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Fluoride-Resistant *Priestia aryabhattai* isolated from Silkworm Feces: A Promising Agent for Fluoride Bioremediation

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Abstract:

Priestia aryabhattai SF-F, a bacterium isolated from the fecal matter of silkworms, demonstrates significant resistance to fluoride, sustaining growth in sodium fluoride (NaF) concentrations as high as 45 mg/L. Its fluoride removal efficiency was assessed using a Fluoride Ion Selective Electrode, which confirmed a substantial capacity for fluoride degradation, suggesting its potential for application in bioremediation of fluoride contaminated environments. Biochemical characterization identified *P. aryabhattai* SF-F as a Gram-positive, motile organism exhibiting positive responses to methyl red and catalase activity tests. Antibiotic susceptibility profiling based on minimum inhibitory concentration (MIC) assays revealed the greatest sensitivity to streptomycin (6 µg), followed by ampicillin (8 µg), while higher MIC values for chloramphenicol and tetracycline (20 µg each) indicated comparatively reduced susceptibility. Morphological analysis using Scanning Electron Microscopy (SEM) indicated cellular alterations under fluoride stress, while Energy Dispersive X-ray (EDX) spectroscopy confirmed the presence of fluoride within the bacterial cells, suggesting intracellular accumulation. Collectively, these findings establish *P. aryabhattai* SF-F as a strong candidate for microbial based fluoride remediation strategies, offering an eco-friendly approach to mitigating fluoride pollution in affected environments.

Keywords: Fluoride-resistant bacteria, Silkworm fecal microbiota, *Priestia aryabhattai*, SEM-EDX analysis, Antibiotic susceptibility.

INTRODUCTION

Fluorine, the 13th most abundant element in the Earth's crust, mainly occurs as fluoride, constituting about 0.09% of the outer layer. Fluoride enters aquatic systems naturally through leaching of fluoride-bearing rocks and via anthropogenic sources. Industrial effluents from semiconductors, ceramics, coal-fired plants, aluminium smelting, and ironworks are major contributors (Singh *et al.,* 2017). At low levels (<0.5 mg/L), fluoride supports dental and skeletal development, but excess exposure (>2.0 mg/L) is toxic, causing dental and skeletal fluorosis (Biswas *et al.,* 2018), whereas inadequate intake may result in decay, enamel weakening, and bone fragility (Bhatnagar *et al.,* 2011). Fluoride contamination is a global health issue, particularly in India, Ethiopia, and China (Maliyekkal *et al.,* 2006), where more than 260 million people are affected (WHO, 2008). Agricultural phosphate fertilizers, coal combustion, mining, and smelting (aluminium, zinc, copper) are major contributors, along with steelmaking, uranium processing, oil refining, and emerging industries like solar panels and lithium batteries (Patrick and Sahu, 2023). Although trace levels (<1.5 mg/L) are beneficial (Kumar *et al.,* 2024), chronic exposure causes fluorosis (Susheela, 1999), with over 20 countries affected and 22 Indian states severely impacted (Zhu *et al.,* 2006). Conventional defluoridation methods reverse osmosis; nanofiltration, electro dialysis, precipitation, and adsorption face limitations such as high cost, waste generation, and sludge disposal. Biological remediation offers a promising alternative due to cost-effectiveness, simplicity, and minimal sludge. Microbes adapt to fluoride-rich environments through biosorption, bioaccumulation, and biotransformation (Messaitfa, 2008), facilitated by cell wall functional groups like amines, carboxylates, thiols, and phosphates (Juwarkar and Yadav, 2010). Bacterial genera such as *Pseudomonas*, *Sphingomonas*, and *Acinetobacter* are known for pollutant degradation (Wuertz and Mergeay 1997; Ebah *et al.,* 2024a,b; Iqbal and Ashraf, 2024; Iqbal and Khalid, 2024). The silkworm (*Bombyx mori*), an agriculturally valuable species, hosts diverse gut microflora with strain-specific immune responses (Chen *et al.,* 2020; Herman *et al.,* 2025), partly mediated by cecropin-like peptides (Chitra *et al.,* 1975) and lysozymes (Morishima *et al.,* 1990). This study investigates fluoride-tolerant bacterial strains from silkworm feces and their efficiency in defluoridation, aiming to uncover sustainable, low-cost remediation pathways.

MATERIALS AND METHODS

Collection of silkworm fecal samples

Fresh fecal matter from silkworms was aseptically gathered using sterile containers from Kudapura village, situated in Challakere Taluk of Chitradurga District, Karnataka, India. The collected samples were transported to the laboratory at room temperature and immediately processed for microbial isolation.

Chemicals and reagents

The study utilized chemicals and reagents of analytical purity. Unless noted differently, all materials were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Isolation and screening of Fluoride-tolerant bacteria

Silkworm fecal samples were serially diluted in sterile distilled water, and 0.1 mL from each dilution was plated on nutrient agar containing varying NaF concentrations. Plates were incubated at 37 °C for 24 h, and bacterial growth was assessed. Strains surviving up to 45 mg/L NaF were selected and re-streaked on the same concentration to confirm fluoride resistance (Powning and Davidson, 1973).

Fluoride concentration measurement

After incubation, culture broths were centrifuged to separate the biomass from the supernatant. Residual fluoride levels in the supernatant were observed using a Fluoride Ion Selective

Electrode (Thermo Scientific) at the Water Testing Laboratory, Indian Institute of Science (IISc), Bengaluru. Out of six isolates tested, three designated SF-B, SF-C, and SF-F exhibited noticeable fluoride reduction after 24 hours of incubation (Sharma *et al.,* 2019).

Molecular identification via 16S rRNA gene sequencing

The SF-F strain was molecularly identified using 16S rRNA analysis. DNA was extracted from overnight cultures using a Chromgene kit (Bangalore, India) and quality-checked on 0.8% agarose gel. About 10–12 ng of DNA was PCR-amplified with universal primers 27F and 1492R, yielding ~1.5 kb products, which were purified (QIAquick, Qiagen) and Sanger-sequenced (ABI, Applied Biosystems, Mumbai). The sequence was aligned with NCBI GenBank entries, and a phylogenetic tree was constructed using the maximum likelihood method via Phylogeny.fr (Pal *et al.,* 2014; Kousar *et al.,* 2022).

Biochemical characterization of SF-F strain

The biochemical profile of the SF-F isolate was evaluated using standard tests: indole, methyl red, citrate utilization, arginine hydrolysis, casein degradation, motility, catalase activity, Gram staining, starch and gelatin hydrolysis, along with hydrogen sulfide (H_2S) and carbon dioxide (CO_2) production assays. These tests were performed according to established microbiological protocols (Kousar *et al.,* 2022).

Antibiotic susceptibility testing

The SF-F strain's antibiotic resistance was evaluated using the disc diffusion method. A 24-hour nutrient broth culture was spread on Mueller-Hinton agar, and Hexa G-plus 6 discs (ampicillin, chloramphenicol, penicillin-G, streptomycin, sulphatriad, tetracycline; HiMedia, Mumbai, India) were applied under sterile conditions. Plates were incubated at 37 °C for 24 h, and inhibition zones were measured to determine sensitivity according to standard guidelines (Raghavendra and Neelagund, 2012; Saleem *et al.,* 2018; Edrees and Anbar, 2021; Morsi *et al.,* 2022).

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the SF-F strain was assessed by agar well diffusion. Standardized inoculum (OD at 660 nm) was spread on nutrient agar plates, and wells were loaded with different concentrations of ampicillin, chloramphenicol, penicillin-G, streptomycin, sulphatriad, and tetracycline (HiMedia, Mumbai, India) prepared in phosphate buffer (pH 7.4). Plates were incubated at 37 °C for 24 h, after which inhibition zones were measured. Experiments were performed in six replicates, and results expressed as mean ± SE. (Raghavendra and Neelagund, 2012).

Surface morphology and elemental analysis (SEM-EDX)

Field Emission Scanning Electron Microscopy (FESEM) was used to examine the outward structure of treated and untreated SF-F cells. Bacterial samples were mounted on coverslips, air-dried, and coated with a ~15 nm thick layer of gold–palladium using a sputter coater for 30 minutes to enhance conductivity. Imaging and elemental analysis were performed using an FEI ESEM QUANTA 200 microscope equipped with an Energy Dispersive X-ray Spectroscopy (EDX) detector, which allowed assessment of morphological and compositional differences between fluoride-treated and untreated samples (Wasewar *et al.,* 2009).

Statistical analysis

Data were analysed using one-way ANOVA with Tukey's post hoc test at $P < 0.05$ (Cowan, 1974; Raghavendra and Neelagund, 2012).

RESULTS

Screening of Fluoride-tolerant bacteria

Bacterial isolates were obtained by culturing serially diluted silkworm fecal matter on nutrient

agar plates containing varying concentrations of sodium fluoride (NaF). Growth was observed up to 45 mg/L NaF, with no viable colonies at ≥50 mg/L. Colony numbers declined progressively beyond 30 mg/L, and isolates showing stable growth at 45 mg/L were repeatedly subcultured to confirm resistance. Six distinct fluoride-tolerant isolates, designated SF-A to SF-F, were selected for further analysis.

Assessment of Fluoride depletion in growth medium

The capacity of the selected isolates to reduce fluoride levels was assessed using a Fluoride Ion Selective Electrode. After 24 hours of incubation in fluoride-enriched media, three strains SF-B, SF-C, and SF-F exhibited a substantial decline in fluoride concentration compared to the control group. Among these, SF-F showed the most substantial fluoride removal, highlighting its potential as a strong candidate for fluoride bioremediation applications (Table 1).

Table 1. Screening of Fluoride-tolerant bacterial strains.

Bacterial strains	Concentration of F^- (ppm) 1 mg/L= 1 ppm
SF-B	18.3 mg/L
SF-C	18.1 mg/L
SF-F	10.3 mg/L
Control	45 mg/L

Molecular Identification and Phylogenetic Placement

The SF-F strain, selected for its notable ability to eliminate fluoride, underwent inherited characterization. A portion of its 16S rRNA gene was sequenced, and the resulting data was deposited in the NCBI GenBank under the accession number PV902311. By comparing this sequence with others and analysing observable traits, the strain was identified as *Priestia aryabhattai*. This identification was validated through the construction of a phylogenetic tree using closely related sequences, which confirmed its evolutionary and taxonomic position (Figure 1).

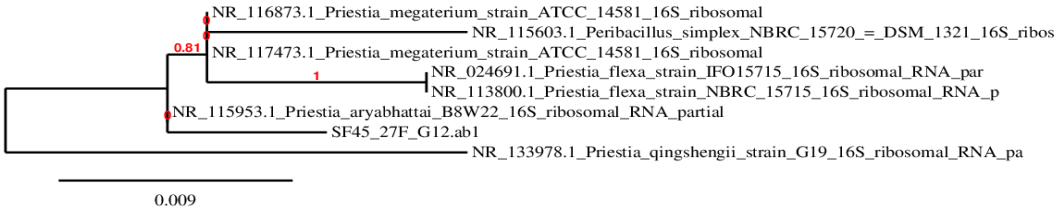

Fig. 1. Phylogenetic tree based on 16S RNA gene sequence showing the relationship of SF-F strain to closely related taxa. The SF-F strain was indentified as *Priestia aryabhattai* and clustered accordingly.

Biochemical profiling of isolate SF-F

The biochemical profile of SF-F, cultivated at 37 °C for 24 h, showed negative results for indole formation, citrate utilization, arginine and casein hydrolysis, and starch, gelatin, H_2S, and CO_2 production. Positive results were observed for methyl red and catalase tests, indicating mixed acid fermentation and catalase activity. The bacterium was motile and Gram-positive (Table 2).

Table 2. Biochemical characteristics of SF-F strain.

Biochemical characteristics	SF-F strain
Indole production	–
Methyl red	+
Citrate utilization	–
Arginine hydrolysis	–
Casein utilization	–
Motility	Motile
Catalase	+
Gram staining	Gram +ve
Starch hydrolysis	–
Gelatinase hydrolysis	–
H_2S test	–
CO_2 test	–

Antibiotic Resistance Profile

Antibiotic sensitivity testing using the disc diffusion method showed varied responses to six different antibiotics. The SF-F strain was highly susceptible to tetracycline and streptomycin, showed moderate sensitivity to ampicillin and chloramphenicol, and exhibited resistance to both penicillin-G and sulphatriad. The mean inhibition zone diameters (± standard error) are presented in (Table 3).

Table 3. Antibiotic susceptibility test results by disc diffusion method.

Antibiotics	SF-F strain
1) Ampicillin	1) 20.80±0.85
2) Chloramphenicol	2) 19.67±0.12
3) Penicillin-G	3) 9.16±0.02
4) Streptomycin	4) 26.70±0.37
5) Sulphatriad	5) 8.70±0.33
6) Tetracycline	6) 28.15±0.26

The value of each constituents consisted of mean±SE of three replicates
Value is significantly different when p<0.05

Minimum Inhibitory Concentration (MIC) of SF-F Strain against Selected Antibiotics

The minimum inhibitory concentration (MIC) assay was conducted to determine the susceptibility profile of the SF-F strain against four selected antibiotics. The strain exhibited the greatest sensitivity to streptomycin, with an MIC of 6 µg, followed by ampicillin (8 µg). In contrast, comparatively higher MIC values were observed for chloramphenicol and tetracycline (both 20 µg), suggesting reduced susceptibility to these agents. The detailed results are presented in (Table 4).

Table 4. Minimum inhibitory concentration (MIC) of SF-F bacterial strain against different antibiotics.

Antibiotic	Cncentration (µg)	Zone of inhibition
1) Ampicillin	2	14.23±0.10
	4	16.00±0.60
	6	18.30±0.45
	8	20.00±0.24
	10	20.57±0.31
2) Chloramphenicol	5	12.80±0.36
	10	14.60±0.39
	15	17.77±0.01
	20	19.40±0.77
	25	19.35±0.07
3) Streptomycin	2	20.77±0.42
	4	23.46±0.18
	6	26.67±0.11
	8	26.72±0.33
	10	26.77±0.21
4) Tetracycline	5	22.13±0.18
	10	24.17±0.01
	15	26.17±0.32
	20	28.11±0.25
	25	28.40±0.23
Control (PBS)		0.14±0.02

The value of each constituents consisted of mean±SE of three replicates
Value is significantly different when p<0.05
Control: phosphate buffer solution (PBS) of 0.02 N of pH 7.4

Morphological Observations via Scanning Electron Microscopy (SEM)

SEM imaging of SF-F cells revealed distinct structural changes before and after fluoride exposure. Untreated cells (Figure 2a and 2b) displayed smooth surfaces with fine, short filaments, indicating intact morphology. In contrast, fluoride-treated cells (Figure 2c and 2d) exhibited visible surface irregularities, including ruptures and deformation, suggesting that exposure to fluoride altered the cell envelope and induced microstructural damage. These findings imply that fluoride stress leads to significant morphological alterations.

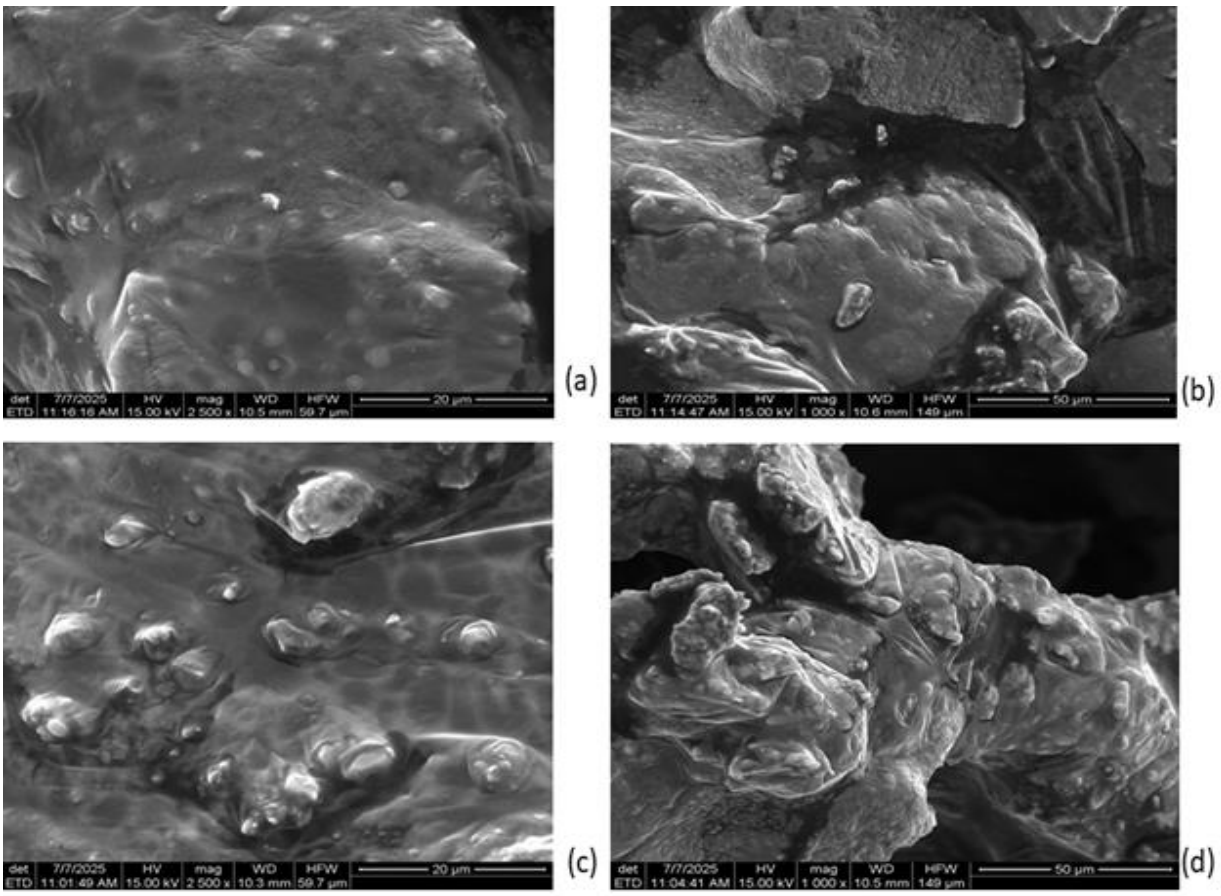

Fig. 2. SEM images of SF-F cells before (a,b) and after (c,d) fluoride treatment. Fluoride exposure caused visible surface damage and deformatiob, indicating fluoride-induced morphological stress.

Elemental Composition Analysis Using EDX

Energy Dispersive X-ray Spectroscopy (EDX) analysis compared untreated and fluoride-treated SF-F cells. In the treated sample (Fig. 3b), fluorine was detected at approximately 1.2% by weight, whereas it was absent in the untreated cells (Fig. 3a). The appearance of fluorine in the treated strain indicates successful biosorption, confirming the strain's interaction with and uptake of fluoride ions from the surrounding environment.

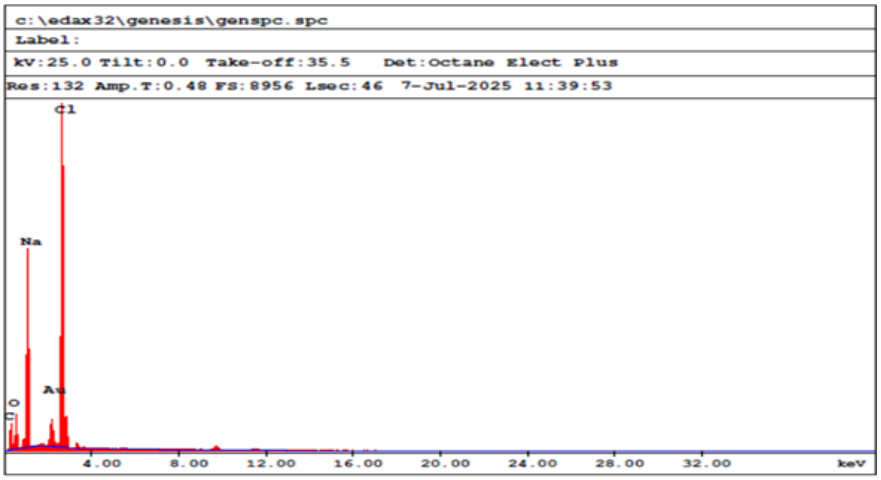

Fig. 3a. EDX spectrum of untreated SF-F cells showing no detectable fluorine signal.

Fig. 3b. EDX spectrum of untreated SF-F cells indicating the presence of fluorine signal, validating successful fluoride uptake through biosorption.

DISCUSSION

Fluoride contamination in groundwater remains a major public health concern, with reported levels of 0.4–11 mg/L often exceeding the WHO guideline of 0.5–1.5 mg/L and causing dental and skeletal fluorosis; even sub-threshold exposure is linked to adverse health outcomes (Mukherjee *et al.,* 2017; Chellaiah *et al.,* 2021). This study isolated fluoride-tolerant bacteria from *Bombyx mori* feces, an underexplored microbe-rich source. Among six isolates (SF-A to SF-F), SF-F exhibited the highest tolerance, growing up to 45 mg/L NaF, suggesting adaptations such as ion regulation, efflux mechanisms, or membrane modifications, consistent with previous reports on *Bacillus* and *Pseudomonas* (Thivya *et al.,* 2017; Sharma *et al.,* 2019). Quantitative assays confirmed its ability to reduce fluoride via biosorption, intracellular uptake, or enzymatic transformation, as seen in *Bacillus cereus* FT1 and *Bacillus marisflavi* FT2 (Pal *et al.,* 2014). 16S rRNA sequencing identified SF-F as *Priestia aryabhattai*. While previously associated with plant growth promotion and stress tolerance, this is the first report of fluoride resistance in *P. aryabhattai*, highlighting insect gut microbiota as reservoirs of stress-adapted microbes.

Biochemically, SF-F was catalase and methyl red-positive, indicating oxidative stress defence and mixed acid fermentation, with limited hydrolytic activity suggesting energy-efficient metabolism under stress.

Antibiotic profiling revealed sensitivity to tetracycline and streptomycin, intermediate susceptibility to ampicillin and chloramphenicol, and resistance to penicillin-G and sulphatriad, a narrow profile advantageous for environmental applications given concerns over resistant *Aeromonas*, *Pseudomonas*, and *Enterobacteriaceae* in aquatic systems (Banerjee *et al,.* 2016). Minimum inhibitory concentration (MIC) assays provided further resolution, with the lowest MIC for streptomycin (6 µg), followed by ampicillin (8 µg), whereas higher MICs for chloramphenicol and tetracycline (20 µg each) indicated reduced susceptibility. These results validate the disc-diffusion findings and suggest that although SF-F is not broadly multidrug-resistant, it maintains selective tolerance mechanisms likely linked to stress adaptation pathways. SEM showed cell ruptures and dents under fluoride exposure, while EDX confirmed intracellular fluoride, likely facilitated by cations such as Na^+, Ca^{2+}, and Mg^{2+} (Wasewar *et al.,* 2009). Overall, *P. aryabhattai* SF-F demonstrates strong potential for biological fluoride remediation, combining high tolerance, detoxification ability, and low antibiotic resistance. Future work should explore genomic and transcriptomic pathways of fluoride resistance and scale-up trials in bioreactors and natural fluoride-contaminated systems.

CONCLUSION

This study identified *Priestia aryabhattai* SF-F from *Bombyx mori* feces as a promising candidate for fluoride bioremediation. The strain tolerated up to 45 mg/L NaF and showed effective fluoride removal, with SEM-EDX confirming cell–fluoride interaction, likely through biosorption. Its metabolic profile and limited antibiotic resistance support safe environmental application. Further genomic and pilot-scale studies are needed to validate its performance under real-world conditions.

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CONFLICT OF INTEREST

The authors affirm that there are no conflicts of interest related to this work.

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