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ORIGINAL ARTICLE

Characterization of a fluoride-resistant bacterium *Acinetobacter* sp. RH5 towards assessment of its water defluoridation capability

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Abstract The present study investigates the defluoridation capability of fluoride-resistant bacteria from contaminated groundwater collected from Asanjola and Madhabpur, West Bengal, India. Seven strains of fluoride-resistant bacteria were isolated employing culture media containing 10–250 mg/L of fluoride to evaluate their ability in reducing fluoride concentration in water. Five isolates exhibited significant amount of reduction in fluoride. Isolate RH5 achieved a maximum fluoride removal of 25.7 % from the media at 30 °C and pH 7 after 8 days of incubation. Based on morphological, physiological characteristics and analysis of 16S rDNA gene sequence, isolate RH5 was identified as *Acinetobacter* sp. RH5. Growth of RH5 was analysed at a diverse pH range, and it could thrive at pH 5–10. The present investigation revealed that the selective pressure of fluoride results in growth of fluoride-resistant bacteria capable of secreting high-affinity anion-binding compounds. This bacterium played a dominant bioremediative role by concentrating the anions so that they become less available. Hence, the fluoride-resistant bacteria, *Acinetobacter* sp. RH5, could be used as a promising strain for application in water defluoridation from contaminated sites.

Keywords Bioremediation · Defluoridation · Xenobiotic · Characterization · *Acinetobacter*

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Introduction

Trace elements have toxic effects when their level of permissibility is exceeded. The permissible limits for the discharge of toxic metals into the water bodies have been prescribed by various regulatory bodies (Sud et al. 2008). The health hazards and environmental degradation caused due to addition of the metal ions at a much higher concentration than the permissible limits are given in Table 1. For example, the fluoride ion has got some side effects which are toxic to the protoplasmic content of the cell affecting its biochemical content when its levels exceed 1.5 mg/L in drinking water (Annadurai et al. 2014).

Fluorine accounts for almost 0.3 g/kg of the earth's crust and exists in the form of fluoride in minerals such as fluorspar, cryolite and fluorapatite (Ghosh et al. 2013). The permissible limit for fluoride in drinking water as prescribed by World Health Organization (WHO), Indian Standard (IS), US Environment Protection Agency (USEPA) and European Union (EU) is 1.5, 1.0, 4 and 1.5 mg/L, respectively, with a target of between 0.8 and 1.2 g/L to maximize benefits and minimize adverse effects (WHO 2006; Edmunds and Smedley 2013). The effects of varying concentrations of fluoride on human health are given in Table 2.

In recent years, high levels of fluoride in groundwater (McDonagh et al. 2000) have resulted in vitiation of water quality, which has led to widespread anxiety. In West Bengal, India, excess amount of fluoride in groundwater has been detected across seven districts, viz. Purulia, Birbhum, Bankura, Malda, South Dinajpur, North Dinajpur and South 24-Parganas. Literature review by Bhattacharya and Chakrabarti (2011) confirms the existence of fluoride as a complex ion in a naturally occurring mineral called apatite which is a fluorinated calcium phosphatic

Table 1 Permissible limits and health effects of various toxic heavy metals

Metal contaminants	IS: 2490 (1974) For surface water (mg/L)	IS: 3306 (1974) for public sewers (mg/L)	IS: 3307 (1974) for irrigation (mg/L)	WHO (μg/L)	USEPA (μg/L)	Adverse health effects
Arsenic	0.20	0.20	0.20	10.00	50.00	Carcinogenic, liver tumours, skin and gastrointestinal effects
Mercury	0.01	0.01	–	1.00	2.00	Dermatitis, anorexia, kidney damage and muscle pain
Cadmium	2.00	1.00	–	3.00	5.00	Carcinogenic, lung fibrosis, dyspnoea and weight loss
Lead	0.10	1.00	–	10.00	5.00	Loss of appetite, anaemia, sterility, kidney problem, high blood pressure
Chromium	0.10	2.00	–	50.00	100.00	Lung tumours, allergic dermatitis
Nickel	3.00	3.00	–	–	–	Chronic bronchitis, lung cancer, nasal sinus
Zinc	5.00	15.00	–	–	–	Metal fume fever, restlessness
Copper	3.00	3.00	–	–	1300.00	Irritation of nose, mouth, eyes, headache diarrhoea
Fluoride	1.0	1.0	–	0.0015	0.004	Dental fluorosis, skeletal fluorosis

Table 2 Effects of varying concentrations of fluoride on human health

Level in water	Effects
Nil	Limited growth and fertility
0.8–1.2 mg/L	Prevention of tooth decay, strengthening of skeleton
Above 1.5 mg/L	Fluorosis: pitting of tooth enamel and deposits in bones
Above about 10 mg/L	Crippling skeletal fluorosis

compound. It has been observed from experimental investigations that various physico-chemical factors are found to be responsible for fluoride contamination of water from the fluoride-bearing rock into the saturated zone of groundwater (Datta et al. 2014). Analysis of water samples revealed that the presence of Na–K–Ca–Mg–HCO_3 favours continuous erosion from the fluoride-rich host rock under alkaline pH condition of the circulating water as revealed by Saxena and Ahmed (2003). Due to highly fragile economic structure, the absence of any treatment or monitoring systems the rural populace is compelled to drink fluoride contaminated water in these places (Singh et al. 2001). Fluoride is an oligo-element indispensable to bone and teeth development when ingested below the specified limit of 1.5 mg/L (Li et al. 2001; Susheela 1999) but poses a threat to human health when the prescribed limit is breached. Fluoride intake in excess leads to dental, skeletal and non-skeletal fluorosis (Gopalakrishnan et al. 1991; Zhu et al. 2006). It has been reported to be endemic in 20 countries of the world (Messaitfa 2008) with 22 states of India bearing the brunt of fluoride contamination (Susheela 1999). Several methods have been reported to treat fluoride contaminated water such as osmosis, nanofiltration, electrodialysis, precipitation and adsorption. These methods have certain drawbacks like secondary pollution, high sludge generation and high capital investment. The advantages of biological processes are functional simplicity, cost-effectiveness, less sludge production, facile regeneration of the biomass. Microbes have the capability of developing resistance to their surroundings through several processes such as bioaccumulation, biotransformation and biosorption (Juwarkar and Yadav 2010) since the bacterial cell wall comprises metal binding groups like amines, carboxylates, sulfhydryl and phosphates which aid in the interaction of metal ions (Kleinubing et al. 2011; Wuertz and Mergeay 1997). Biological degradation of petroleum wastes has been reported by bacteria of the genus *Pseudomonas* (Chung et al. 2003), *Sphingomonas* (Liu et al. 2009) and *Acinetobacter* (Ahmad et al. 2012). *Acinetobacter* strains have been explored for degradation of persistent organic pollutants (POPs) like atrazine (Cail et al. 2003). In 2012, five fluoride-resistant strains were isolated and a maximum fluoride removal of 22.1 % was achieved by *Pseudomonas aerugenosa* (Chouhan et al. 2012). Table 3 gives a comparative study of fluoride removal by processes other than bioremediation.

A freshwater unicellular microbial species, *Acinetobacter* sp. RH5, was isolated from Asanjola, India. The aim was to characterize fluoride-resistant bacterial strains from oligotrophic and heterotrophic niches and to utilize these strains towards defluoridation of contaminated water.

Table 3 Comparative study of fluoride removal by various techniques

Sl. no.	Techniques	% Removal of fluoride/adsorption capacity	References
1.	Adsorption by activated alumina	69.5 %	Ghorai and Pant (2005)
2.	Adsorption by carbon materials $KMnO_4$ modified activated carbon	15.9 mg/g	Daifullah et al. (2007)
3.	Co-precipitation (Nalgonda technique)	2.1–0.7 mg/L	Dahi et al. (1996)
4.	Precipitation with calcium and phosphate compounds	15 mg/L	Saha (1993)
5.	Ion exchange (modified zeolite)	94 %	Samatya et al. (2007)
6.	Reverse osmosis	98.9 %	Diawara et al. (2011)
7.	Nanofiltration		
	Brackish water	63.3 %	Diawara et al. (2011)
	Fluorinated drinking water	71 %	
8.	Biosorption (powdered biomass *Tinospora cordifolia*)	25 mg/g	Pandey et al. (2012)
9.	Biosorption (powdered activated carbon of *Eichhornia crassipes*)	70 %	Halder et al. (2014)
10.	Bioremediation (*Pseudomonas aerugenosa*)	22.1 %	Chouhan et al. (2012)
11.	Bioremediation (*Acinetobacter* RH5)	25.7 %	Present study

Materials and methods

Sample collection and fluoride concentration measurement

Asanjola and Madhabpur villages in Rampurhat tehsil (24.17°N, 87.78°E), Birbhum district, West Bengal, India, were selected for collecting the samples as this district is known to be endemic to fluoride where the rural population is dependent only on groundwater resources for their daily consumption (Susheela 1999). Due to highly fragile economic structure, the absence of any treatment or monitoring systems causes the rural populace to drink fluoride contaminated water in these villages; 50 mL of water samples was collected in presterilized containers and analysed for their fluoride concentration.

A fluoride ion-selective electrode (Orion, Thermo Scientific, USA) was employed for measuring the fluoride concentration of water samples. Calibration of the instrument was performed with standard solutions containing fluoride concentrations of 0.1, 1, 10 and 100 ppm (mg/L). A total ionic strength adjustment buffer (TISAB) was added to each sample before measuring the fluoride concentration to equalize the pH and ionic strength of the samples and the standards. It also facilitates removal of any interference by Fe(III) and Al(III) by forming a complex with these ions, hence releasing fluoride ions into the solution (Schamschula et al. 1985; NIOSH 1994).

Isolation and adaptation of bacterial cultures on media containing sodium fluoride

All the glassware was acid washed with 10 % hydrochloric acid prior to use. The collected water samples were allowed to stand at room temperature for 3 h to permit sedimentation of debris. Luria–Bertani (LB) medium with 2 % agar was used for plating and was sterilized by autoclaving at 121 °C and 15 psi pressure for 15 min; 100 µL of the supernatant was inoculated on plates containing sterilized LB agar media using spread plate technique and incubated for 24 h at 30 °C. Petri plates exhibiting bacterial growth were selected, and distinct colonies were streaked on a new set of LB agar media and incubated for 24 h at 30 °C.

LB agar media containing 10 mg/L fluoride was prepared using sodium fluoride (NaF), and the bacterial cultures were streaked onto these plates. It was followed by incubation of these plates at 30 °C for 24 h. Resistant micro-organisms were inoculated in LB broth containing the same concentration of fluoride as in the source plate media and incubated for 24 h at 30 °C in a rotary shaker at 120 rpm. Low concentration of fluoride was initially selected to allow the culture micro-organisms to adapt on media containing fluoride and to prevent inhibition of growth (Zhang et al. 2013). The microbes were then subjected to an increased fluoride concentration when broth cultures were further subcultured on LB agar plates containing 50 mg/L fluoride concentration, followed by incubation and then inoculation in LB broth containing 50 mg/L of fluoride. The same procedure was repeated for media containing 150 mg/L and 250 mg/L fluoride. After three subcultures, isolated bacterial cultures were obtained.

Determination of strain performance and selection of initial pH of selective medium

In order to determine the fluoride degrading capability, the isolated strains were inoculated in 250-mL conical flasks in triplicates containing LB broth with 20 mg/L fluoride concentration and incubated at 30 °C on a rotary shaker at

120 rpm. Broth containing 20 mg/L fluoride was selected due to the reason that maximum fluoride concentration in contaminated groundwater was observed to be 19.2 mg/L. Samples were taken from these flasks and analysed for fluoride concentration and growth. Ten millilitres of sample was taken at a time for fluoride analysis and centrifuged at 4500 rpm for 15 min, and the supernatant was subjected to fluoride concentration analysis using a fluoride ion-selective electrode. The bacterial isolate designated as RH5 showing the maximum fluoride removal percentage was chosen, and effects of initial pH on growth in LB broth were studied for this isolate. Initial pH was adjusted to 5, 6, 7, 8, 9, and 10, respectively, by adding 0.1 mol/L sodium hydroxide or 0.1 mol/L hydrochloric acid solutions. Study of bacterial growth by means of spectrophotometric analysis at 600 nm gave the optimum pH for the isolate RH5.

Biochemical characterization and growth kinetics of isolate RH5

Methyl red test

The methyl red test was conducted to verify the ability of isolate RH5 to perform mixed acid fermentation (Palitzsch 1911; MacFaddin 1980); 100 µL of inoculum of isolate RH5 was inoculated in a tube containing MRVP (methyl red Voges–Proskauer) broth and incubated at 35 °C for 24 h. Five drops of methyl red were added after incubation, and the result was recorded. A red colour change indicated the presence of mixed acid fermentation.

Voges–Proskauer test

Voges–Proskauer test was performed to determine whether the isolate RH5 produced 2,3-butanediol as a fermentation product from glucose. Since 2,3-butanediol cannot be easily detected, the test targets acetoin, which is an intermediate in the pathway. An inoculum from a culture of isolate RH5 was inoculated in a tube containing MRVP (methyl red Voges–Proskauer) broth and incubated for 24 h at 35 °C. Five drops of Barritt's A reagent were added to the tube after incubation followed by addition of five drops of Barritt's B reagent. The tube was then allowed to stand in a slant position for half an hour. Development of red colour indicated a positive test (MacFaddin 1980).

Catalase test

This test was accomplished using the method of Evans and Kloos (1972). A sterile inoculating loop was used to collect a single 24-h-old colony of isolate RH5 culture from LB agar medium and placed on a slide. A single drop of 3 % H_2O_2 was placed on the smear and covered with a petri plate. The presence of catalase was signified by the formation of bubbles.

Oxidase test

Overnight grown individual colonies were removed using a sterile, plastic loop. The cells were rubbed onto a moistened strip impregnated with oxidase reagent (1 % *N*,*N*,*N*,*N*-tetramethyl *p*-phenylenediamine dihydrochloride). This chemical replaces oxygen as a recipient for the electrons from the oxidase cytochrome. The additional electrons turn the oxidase reagent from colourless to purple. If oxidase is not present, no colour change is observed.

Nitrate reduction test

Nitrate reduction test was executed to determine the ability of the isolate to reduce nitrate to nitrite using the enzyme nitrate reductase. Nitrate broth containing nutrients and potassium nitrate was inoculated with the test micro-organism. A second tube containing only uninoculated nitrate broth was used as control. Both inoculated and control tubes were incubated for 24 h at 30 °C. After incubation, a drop of reagent A (0.8 g sulfanilic acid and 100 mL 30 % acetic acid) and reagent B (500 mg *N*,*N*-dimethyl-1-naphthylamine and 100 mL 30 % acetic acid) each was added. Then a small amount of powdered zinc was added and results were recorded. Development of red colour after the addition of sulfanilic acid and *N*,*N*-dimethyl-1-naphthylamine, followed by its disappearance on addition of zinc, indicates a positive result. Appearance of no colour after the addition of the same reagents followed by appearance of red colour on addition of zinc indicates a negative result.

A spectrophotometer (UV-2300, Techcomp Ltd., Singapore) was employed to measure the growth of isolate RH5 in LB broth. Absorbance of the bacterial isolate was measured at 600 nm at intervals of 4 h. The absorbance readings were then plotted against time to determine the growth phases and to establish a link between fluoride removal and growth of the isolate RH5 (Campbell et al. 2006).

Identification and phylogenetic analysis of isolate RH5

Identification by means of 16S rDNA sequencing was carried out for identification of isolate RH5. Genomic DNA was isolated from the plate culture of isolate RH5 using AMpurE Bacterial Genomic DNA Mini kit (Amnion Biosciences Pvt. Ltd., India). Using consensus/universal oligos, the ~1.5-kb 16S rDNA fragment from isolated gDNA was amplified using *Taq* DNA polymerase, employing the primers 5′-CWG RCC TAN CAC ATG SAA GTC and 5′-GRC GGW GTG TAC NAG GC (R = A or G; S = C or

G; W = A or T; N = A or C or G or T; W = A or T). In brief, the PCRs (polymerase chain reaction) were performed under the following set of conditions: 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min 30 s. Sanger dideoxy sequencing was performed by using BigDye Terminator v.3.1 Cycle Sequencing kits (Applied Biosystems, USA). Purified cycle sequencing products were analysed on an ABI Prism 3130 xl Genetic Analyzer. The PCR product was bi-directionally sequenced using the forward, reverse and/or an internal oligos. The PCR product was loaded on 1.0 % agarose gel along with a 500-bp DNA ladder (Amnion Biosciences Pvt. Ltd., India). Sequence data were aligned and analysed for finding the closest homologous micro-organisms using nucleotide Basic Local Alignment Search Tool (NCBI-blastn).

Results and discussion

Fluoride concentration of samples

Water samples from Asanjola were highly contaminated with fluoride with 3 out of 4 groundwater samples containing fluoride concentration above 15 mg/L. The rock types present in the areas surrounding Asanjola are mainly composed of biotite which itself produces dissolved fluoride concentrations beyond 4 mg/L (Gupta et al. 2012). Madhabpur fared a little better with only one sample exceeding the prescribed fluoride limit. In the preliminary experiments, we found that isolate RH5 could grow in 100 mg/L of NaF and possess the ability of accumulating large amounts of fluoride. But, there are no systematic studies regarding the application of this strain for the defluoridation of water (Table 4).

Strain isolation and adaptation on media containing fluoride

A total of seven bacterial isolates were obtained from samples collected from Rampurhat. The isolates were labelled as VF1, VF2, VF3, VF4, VF5, R3 and RH5. These isolates were differentiated on the basis of their colony characteristics and subjected to Gram staining. The isolated strains were subjected to different fluoride concentrations and all exhibited growth till 100 mg/L of fluoride concentration after incubation at 30 °C for 24 h. The resistant isolates were then inoculated in LB broth with the same concentration of fluoride. Five isolates were able to withstand maximum fluoride concentrations up to 250 mg/L.

Table 4 Fluoride concentration of samples of Asanjola and Madhabpur

Area	Sites	Fluoride concentration (mg/L)
Asanjola	Hand pump1	19.2
	Primary school	15.4
	Hand pump2	19.0
	Pond	0.512
Madhabpur	Primary school	16.3
	Hand pump	0.418
	Pond	0.630

Identification of strain performance and determination of initial pH of selective medium

All of the isolates were analysed for fluoride reduction capability by measuring fluoride concentration of the broth after centrifugation at 4500 rpm for 15 min. Percentage reduction in fluoride content was determined for each alternate day, viz. days 0, 2, 4, 6, 8 and 10. Reduction in fluoride concentration was observed till day 8 after which there was no considerable defluoridation which can be attributed to reduction in cell viability. Isolates VF1, VF4, VF5, R3 and RH5 exhibited significant fluoride removal. Isolates VF2 and VF3 were not able to remove fluoride in significant amounts. Isolate RH5 obtained from water sample taken from Asanjola showed the maximum fluoride reducing capability of 25.7 % and was selected for identification and characterization. The growth of isolate RH5 can be due to high levels of fluoride which might produce a situation closer to that of using an antibacterial agent, in which a resistant organism is released from ecological restrictions of the community and can, therefore, grow in higher levels in the presence of fluoride. Previously, bacterial species have been reported to achieve a maximum of 22.1 % fluoride removal (Xu et al. 2011). Initial pH of medium was adjusted to 5, 6, 7, 8, 9 and 10, respectively, to study the growth of the isolate and uptake of fluoride by the isolated strain. The absorbance recorded at 600 nm was least for LB broth at pH 10 and maximum for pH 7 which is shown in Fig. 1. Therefore, pH 7 was deemed to be the optimum for growth of isolate RH5 (Table 5).

Biochemical characterization and growth kinetics of isolate RH5

Isolate RH5 obtained from water sample taken of Asanjola, Rampurhat, is a Gram negative, non-motile, coccobacillary bacterium. It is oxidative and aerobic in nature. The strain could grow at a diverse range of pH (pH 5–10) conditions, with optimum pH being 7. It could produce acid from galactose, glucose and maltose but not from mannitol and sucrose. The strain tested negative for methyl red, Voges–Proskauer, oxidase and nitrate reduction tests and positive for catalase test.

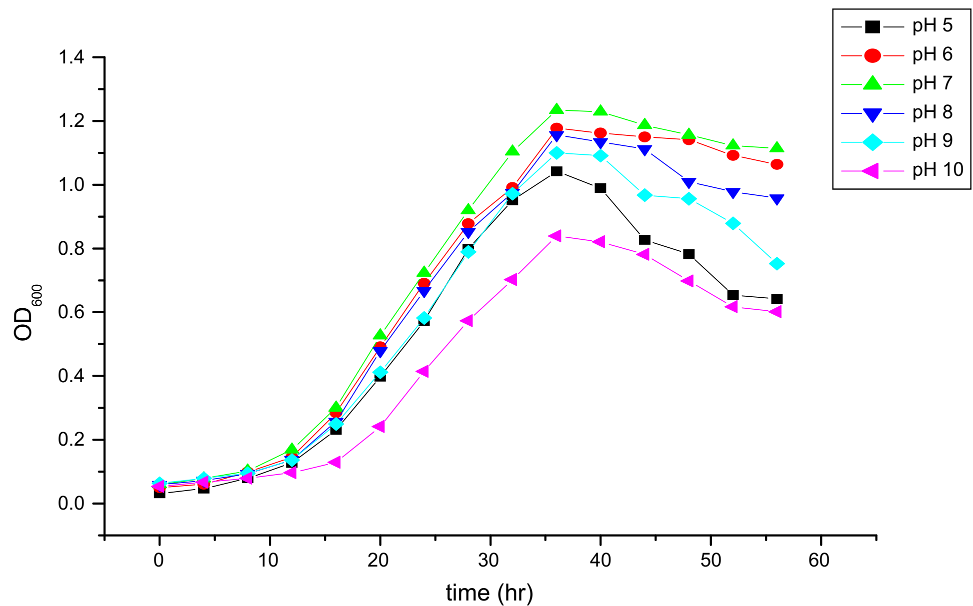

Fig. 1 Effect of pH on growth of *Acinetobacter* sp. RH5; the bacterium was able to thrive on a diverse pH range

Table 5 Percentage reduction in fluoride by isolates

Isolate	Fluoride reduction (%)				
	Day 0	Day 2	Day 4	Day 6	Day 8
VF1	0.0	4.5	7.2	9.1	15.7
VF2	0.0	1.6	2.7	3.7	5.1
VF3	0.0	2.1	3.9	5.1	7.4
VF4	0.0	8.5	14.7	16.8	20.5
VF5	0.0	7.6	12.6	14.2	19.1
R3	0.0	6	11.4	17.4	18.5
RH5	0.0	9.5	18.1	21	25.7

The strain RH5 exhibited a diminutive lag phase, lasting for only 6 h, implying its ability to adapt to the medium of high fluoride concentration. After 6 h, the strain reproduced at a rapid rate, which was indicated by a sharp increase in OD_{600} values. Figure 2 shows that the maximum growth rate was observed between 16 and 36 h.

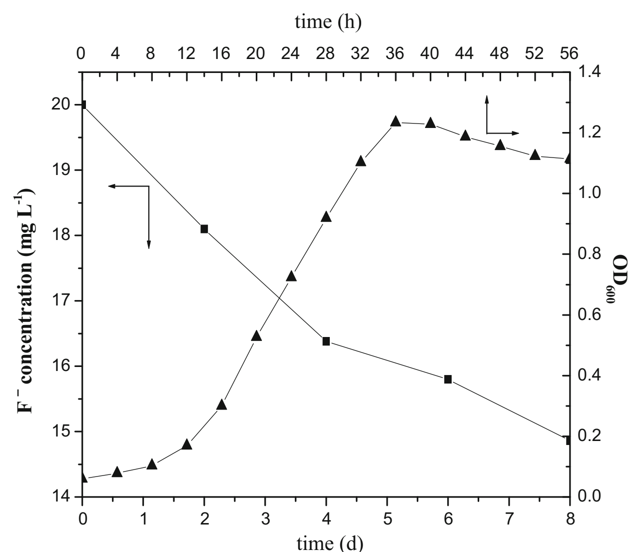

Fig. 2 Growth and fluoride removal efficiency of *Acinetobacter* sp. RH5; lag phase lasted for 6 h; fluoride concentration decreased rapidly till fourth day after which retardation in the fluoride removal rate was encountered

PCR identification of 16S rDNA and phylogenetic analysis of isolate RH5

A 1.5-kb 16S rDNA fragment obtained by PCR was sequenced, and phylogenetic analysis based on 16S rDNA gene sequences suggested that isolate RH5 formed a phylogenetic lineage with members of the genus *Acinetobacter* and demonstrated the maximum 16S rDNA similarity of 99 % with *Acinetobacter calcoaceticus* as shown in Fig. 3. The isolate RH5 was, therefore, found to be a member of *Acinetobacter* genus and named *Acinetobacter* sp. RH5.

Bioremediation of toxic xenobiotics has many advantages over other techniques as it is cheap and non-destructive. Many studies have confirmed that microbes have high affinity for metals and non-metals through a variety of mechanisms. As reported by Doble and Kumar (2005), certain bacterial species secrete high-affinity anion-binding compounds called ionophores. These ionophores bind to certain specific forms of anions and make a complex that can be utilized by the bacteria. So, the reduction in the

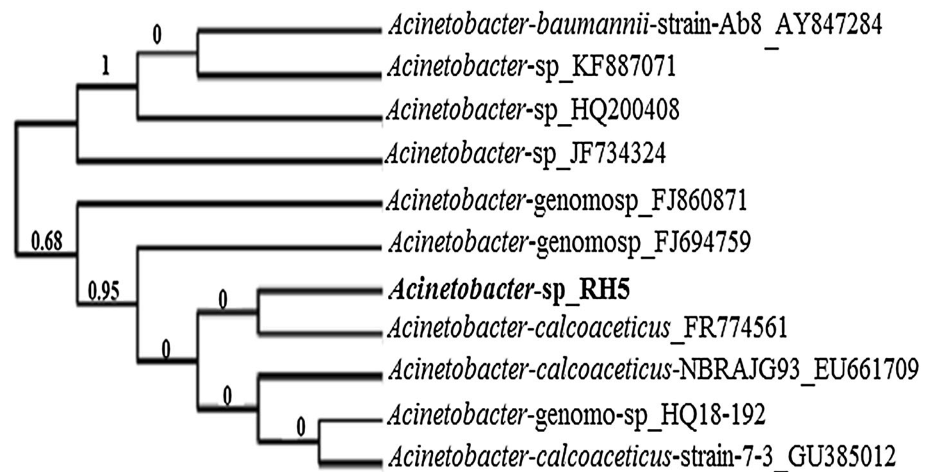

Fig. 3 Phylogenetic analysis of isolate RH5 and related species

fluoride concentration of the media in our present study can be attributed to this fact. Fluoride mostly exists with a proton (HF). Hydrogen fluoride can readily cross cell membrane and acts like a proton conductor. In this way, micro-organisms having the adapting mechanisms concentrate fluoride from the surroundings making it less available in the environment.

Conclusions

Through the current study, it can be convincingly implied that the problem of fluoride contamination can be alleviated by use of bioremediation technologies. *Acinetobacter* sp. RH5 had better defluoridation capability than other isolates obtained from natural and anthropogenic sources for defluoridation of water. The isolate showed maximum resistance to fluoride as well as the maximum fluoride removal capability of 25.7 % at 30 °C. The growth characteristics were tested for a wide pH range, wherein pH 7 exhibited maximum growth of isolate RH5. The strong adaptability of strain RH5 to high concentration of fluoride made it a promising candidate for treating fluoride contaminated water. Though this study dwelt only in the realm of identifying fluoride removal capabilities of bacteria, still it can be worthwhile to infer that development of a bioremediation system for defluoridation can be achieved either by immobilization of *Acinetobacter* sp. RH5 to prevent washing away by flowing water, or by use of sequence batch reactors to achieve longevity and maintenance of the bacteria. In order to determine the sustainability and pragmatism of the bioremediation process, further experiments are being conducted.

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