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SHORT NOTE

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF A FLUORIDE RESISTANT *Bacillus* SPECIES ISOLATE, H2

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Fluorine exists in the earth's crust as fluorides (F) in fluorspar, fluorapatite and cryolite minerals (Ghosh *et al.*, 2013). The F ion is toxic to the protoplasmic content of cell as it affects biochemical contents when its level in drinking water exceeds 1.5 mg L^{-1} (Annadurai *et al.*, 2014). World Health Organization and European Union have set the permissible limit of F in drinking water as 1.5 mg L^{-1}, while US Environment Protection Agency has set it as 4 mg L^{-1}, and Indian Standard (IS) as 1.0 mg L^{-1} (WHO, 2006; Edmunds *et al.*, 2013). Fluoride reportedly has antimicrobial activity, and might contribute to the anticaries activity by decreasing the growth and metabolism of microbes like *Streptococcus mutans* (Hamilton, 1990), due to which F is extensively used in oral hygiene products and even added to the municipal water supplies (Slayton *et al.*, 2006). To avoid the ill effects of F, different bacteria undergo changes in their genomes (Ying *et al*., 2017) and play a role in bioaccumulation (Juwarkar and Yadav, 2010). Fluoride enters a bacterial cell at low pH as HF and gets dissociated on exposure to neutral pH (Marquis *et al.*, 2003). Fluoride ion once inside the bacterial cells inhibit the important enzymes like enolase, F-ATPase and urease which can lead to the decrease in bacterial growth and metabolism (Pandit *et al.*, 2013). A F-resistant *Pseudomonas aeruginosa* achieved 22.1% removal of F (Chouhan *et al.*, 2012), whereas strain RH5 removed 25.7% F from growth medium (Mukherjee *et al*., 2017). Two F-resistant *Bacillus* strains KT201599 and KT201600 could survive at 2000-2500 µg sodium fluoride (NaF) mL^{-1} and were able to remove F from media by 16.7 and 24.7%, respectively (Dutta *et al.*, 2020). A F-tolerant halophilic *Bacillus flexus* NM25 could tolerate 1,500 ppm F in brain-heart infusion agar medium and reduced F concentration up to 67.45% (Pal *et al*., 2014) and *Acinetobacter* sp. could eliminate 57% F from the medium at specified conditions (Shiva Shanker *et al.*, 2020). Microorganisms behave variably to different F ion content in media. Fluoride tolerant bacterial strains *B. cereus* FT1 and *B. marisflavi* FT2 showed higher F absorption efficiency in tryptone soya broth (Goutam *et al.*, 2016). A F-resistant strain c180-2FR got mutated for two glycolytic enzymes, pyruvate kinase and enolase (Ying Liao *et al.*, 2018). Mutations were also noted in promoter *mutp* which led to the upregulated expression of downstream F antiporters. However, decrease in enolase activity was not found associated with decrease in *S. mutans* growth in NaF-enriched cultures (Mitsuhata *et al.*, 2014). Fluoride contamination in ground water is a major problem in some areas of Nalgonda district, Telangana (India) as the water intake with high F leads to dental and skeletal fluorosis. To prevent these health issues, defluoridation of water is done before consumption. The F-resistant wild strains with the ability to bioremediate F exist in nature, which can be exploited and utilized. The present study was aimed to isolate and characterize a high F-resistant bacterium from ground water of Narketpally, Nalgonda district, Telangana (India).

Ground water samples were collected from various hand- and bore-pumps near Narketpally area (17°19`N79°20`E) in Nalgonda, Telangana (India) so as to isolate fluoride resistant organisms. The samples were subjected to serial dilution and then plated onto Luria-Bertani (LB) agar medium with ingredients (g L^{-1}): casein enzyme hydrolysate (tryptone) (10)**,** yeast extract (5), NaCl (5) and agar

(15), pH 7.0 ± 0.2**.** The Petri-plates were incubated at 37°C for 24 h. The bacterial isolates were randomly selected and purified. The purified bacterial isolates were plated onto LB medium containing varied concentrations of 100-4500 mg NAF L^{-1} in ascending concentrations and incubated at 37°C for 24 h. Exposing isolates to high concentration of F directly may cause suppression of their growth, so, the isolates were initially grown in LB medium with low NaF, then transferred to high concentrations of NaF so that isolates could adapt to new environments. The highly F-resistant isolate was selected for further characterization and identification. The morphological, and physio-biochemical characterization of high F-resistant isolate H2 was done as per Logan and De Vos (2015). The colony morphology and Gram's test were performed. F-resistant isolate H2 was assessed for optimum temperature and pH for growth in LB broth. The isolate was grown on LB broth at 4, 25, 37 and 45°C for 48 h. Also, the isolate was grown in LB broth at different pH (5-10) for 48 h at 37°C. The effect of variable salinity concentration on the growth of isolate was observed using NaCl ranging from 2 to 12% in LB broth. Growth of F-resistant isolate was assessed spectrophotometrically (Systronics). Optical density of isolate was measured at 600 nm randomly from 6 to 78 h to monitor the log phase of isolate. Biochemical tests like methyl red, Voges-Proskauer, oxidase, catalase and nitrate reduction tests were performed (Logan and De Vos, 2015). Himedia Hicarbo kit was used to study different carbohydrate utilization by the isolate. Antibiotic sensitivity of isolate was performed using various antibiotic discs.

16S rDNA gene amplification and its sequencing was performed to identify fluoride resistant isolate (Johnson *et al.*, 2019) by constructing a phylogenetic tree. The DNA of isolate was extracted by heat treatment and polymerase chain reaction performed in a PCR machine to amplify its 16S rDNA gene by using two primers, forward 27F (5′-AGAGTTCCTGCTGCAG-3′) and reverse 1492R (5′-GGTTACCTTGACTTTT-3′) respectively (Chen, 2015). The PCR programme applied was: initial denaturation at 95°C for 5 min, then denaturation at 95°C for 30 sec, followed by annealing of primers at 46.2°C for 1 min, and extension at 72°C for 1 min for 30 cycles. The final extension was done at 72°C for 5 min and amplicons were stored and maintained at 4°C. After performing agarose gel electrophoresis of PCR products, a 1.5 kb size PCR product was eluted from agarose gel, purified and sequenced. To identify the isolate, EZ Biocloud public data and analytics portal was used (*https://www.ezbiocloud.net/apps*). The 16S rDNA gene sequence of isolate was submitted to the NCBI Gene bank. The phylogenetic tree was constructed by using MEGA11 software (Tamura *et al*., 2021).

Two high F-resistant strains, H1 and H2 were isolated and purified from the highly F containing ground water sample of Narketpally, Nalgonda, Telangana (India). The isolate H1 has been reported earlier (Mothe *et al.*, 2022), so only isolate H2 is reported here which could resist the F content of 4.4 g L^{-1} in LB broth and 2.8 g L^{-1} in LB agar. The colonies of H2 isolate were white in colour and the cells were Gram +ve rod. The isolate showed optimum growth at 37°C and at pH 8. The isolate tolerated up to 10% NaCl. The F-resistant isolate, H2 showed exponential growth at 54 h (Fig. 1). The biochemical characterization revealed the isolate was negative to methyl red and nitrate reduction tests and positive to oxidase, catalase and Voges Proskauer tests. Biochemical test revealed that the isolate was positive to mannitol, adonitol, arabitol, fructose dextrose; xylose, maltose, galactose, trehalose, sucrose, L-arabinose, inulin, sodium gluconate, salicin, dulcitol, inositol, erythritol, esculin and citrate, but negative to lactose, raffinose, mannose, glycerol, sorbitol, α-methyl- D-glucoside, rhamnose, cellobiose, melizitose, α-methyl-D-mannoside, xylitol, ONPG, D-arabinose, malonate and sorbose Out of the 12 antibiotic sensitivity tests performed, the isolate showed resistance to 4 antibiotics: ampicillin10 (µg),

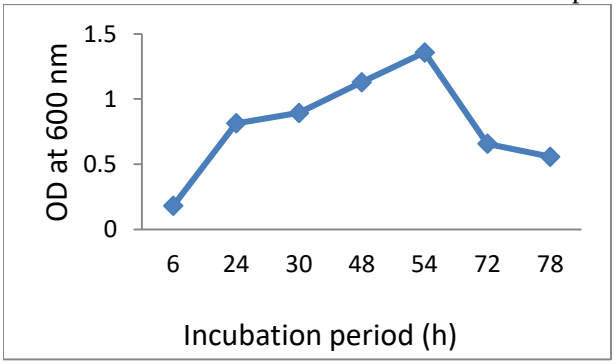

Fig. 1: Growth curve of fluoride-resistant *Bacillus* isolate, H2

nalidixic acid 30 (µg), penicillin 10 (µg) and chloramphenicol 30 (µg). The isolate was sensitive to erythromycin (15 µg), tetracycline (30 µg), amikacin (30 µg), kanamycin (30 µg), vancomycin (30 µg), gentamycin (10 µg), streptomycin (10 µg) and ciprofloxacin (5 µg). In comparison isolate H1 was resistant to 5 antibiotics: chloramphenicol (30 µg), erythromycin (15 µg), penicillin (10 µg), nalidixic acid (10 µg) and ampicillin (10 µg), and sensitive to tetracycline (30 µg), amikacin (30 µg), kanamycin (30 µg), vancomycin (30 µg), gentamycin (10 µg), streptomycin (10 µg) and ciprofloxacin (5 µg).

16S rDNA gene amplification, sequencing and phylogenetic analysis of isolate H2 revealed the bacterium as *Bacillus* sp. which showed 99.93% similarity with *Bacillus safensis* subsp. *safensis* (Fig. 2).

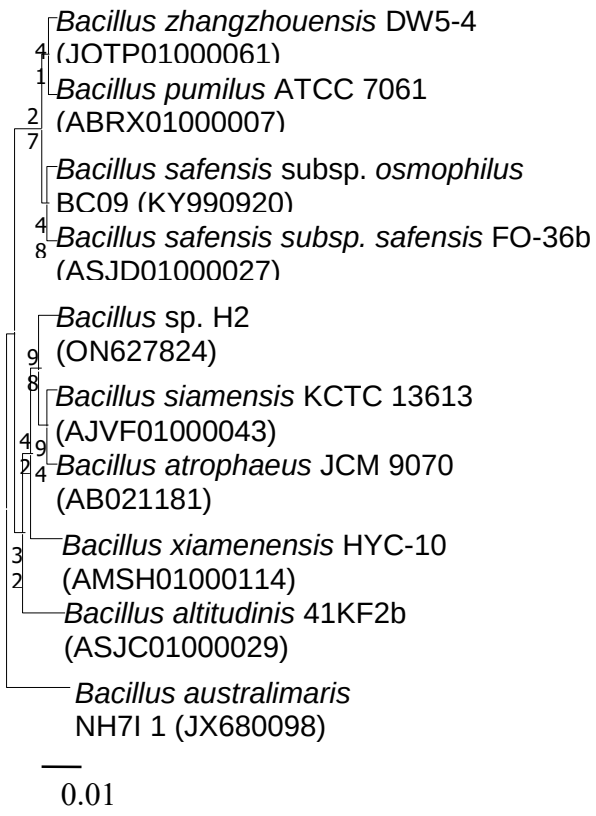

Fig. 2: Phylogenetic tree of high fluoride resistant *Bacillus* isolate H2

The isolate was deposited in NCBI GenBank under Accession No ON627824. The fluoride content in ground water of Nalgonda district has earlier been studied (Brindha *et al.*, 2011; Shaik Arshad *et al.*, 2015). However, scanty attempts have been made to isolate F-resistant bacteria from ground water. One of the high F-resistant *Bacillus* isolate H1, could resist F concentration of 4.8 g L^{-1} (Mothe *et al.*, 2022), but, the present isolate H2 could resist a little low i.e. 4.4 g L^{-1} F concentration in LB broth. H1 isolate on LB agar medium could resist F concentration of 4 g L^{-1} (Mothe *et al.*, 2022), and H2 isolate could resist 2.8 g L^{-1} F only. F-resistant organisms have a role in F bioremediation. Mothe *et al.* (2022) reported that high fluoride resistant strain H1 could achieve 38.2% bioremediation after 72 h incubation with 250 mg L^{-1} F concentration at 37°C, initial pH 7. Hence, isolate H2 could be exploited for bioremediation.

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