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Isolation and identification of *Trichoderma harzianum* from groundwater: An effective biosorbent for defluoridation of groundwater

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Abstract

The ability of non-viable form of *Trichoderma harzianum*, isolated from fluoride rich groundwater, was investigated as biosorbent for defluoridation of groundwater. Biosorption experiments were carried out at laboratory scale for removal of fluoride from groundwater. Significant effect of operational parameters on fluoride biosorption using *Trichoderma harzianum* as biosorbent was evaluated by varying operational parameters such as: initial fluoride concentration (2-8 mgl^{-1}), biosorbent dose (0.4-1.6g/100ml), groundwater pH (6-10), temperature (30-50°C) and biosorption time (30-120 min). The fluoride adsorption isotherms were modeled by Langmuir and Freundlich isotherms. Our result showed that fluoride biosorption, significantly increased with increase in groundwater pH, biosorbent dose, temperature and biosorption time, whereas increase in initial fluoride concentration reduced fluoride removal. The fluoride biosorption was rapid and maximum fluoride uptake was attained with 1.6g 100ml^{-1} biosorbent within 60 min. Optimal pH 10 and temperature 50°C gave maximum defluoridation efficiency. Freundlich isotherm fits well for defluoridation of groundwater using *Trichoderma harzianum* as biosorbent which indicated that biosorbent surface sites were heterogeneous in nature and fitted into heterogeneous site binding model.

Key words

Adsorbate, Adsorption, Biosorbent, Defluoridation, Freundlich Isotherm

Introduction

Fluoride contamination in groundwater from different geographical regions of the world is a cause of great concern. Fluoride exists in environment as a result of both natural and anthropogenic activities. The permissible limit of fluoride in drinking water, according to the World Health Organization, Bureau of Indian Standards and Indian Council for Medical Research is 0.5 - 1.5mgl^{-1}, 1-1.5 mgl^{-1} and 1-2 mgl^{-1} respectively. Fluoride is an essential mineral; within the permissible limits it promotes teeth mineralization as well as bone formation. Above the permissible limit, it causes multidimensional health manifestations *i.e.*, dental and skeletal fluorosis (Yunus *et al.,* 2002). To prevent these health hazards, drinking water fluoride concentrations have to be brought down to < 1.5 mgl^{-1} by a quick and safe treatment method. Several methods for removal of fluoride from water have been reported by many researchers *i.e.* precipitation (Islam and Patel, 2007), ion exchange (Castel *et al.,* 2000) adsorption (Huo *et al.,* 2011), membrane process (Hou *et al.,* 2010), electodialysis (Amor *et al.,* 2001), and Donnan dialysis (Kabay *et al.,* 2008). Invariably, most of these methods have disadvantages such as high energy consumption, complex operation, huge sludge formation, water quality detrimental after effects, un-sustainable and non-ecofriendly.

In recent years, biosorption emerged as an effective technique for removal of fluoride from water using ubiquitous biomaterials. Biosorption has several advantage such as simple, easy and low cost operation, abundantly available and low cost biomaterials, biosorbent regeneration, fluoride selective, lesser chemical and/or biological sludge formation, high efficiency in dilute effluents, environmental

friendly, high accessibility and economically viable. Various biosorbents have been investigated for fluoride removal using various biomass such as sugarcane charcoal (Mondal *et al.,* 2013a), *Aspergillus* and Ca-pretreated *Aspergillus* biomass (Mondal *et al.,* 2013b), tea ash (Mondal *et al.,* 2012a), activated rice husk ash (Mondal *et al.,* 2012b), eggshell powder (Bhaumik *et al.,* 2012), neem and kikar leaves (Kumar *et al.,* 2008), water hyacinth (Simha *et al.,* 2002), *Anabaena fertilissima* and *Chlorococcum humicola* (Bhatnagar *et al.,* 2002), Spirogyra IO1 sp.(Mohan *et al.,* 2007a), Spirogyra IO2 sp. (Mohan *et al.,* 2007b) and *Pleurotus ostreatus* 1804 (Ramanaiah *et al.,* 2007). However, fluoride removal using microbial biosorbent is still unexplored. The filamentous fungi are capable developing significantly higher mass as compared to bacteria, thus, they can be used in remediation biotechnology to remove fluoride from contaminated groundwater.

The present study explored the possibility of fluoride removal from groundwater using fungal biosorbent. The primary objective of the present study was to isolate fluoride absorbing fungi from fluoride contaminated groundwater. The secondary objective was to investigate the effect of pH, biosorption time, initial fluoride concentration, temperature and biosorbent dose on defluoridation of groundwater using *Trichoderma harzianum* as biosorbent. The equilibrium nature of biosorption process was studied by using Langmuir and Freundlich isotherm model.

Materials and Methods

All the chemicals used in the study were of HiMedia. Standard fluoride adsorbate solutions of 1000mgl^{-1} were prepared by dissolving 2.21g of NaF in 1000 ml double distilled water and all working solutions were prepared by appropriate dilution of freshly prepared stock solution with groundwater. Fluoride contaminated groundwater samples were collected from four different places in Karnataka India *i.e.*, Manahalli, Hosur, Bomnal, and Bommanigere, which contained fluoride 1.81, 3.9, 4.4 and 5.2 mgl^{-1} respectively.

Fluoride analysis: The concentration of fluoride in groundwater sample was analyzed by SPADNS method using Aqualytic Fluoride Ion Analyzer (APHA, 2005).

Isolation and identification of fluoride adsorbing fungi: All the four samples were serially dilute to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and blank (distilled water without sample) was prepared. 100 µl from each dilution was inoculated in Potato dextrose agar (PDA) containing 2 mgl^{-1} fluoride with spread plate technique and incubated at 25°C for 72 hr. Fragments of individual colonies were transferred separately to the same medium (PDA containing 2 mgl^{-1} fluoride) and growth was accompanied for 72 hr at 25°C. The isolated fungal strains were again subcultured in PDA plates of varying fluoride concentration (2, 4, 6 and 8 mgl^{-1}) to select fluoride resistant fungi from the isolated fungal strains. The fluoride resistant fungal strains were subjected to preliminary batch biosorption with fluoride spiked groundwater at operating condition, 4 mg l^{-1} initial fluoride concentration; 0.4g 100ml^{-1} biosorbent dose pH 7, 30 °C temperature and 100 rpm agitation speed to select fluoride adsorbing fungi from fluoride resistant fungi.

Morphological identification: The strains were identified after growth on Potato Dextrose Agar (PDA) medium, by observing its macroscopic characteristics (colony color, texture appearance and diameter of the colonies) and microscopic characteristics.

Molecular identification by PCR amplification of ITS region: Fungal DNA was extracted using E5038 SIGMA plant/fungal DNA isolation kit and subjected to an internal transcribed spacer (ITS) PCR assay for identification. The target regions of rDNA ITS1, ITS2 regions and 5.8S gene were amplified symmetrically using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplifications were performed in a total reaction volume of 25 µl containing 0.4 mM of dNTP mix, 10 pmol µl^{-1} of each primer, 2.5 µl of 10X PCR buffer, 0.5 mM $MgCl_2$, 1.5 unit Taq DNA polymerase (Bangalore Genei, India) and 50 ng of template DNA. PCR amplifications were performed in a thermal cycler (Eppendorf, Germany) with an initial denaturing step of 95°C for 3 min, followed by 35 amplification cycles of 95°C for 30 Sec, 50°C for 45 Sec and 72°C for 90 Sec and a final extension step of 72°C for 10 min. PCR amplification products were electrophoretically separated on 1.8% (w/v) agarose gels and visualized under 300 nm UV light and photographed using a Molecular Imager, imaging system (Gel-Doc XR$^+$, BIORAD, USA). A 100 bp size marker was used as reference (Bangalore Genei, India). PCR amplified sample was sequenced using Sanger's di-deoxy chain Termination sequencing chemistry with Sequencing Machine - ABI 3500xL Genetic Analyzer. Base calling of received sequence data was done using Chroma Lite v2.01 software (http://technelysium. com.au), with conversion to FASTA format. Species were identified by searching databases using the National Center for Biotechnology Information (NCBI) BLAST (Basic Local Alignment Search Tool) sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS sequence was compared using nucleotide BLAST with default settings and megablast (highly similar sequences) as the selected program. Species identification was determined from the lowest expected value (E-value) of the BLAST output and similarity percentage.

Biosorbent development: A non-viable form of *Trichoderma harzianum* was studied as biosorbent to evaluate its potential to adsorb fluoride from groundwater. To avoid secondary contamination of treated water, non-viable form of *Trichoderma harzianum* was used as biosorbent. Isolated fungus strain was mass cultured in potato dextrose broth at 25 °C for 3 d. Fresh biomass was heat killed in an autoclave at 121°C, 15 lb for 15 min. Heat killed biomass was collected by filtration and dried in vacuum desiccators for 24 hr.

Biosorption study: The batch biosorption was carried out using spiked pre-characterized groundwater having initial fluoride concentration of 2, 4, 6 and 8 mgl^{-1}. Sodium fluoride of HiMedia grade was used to spike groundwater samples to obtain the desired initial fluoride concentration. A series of batch biosorption experiments were carried out to explore the effect of operational parameters, such as pH, biosorption time, biosorbent dose, temperature and initial fluoride concentration on defluoridation of groundwater following the methods of Ramanaiah *et al.* (2007).

Four 250 ml stoppard glass bottles with 0.4 g of dry fungal biosorbent were taken and 100 ml of groundwater containing fluoride concentration of 2, 4, 6 and 8 mgl^{-1} were added in separate bottles and biosorption study was carried out at constant 30°C temperature, 100 rpm agitation speed, 60 min biosorption time, 0.4g biosorbent dose and pH 7.0. The influence of groundwater pH on fluoride biosorption was evaluated at various pH (6.5, 7.5, 8.5 and 9.5) of groundwater by keeping other biosorption parameters constant (groundwater volume: 100 ml, biosorbent dose: 0.4 g, initial fluoride concentration: 4 mgl^{-1}, temperature: 30 °C, agitation speed: 100 rpm and biosorption time: 60min). pH of suspension was adjusted with 0.01 M HCl and 0.01 M NaOH. To determine the effect of biosorbent dose on sorption capacity and intensity, the biosorbent dose varied as 0.4, 0.6, 0.8 and 1.6 g and biosorption study was carried out at constant 30°C temperature, 100 rpm agitation speed, 60 min biosorption time, 4 mg l^{-1} initial fluoride concentration and pH 7.0. The effect of reaction temperature on fluoride biosorption was carried out at three different temperatures (30, 40 and 50 °C) by keeping other biosorption parameters constant (groundwater volume: 100 ml, biosorbent dose: 0.4 g, initial fluoride concentration: 4 mgl^{-1}, pH: 7, agitation speed: 100 rpm and biosorption time: 60 min. Sorption kinetics was determined by analyzing fluoride removal from groundwater at different time intervals of 30, 60, 90 and 120 min.

Results and Discussion

In the present study, three fungal strains were isolated from fluoride contaminated groundwater and subjected to preliminary batch biosorption. Out of three fungal isolates, only one fluoride resistant strain was able to remove fluoride with 21% fluoride removal efficiency. The fluoride adsorbing fungal strain was identified as *Trichoderma harzianum* strain on the basis of morphological and molecular identification as given below.

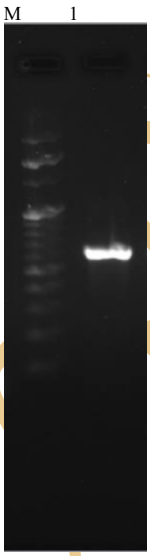

Fig. 1.: Gel electrophoresis result of PCR amplified sample

Morphological characteristic of *Trichoderma harzianum is as rapidly growing strain o*n PDA plate (within a 42 hr whole PDA plate covered with mycelium) and form white cottony mycelium with green conidial production. The conidia production was denser in center then towards the margins. The spores were ellipsoidal oval in shape and conidiophores were septate and highly branched.

The presumptive fungal specie (after morphological identification) was genomically aligned with the nucleotide sequence obtained for molecular identification (Fig. 1). The isolated fluoride adsorbing *Trichoderma* species was 100% identical to *Trichoderma harzianum* TH8 (Gene Bank accession number JN039051.1) genomic alignment was confirmed (verified with BLAST algorithm in NCBI).

The effect of biosorbent dose on fluoride removal is a significant parameter because this concludes the capacity of a biosorbent for given initial concentration of adsorbate at the constant operating conditions. It was observed that fluoride removal increased with an increase in biosorbent dose. The maximum adsorption capacity of fluoride was found to be 0.2 mg g^{-1} at biosorbent dose of 0.4 g 100ml^{-1}. The results showed that the biosorbent dose at 1.6 g binds maximum 38% of fluoride from groundwater and 29% to 20% fluoride removal

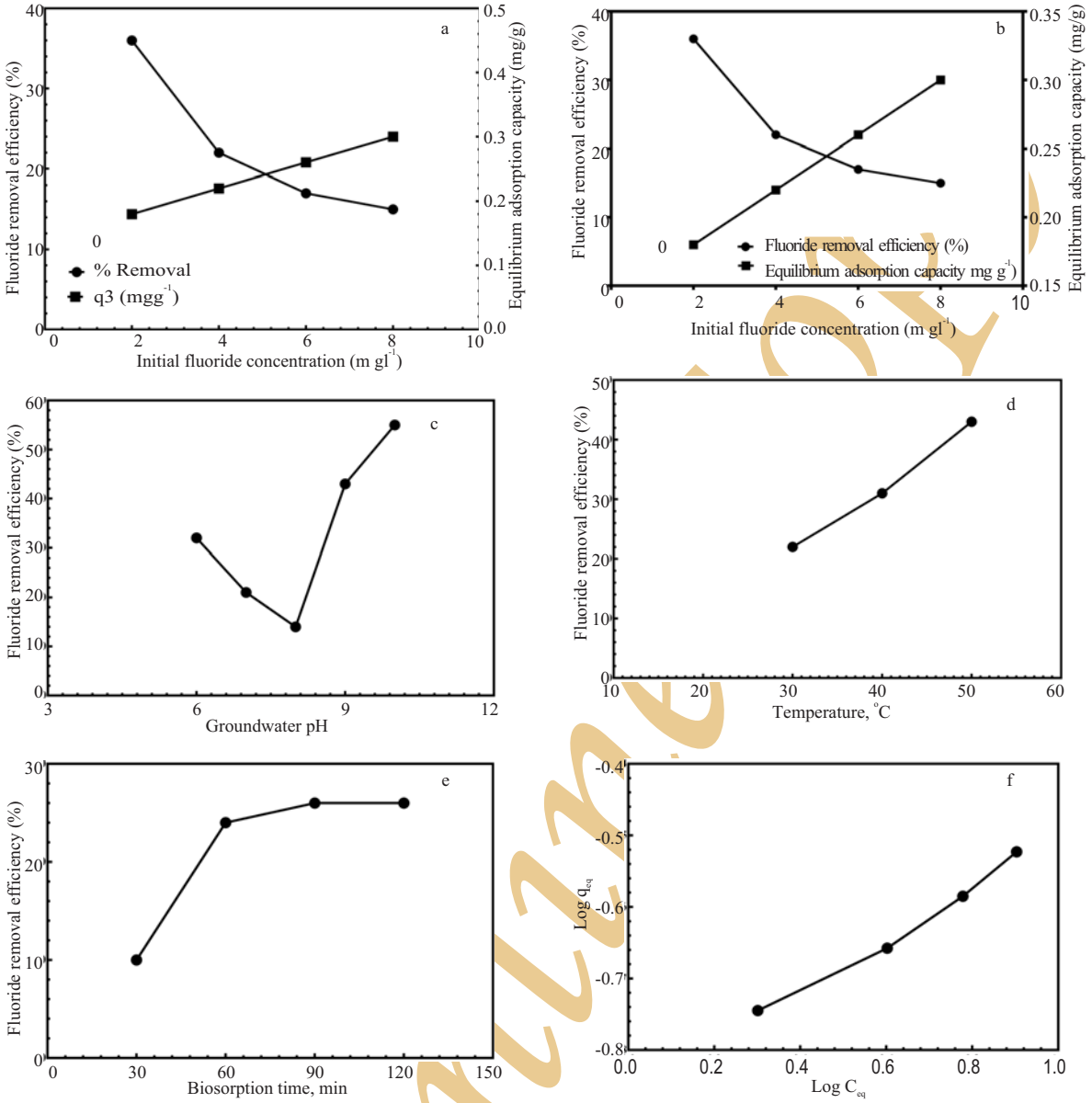

Fig.1: Effect of (a) biosorbent dose, (b) initial fluoride concentration, (c) ground water pH, (d) temperature and (e) biosorption time on fluoride removal.

was observed with 0.8g and 0.4 g of biosorbent, respectively (Fig 2a). The increase in fluoride removal efficiency with increase in biosorbent dose was due to increase in number of active sites with an increase in amount of biosorbent. Similar result was reported by Kumar *et al.* (2008) and Ramanaiah *et. al.* (2007) using neem and kikar leaves and *Pleurotus ostreatus*1804 as biosorbent, respectively. It can also be seen from Fig 2a. that increase in biosorbent dose lead to gradual decrease in adsorption capacity (amount of fluoride adsorbed per unit weight of biosorbent) due to increasing mass of sorbent per unit volume known as solid concentration effect *i.e.*, over crowding of particles (Mehrotra *et al.,* 1999).

Biosorption of fluoride also varied with varying range of initial fluoride concentration. It was observed that fluoride removal efficiency decrease gradually from 36% to 15% with increase in groundwater fluoride concentration from 2 to 8mgl^{-1} (Fig.2b). At fixed biosorbent dose, limited number of active sites are available which gets saturated at higher concentration of fluoride, resulting in lower fluoride removal

efficiency as reported by Jamode *et al.* (2004). Therefore, low initial fluoride concentration is favourable for higher defluoridation efficiency. It was also evident from Fig. 1b that increase in initial fluoride concentration at fixed biosorbent dose, increased the amount of fluoride adsorbed per unit weight of biosorbent, which result in higher equilibrium adsorption capacity.

Groundwater pH is one of the operating parameters which strongly influence fluoride removal by biosorption. Biosorption is a surface phenomenon, therefore sorption capacity of biosorbent depends on charge and ionization state of functional groups present on the biosorbent surface. Ionization of biosorbent surface functional group, as well as, the ionic form of fluoride depends on pH of aqueous medium (Yadav *et al.,* 2013). Fungal cell wall consist of high amount of polysaccharide (*i.e.*, chitin, chitosan), protein and small amount of lipid and inorganic salts like Ca^{++}, K^{+} (Herrera, 2012). It was found that fluoride removal efficiency decreased with increase in pH of solution from 6 to 8. It was also observed that fluoride removal increased with increase in pH from 9 to 10. Highest biosorption values were observed at pH 10 (55%). Dobaradaran *et al.* (2014) and Shams *et al.* (2010) also reported high fluoride removal efficiency at higher pH. High acidic (2-5) medium was not studied for fluoride biosorption as chitosan solubility increases due to protonation of amino groups in acidic medium (Agnihotri *et al.,* 2004) and high concentration of free H^{+} attribute to hydrofluoric acid formation which reduces fluoride biosorption.

An increase in biosorption efficiency was observed with increase in temperature from 30°C to 50 °C (Fig. 1d). Mohan *et al.* (2007) and Ramanaiah *et al.* (2007) reported that availability of active surface sites increased the number for sorption on the biosorbent or due to thinning of boundary layer surrounding the biosorbent, so that the mass transfer resistance of adsorbate in the boundary layer decreased attribute to an apparent increase in biosorption efficiency with increase in temperature.

The effect of biosorption time on fluoride removal efficiency was studied. It is found that removal of fluoride ions increased with increase in biosorption time to some extent. Further increase in biosorption time did not increase the fluoride removal due to deposition of fluoride ions on the available active sites on fungal biosorbent (Fig. 2e). It was observed that faster biosorption rate was at first 60min which reached to equilibrium at 90min and later there was no increase in biosorption i.e. biosorption reached saturation. The availability of adsorbent sites for coordination of anion, electrostatic affinity, ion-exchange and high solute concentration gradient may attribute to rapid initial uptake of fluoride from biosorbent (Mariappa *et al.,* 2002), but after the equilibrium point, the rate of fluoride uptake was constant due to decrease in fluoride concentration in the solution.

A theoretical plot of Freundlich's adsorption isotherm model [redacted] with the experimental data is shown in Fig. 2. The plot of log q_{eq} against Log C_{eq} was linear at various initial concentrations of fluoride fit with a relatively good correlation coefficient (R^2 0.98), justifying the acceptability of Freundlich adsorption isotherm model for the studied fungal–fluoride system. The value of Freundlich's constant k_f for fluoride ion was found to be 1.14, which indicated greater affinity for fluoride ion and value of n was 2.75, showed adsorption intensity of fungal biosorbent. The value of n between 1 to 10 indicated favorable adsorption. The experimental data with Langmuir isotherm model did not lead to linearization (R^2 0.91), indicating non-acceptability of isothermal model.

Desorption studies gives perception about the nature of adsorbent–adsorbate bonding. The effective desorption of fluoride ions from biosorbent was found in acidic solution. However, desorption, in the case of 0.1N NaOH, was less as compared to desorption with 0.1N HCl in all the cycles studied. It was observed that 82% desorption could be achieved successfully.

The present study ascertains the defluoridation potential of *Trichoderma hezardium*. *Trichoderma hezardium* as biosorbent gives high defluoridation efficiency at higher adsorbent dose and low initial fluoride concentration in alkaline medium. The process of fluoride adsorption on *Trichoderma hezardium* biosorbent from groundwater followed Freundlich isotherm, which indicates heterogeneous in nature of surface binding sites. The value of adsorption capacity (k_f) and intensity of adsorption (1/n) indicate great affinity for fluoride. This is the first report indicating that *Trichoderma hezardium* as an effective biosorbent for the removal of fluoride from groundwater.

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