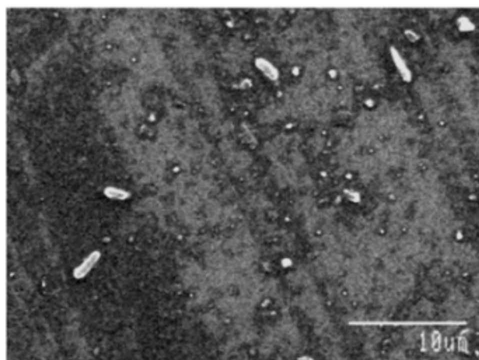
(a) Control



(a) 1500 ppm NaF



(b) Control



(b) 1500 ppm NaF

Figures 4a and 4b. Scanning electron micrographs of the two bacterial strains in the presence of 1500 ppm NaF. a: the morphology of *B. cereus* FT1 (3000 ×) and b: the morphology of *B. marisflavi* FT2 (3000 ×).

DISCUSSION

In recent years, water pollution has become a major threat for both animals and human beings in many parts of the world. Due to overwhelming globalization and competition, most industries directly deposit their waste without prior treatment which increases the concentration of toxic metals such as Pb, Cd, Ni, and Co, etc. in water bodies.[27] Despite F toxicity being quite different to the toxicity of these toxic metals and F being very difficult to remove from water bodies, several relatively inexpensive mechanical and chemical procedures have been introduced for F removal, such as electro-chemical methods, adsorption processes, and ion exchange processes.[28] Researchers have reported that there are some advantages with methods of F removal involving the accumulation or absorption of F using different material matrices like activated carbon, alumina, and zeolites.[29-31] In the present study, we have characterized two potent bacterial strains, *B. cereus* FT1

and *B. marisflavi* FT2 isolated from F contaminated soil, where the average F concentration was 1.5 mg/kg of soil.[25] Both of these bacterial strains can tolerate a high level of F in the form of NaF and can actively absorb F in a liquid medium. *B. cereus* is a Gram positive, soil dwelling, spore forming, and facultative anaerobic bacterium whereas *B. marisflavi* is an endospore forming, Gram positive, aerobic, and non-pathogenic bacterium. In general, bacteria possess different types of mechanisms to deal with toxic components. In the case of F, bacteria possess a special type of channel protein named putative F-transporters that help them to reduce the toxic effect of F on the bacterial cell.[32] In fact, these genes are riboswitches which are special type of metabolite binding RNA structure and are activated by high F concentrations. It has also been evaluated that the channel transporter has a high affinity with the fluoride ion but rejects other negative ions like chloride.[32] In the present study, it was observed that both of these isolated bacterial strains could tolerate very high concentrations of F. Investigators have also reported the F tolerance by *Acinetobacter* sp. isolated from soil samples.[33] The F tolerance of these bacterial strains is due to the expression of riboswitch genes or some other mechanisms. Further studies are necessary to understand the tolerance mechanism in these two bacteria isolated by our group. The F absorption efficiency exhibited by these two bacterial isolates *B. cereus* FT1 and *B. marisflavi* FT2 was very high. However, no significant absorption was detected with low concentrations of F. This might be due to the limitation of the detection limit in the estimation method. In a similar study, researchers reported the absorption of F by the bacterial strain *Bacillus flexus* MN25 (Acc. No. HQ875778).[25] There are several reports regarding the bioremediation of toxic metals by bacterial isolates, but only a few studies have been conducted on F absorption.[34] A F resistant mutant *Streptococcus mutans* GS-5 isolated at low pH was also reported previously.[35] In a similar study, researchers reported the bioabsorption of F by five bacterial strains namely, *Micrococcus luteus*, *Aeromonas hydrophila*, *Micrococcus varians*, *Pseudomonas aerugenosa,* and *Escherichia coli*.[24] Bacteria can absorb F up to a certain degree and the absorbed F is not toxic for its cell division and survival. The present investigation demonstrated the bacterial growth curve at two different concentrations of NaF, 1500 and 3000 ppm. It was observed that in both treatments, the lag phase was extended compared to the control, but after a certain time period they started dividing actively. The effect of F on the bacterial cell morphology was also observed using scanning electron microscopy. Both the bacterial strains were able to maintain their normal cellular morphology, although slight bulging characteristics were detected in a few bacterial cells.

CONCLUSION

In conclusion, the F tolerance efficiency of these two bacterial isolates was high and this property might be useful for investigating the F related expression of membrane channel protein, which will be important for developing F removing engineered bacteria in the future. To the best of our knowledge, it is the first report on F absorption by the bacterial strains, *B. cereus* and *B. marisflavi*.

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