4. Effect of various carbon sources, nitrogen sources, pH and temperature on the removal of fluoride in MSM and ground water through bioreactor using *Bacillus licheniformis* (PPR8)

4.1. Introduction

The advances in organic production have led to the beginning of many new organic compounds into the environment. Fluoro-aromatics compounds are being gradually more used in a wide range of agrochemical and pharmaceutical products due to the requirement to develop the environmentally suitable alternatives to chlorinated compounds (Key *et al.*, 1997). The diversity, structures and the chemical inertness of many halogenated organic components are particular problems and challenges for microbial degradation (Fewson, 1981).

The microorganisms have acquired a mixture of mechanisms for adaptation of excising toxic elements present in soil and water. Among various adaptation mechanisms, mineralization, metal sorption, accumulation, extracellular precipitation, enzymatic oxidation or reduction to a less toxic form and efflux of xenobiotics from the cell has been reported (Hughes and Poole, 1991; Joshi-Top and Francis, 1995; Hussein *et al.*, 2004). These mechanisms are for a while encoded in plasmid genes facilitate transmit of toxic metal resistance from one cell to another (Silver and Phung, 1996). The detoxifying ability of these resistant microorganisms can be manipulated for bioremediation of toxic elements in wastewater. They are *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Staphylococcus* have been shown to be relatively capable in bioaccumulation of uranium, copper, lead and other metal ions from polluted effluents (Filali *et al.*, 1999; Gupta *et al.*, 2001). The bioremediation of xenobiotics using microorganisms has received a huge agreement of interest in recent years, not only as a scientific novelty but also for its potential application.
in industry. A lot of plants and bacteria produce high affinity anion binding compounds called ionophores. The ionophores bind particular chemical forms of anions (Kim et al., 1998). These anion ionophore complexes are then absorbed by organism for consumption.

Fluorine is considered to be a most electronegative and most reactive among all the chemical elements found in the periodic table. The fluoride contamination in groundwater has drawn worldwide attention due to its important impact on human health. As per previous reports, fluoride concentration beyond the permissible limit of 1.5 ppm has affected more than 260 million people in globally. Fluoride is reportedly endemic in approximately 25 countries includes India, China and Africa being severely affected. There is no treatment for fluorosis; therefore prevention is the only means of controlling the disease. In this study, carbon sources, nitrogen sources, pH and temperature has been used for the bacterial growth and efficient removal of fluoride. Here carbon sources, nitrogen sources were selected as they are representative sources with various concentrations (0.5, 1.0 and 1.5%) and applied for removal of fluoride from aqueous medium. Thus, the objectives of this study were framed:

(i) To study the effect of various concentration of carbon sources and nitrogen sources (0.5, 1 and 1.5%) on the removal of fluoride in mineral salt medium (MSM) by Bacillus licheniformis PPR8.

(ii) To study the effect of various pH (pH 6, 7, 8 and 9) and various temperature (25, 30, 35, 40 and 45°C) on the removal of fluoride in mineral salt medium (MSM) by Bacillus licheniformis PPR8.

(iii) Removal of fluoride from fluoride contaminated ground water through lab scale bioreactor study and analysis of physicochemical parameters in treated water samples.
4.2. Material and Methods

4.2.1. Standard methods for fluoride analysis using SPADNS reagent

Fluoride analysis was carried out by standard methods using SPADNS reagent. SPADNS solution was prepared by dissolving 958 mg SPADNS in double distilled water and diluted to 500 ml. Zirconyl acid reagent was prepared by dissolving 133 mg zirconyl chloride octahydrate in about 25 ml distilled water, then added 350 ml concentrated hydrochloric acid and diluted to 500 ml with distilled water. Acid zirconyl SPADNS reagent was prepared by mixing equal volume of SPADNS solution and zirconyl acid reagent. Reference solution was prepared by adding 10 ml SPADNS solution to 100 ml double distilled water.

For the preparation of standard curve, the standard solution was prepared by dissolving 0.221 g of anhydrous sodium fluoride in water and diluted up to one liter. The stock solution was further diluted to get standard solution having 10 ppm of fluoride. The stock solution was further diluted to get 100 ppm by 10 ml of stock solution was mixed with 90 ml of double distilled water. From the 100 ml of fluoride stock solution, various concentrations of fluoride (2, 4, 6, 8, 10 and 12 ppm) were prepared in 50 ml of double distilled water. From each 50 ml of various concentration of fluoride solution, about 4 ml of samples were withdrawn and mixed with 1 ml of SPADNS solution and allowed to one minute for reaction time. The absorbance of all samples was measured at 570 nm against a reagent blank. Then a calibration plot was constructed by plotting absorbance against fluoride concentrations using UV-Vis spectrophotometer (Model: Cyberlab UV100, USA) (Alwarez et al., 2009).

4.2.2. Microorganism and preparation of inoculums

The colony morphology of the bacterium Bacillus licheniformis PPR8 was observed on the nutrient agar plate after the incubation at 37°C for 24 h. The cell morphology was examined using a scanning electron microscope (SEM: ZEISS, Germany) with 20,000V accelerating voltage and 10,000 times enlargement.
A loopful culture of *Bacillus licheniformis* PPR8 was inoculated in presterilized 100ml nutrient broth. The flask was kept in a shaker at 120 rpm for 12 hrs at 30°C. The culture broth was centrifuged at 4000 rpm for 20 min. Cell suspension was prepared using sterile distilled water and adjusted to 1OD consisting of $10^8$ CFU/ml using UV-Vis Spectrophotometer (Model: Cyberlab UV100, USA). One ml consisting $10^8$ CFU/ml of the above suspension was used as inoculum for the fluoride removal.

### 4.2.3. Effect of various concentrations of glucose on the removal of fluoride

The effect various concentrations of glucose on the removal of fluoride was investigated by using *Bacillus licheniformis* PPR8 (Plate 4.1). About 100 ml mineral salt medium (MSM) was prepared in 250 ml conical flasks with various concentrations (0.5, 1.0 and 1.5%) of glucose and amended with 15 ppm constant fluoride and they were respectively sterilized. After the sterilization, about 1% of *Bacillus licheniformis* PPR8 at 1OD culture was transferred aseptically. Then all conical flasks were kept in a shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, about 5 ml samples were withdrawn from each conical flasks and centrifuged at 3000 rpm for 10 minutes. Then the supernatant was collected from the centrifuge tube and fluoride was estimated by SPADNS reagent method using UV-Vis Spectrophotometer at 570 nm. In relation to fluoride removal, the growth of the bacterial strains was monitored from the aqueous medium. The cell pellets were obtained from centrifugation (3000 rpm for 20 minutes) and re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

### 4.2.4. Effect of various concentrations of cellulose on the removal of fluoride

The effect various concentrations of cellulose on the removal of fluoride was investigated by using *Bacillus licheniformis* PPR8 (Plate 4.2). In 250 ml conical flasks about 100 ml mineral salt medium (MSM) was prepared with various concentrations (0.5, 1.0 and 1.5%) of cellulose and amended with 15 ppm of
fluoride and they were sterilized. To it, about 1% of 1OD Bacillus licheniformis PPR8 was transferred aseptically. Then all conical flasks were kept in shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, about 5 ml samples were collected from the all conical flasks and the amount of fluoride was estimated by SPADNS reagent method in UV-Vis Spectrophotometer at 570 nm. The bacterial strains were monitored in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

4.2.5. Effect of various concentrations of starch on the removal of fluoride

The effect various concentrations of starch on the removal of fluoride was investigated by using Bacillus licheniformis PPR8 (Plate 4.3). In 250 ml conical flasks 100 ml mineral salt medium (MSM) was prepared with various concentrations (0.5, 1.0 and 1.5%) of starch and amended with 15 ppm constant fluoride and they were sterilized. About 1% of 1OD Bacillus licheniformis PPR8 was transferred aseptically to the sterilized medium. Then, all conical flasks were kept in shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, about 5 ml samples were collected from the all conical flasks and the amount of fluoride was estimated by SPADNS reagent method in UV-vis Spectrophotometer at 570 nm. The bacterial growth was checked in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

4.2.6. Effect of various concentrations of beef extracts and 1% cellulose on the removal of fluoride

The effect various concentrations of beef extract and 1% cellulose on the removal of fluoride was investigated by using Bacillus licheniformis PPR8. About 100 ml mineral salt medium (MSM) was prepared in 250 ml flask with various concentrations (0.5, 1.0 and 1.5%) of beef extract amended with 1% of cellulose (presumptively selected) and 15 ppm of constant fluoride and all the flasks were
sterilized. After the sterilization, 1% of *Bacillus licheniformis* PPR8 containing 1OD culture was transferred by aseptically. All conical flasks were kept in shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, 5 ml samples were collected and the amount of fluoride was estimated by SPADNS reagent method in UV-Vis Spectrophotometer at 570 nm. The growth of the bacterial strains was monitored in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

4.2.7. **Effect of various concentration of yeast extract and 1% cellulose on the removal of fluoride**

The effect various concentrations of yeast extract and 1% cellulose on removal of fluoride was investigated by using *Bacillus licheniformis* PPR8. About 100 ml mineral salt medium (MSM) was prepared in 250 ml flasks with various concentrations of yeast extract (0.5, 1.0 and 1.5%) amended with 1% of cellulose with 15 ppm constant fluoride and all flasks were sterilized. After the sterilization, 1% of *Bacillus licheniformis* PPR8 containing 1OD culture was transferred aseptically. All conical flasks were kept in shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, about 5 ml samples were collected and the amount of fluoride was estimated by SPADNS reagent method in UV-Vis Spectrophotometer at 570 nm. The growth of the bacterial strains was monitored in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

4.2.8. **Effect of various concentration of peptone and 1% cellulose on the removal of fluoride**

The effect various concentrations of peptone and 1% cellulose on removal of fluoride was investigated by using *Bacillus licheniformis* PPR8. About 100 ml mineral salt medium (MSM) was prepared in 250 ml flasks with various
concentrations of peptone (0.5, 1.0 and 1.5%) amended with 1% of cellulose with 15 ppm constant fluoride and all flasks were sterilized. After the sterilization, 1% of *Bacillus licheniformis* PPR8 containing 1OD culture was transferred aseptically. All conical flasks were kept in shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, 5 ml samples were collected and the amount of fluoride was estimated by SPADNS reagent method in UV-Vis Spectrophotometer at 570 nm. The growth of the bacterial strains was monitored in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

### 4.2.9. Effect of various pH with 1% of cellulose and 0.5% yeast extract on the removal of fluoride

The effect various pH conditions (pH 6, 7, 8 and 9) with 1% of cellulose and 0.5% yeast extract on the removal of fluoride was investigated by using *Bacillus licheniformis* PPR8 (Plate 4.4). About 100 ml mineral salt medium (MSM) was prepared 250 ml flasks with varying pH (pH 6, 7, 8 and 9) with 1% of cellulose and 0.5% yeast extract and amended with 15 ppm constant fluoride and all flasks were sterilized. The pH of the medium was adjusted by 1N of HCL and NaOH. After the sterilization, about 1% of *Bacillus licheniformis* PPR8 containing 1OD culture was transferred aseptically. All the conical flasks were kept in a shaker at 37°C in 120 rpm for 10 days. Every 24 hours time interval, 5 ml samples were collected and the amount of fluoride was estimated by SPADNS reagent method in UV-Vis Spectrophotometer at 570 nm. The growth of the bacterial strains was monitored in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.
4.2.10. Effect of various temperatures with 1% of cellulose and 0.5% yeast extract on the removal of fluoride

The effect of various temperatures on the removal of fluoride was investigated by using *Bacillus licheniformis* PPR8. About 100 ml mineral salt medium (MSM) was prepared in 250 ml flasks with 1% of cellulose and 0.5% yeast extract amended with 15 ppm of constant fluoride. The pH of the medium was adjusted to 7.0 by using 1N HCL and NaOH and sterilized. After the sterilization, 1% of *Bacillus licheniformis* PPR8 containing 1OD culture was transferred aseptically. Then the individual conical flasks were kept under various temperatures, namely, 25, 30, 35, 40 and 45°C in an orbital shaker at 120 rpm for 10 days. Every 24 hours time interval, 5 ml samples were collected and the amount of fluoride was estimated by SPADNS reagent method in UV-Vis Spectrophotometer at 570 nm. The growth of the bacterial strains was monitored in the medium by the cell pellet obtained from centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

4.2.11. Characterization of bacterial cells before and after treatment with fluoride

The bacterial cells of *Bacillus licheniformis* PPR8 from the fluoride treated and the untreated aqueous medium was characterized by FTIR, SEM and TEM-EDAX. The FTIR was used to find out the functional groups involved in the removal of fluoride by infrared spectra of bacteria amended with and without fluoride was studied. The bacterial cells were dried and mixed with KBr and the infrared spectrum was obtained using an FTIR (Model: Spectrum RX1). The spectra were obtained within a scanning range 400-4000/cm.

The surface morphology and elemental composition of the bacterial cells after fluoride removal was investigated by using a Scanning electron microscope (Model: SEM-ZEISS, Germany) operated at 200 kV accelerating voltage. Transmission electron microscopy (TEM) (Model: JEOL, JEM 3010- Japan)
equipped with EDAX was performed to identify the accumulation of fluoride inside the bacterial cells. For sample preparation, the bacterial strain *Bacillus licheniformis* (PPR8) was grown with 15 ppm fluoride concentration (experimental) and without fluoride as control. After 72 hrs incubation, the bacterial cells were harvested by centrifugation at 10000 rpm for 10 minutes. The centrifuged bacterial cells were washed with phosphate buffer (pH8) for several times and fixed by 3% glutaraldehyde. Further, it was washed several times with phosphate buffer. The sample was dried with various concentrations (10, 20, 30, 50, 70, 90 and 100 %) of ethanol under ambient conditions. Finally, the treated and untreated bacterial cells were re-suspended with 1ml of sodium phosphate saline. Then, the aliquots about 5µl of the bacterial cell suspension were mounted on 400 mesh Cu grids and followed by drying at overnight in a desiccator (Mishra et al., 2012).

4.2.12. Removal of fluoride from fluoride contaminated ground water through a lab scale bioreactor study

The method for fluoride contaminated water treatment was planned and the setup was made as shown in Plate 4.5. This set up was prepared based on the pilot scale water treatment plant chart out by Ayyasamy et al. (2008). The lab scale set up consists of reservoir, reactor tank, settling tank, filtration tank and collection tank. All the tanks are 10 litter capacities and made up of tarson. The reactor tank and settling tanks are fitted with mechanical stirrers. Artificial aerator was connected to both bioreactors for aeration purpose. About 10 liters of fluoride contaminated water containing 13 ppm of fluoride was subjected to primary (bioprocess) treatment inoculated with *Bacillus licheniformis* PPR8. Every 24 hours time interval, about 5 ml samples were collected from both bioreactors. The collected samples were centrifuged at 3000 rpm for 10 minutes. Then the supernatant was collected from the centrifuge tube and the amount of fluoride was estimated by SPADNS method in UV-Vis Spectrophotometer at 570 nm. The growth of the bacterial strains was monitored in the medium by the cell pellet
obtained from the centrifugation was re-suspended with 5 ml distilled water and its absorbance value was noticed at 600 nm for bacterial growth.

The treatment setup was made as shown in the following protocols:

**Reactor 1 (R1):** F-Contaminated ground water + 1% cellulose + 1% Inoculum

**Reactor 2 (R2):** F-Contaminated ground water + 0.5% Yeast extract + 1% Inoculum

### 4.2.12.1. Physicochemical parameters of untreated and treated fluoride contaminated water

The fluoride contaminated water was collected from the Alamelupuram village of pappireddypatti block of Dharmapuri district in Tamil Nadu, India. The sample was taken in sterile container, transported to the laboratory and analyzed various physicochemical parameters like pH, colour, total fluoride, total solids, total suspended solids and total dissolved solids (APHA, 2005). All the physic-chemical parameters of samples before and after treatment were analyzed (Table 4.1).

### 4.2.12.2. Filtration of fluoride contaminated treated water by sand filtration

The bacterially treated fluoride contaminated water filtered by sand filtration and it was shown in Plate 4.6. The glass column having 75 cm in length and 10 cm in diameter was taken. The bottom of the column was closed with a glass stopper. To this, large stones (2.0 to 2.3 cm) were packed in bottom regions followed by small stones (0.7 to 1.5 cm), gravel (0.4 to 0.6 cm), coarse sand (0.05 to 0.1 cm), and fine sand (0.15 to 0.3 mm). The bacterially treated water was transferred to the column. Then the filtered water was collected in a separate sterile conical flask and tested for their toxicity on onion (*Allium cepa*).

### 4.3. Results

#### 4.3.1. SPADNS reagent standard curve

The Fig 4.1 was shown the prepared SPADNS reagent standard curve. Absorbance at various concentrations of fluoride samples were taken OD at 570 nm after setting absorbance of reference solution as zero. Various concentrations
of fluoride vs absorbance were plotted on a graph to find out the concentrations of fluoride in unknown water samples.

**4.3.2. The potential strain of Bacillus licheniformis PPR8**

The Table 4.2 showed the strain *Bacillus licheniformis* PPR8 was gram-positive, citrate and starch hydrolyzing organism. The strain PPR8 utilizes glucose, cellulose and starch as the suitable carbon sources. Among the carbon sources, the cellulose exhibits a significant growth in the cellulose agar medium. The Fig. 4.2 was shown that the morphology and cell size of the potential bacterial strain characterized by SEM. The physiological and biochemical features of strain PPR8 met the typical characteristics of *Bacillus licheniformis* PPR8.

**4.3.3. Removal of fluoride in MSM with various concentration of glucose**

The effect of various concentrations (0.5, 1.0 and 1.5%) of glucose on the removal of fluoride by *Bacillus licheniformis* PPR8 was studied and the results are presented in Fig.4.3a. In this study, the MSM broth containing 15 ppm of fluoride and 1% inoculums was used. In which, by using 0.5% of glucose, there was no significant fluoride removal was observed from 1st to 5th day of the experiment. After the 5th to 8th day, there was better significant fluoride removal (87%) was observed. The maximum fluoride removal (68%) was observed throughout the experiment by using 1% of glucose. About 75% of fluoride removal was observed during 7 to 9th day of the experiment. By using 1.5% of glucose, there was a significant fluoride removal, which was 73% observed from the 3rd day to throughout the period. Overall optimization study in the different concentration of glucose, 1.5% of glucose was showing a very significant fluoride removal. The Fig.4.3b was showed the growth rate of *Bacillus licheniformis* PPR8 at various concentrations of glucose (0.5, 1.0 and 1.5%) in the MSM medium containing 15 ppm fluoride. In this study, 0.5 and 1.5% of glucose showed there was significant bacterial growth in 1st to 10th day of experiment and it influences the fluoride removal. In case of 1% of glucose, there was no significant of fluoride removal.
Both 0.5 and 1.5% of glucose was severely influenced the removal of fluoride as well as the growth rate of Bacillus licheniformis PPR8.

4.3.4. Removal of fluoride in MSM with various concentration of cellulose

The effect of various concentrations (0.5, 1.0 and 1.5%) of cellulose on the removal of fluoride by Bacillus licheniformis PPR8 was studied and the results are presented in Fig.4.4a. In this study, the MSM containing 15 ppm of fluoride and 0.5% of cellulose, there was no significant removal of fluoride from 1st to 5th day of the experiment. After the 5th to 8th day of the experiment, there was better significant of fluoride removal which was 83%. By using 1% of cellulose, the maximum fluoride removal was observed (94%) on 4 to 8th day of the study. By using 1.5% of cellulose, there was a significant fluoride removal (86%) was observed from 6th to 9th day of the experiment. The optimization of cellulose with different concentrations showed that 1% of cellulose was achieved a very significant fluoride removal. The Fig.4.4b was showed the growth rate of Bacillus licheniformis PPR8 under various concentrations of cellulose (0.5, 1 and 1.5%) in the MSM medium containing 15 ppm fluoride. In this study, using 0.5 and 1.0% of cellulose, there was significant growth was noted in from 1st to 10th day of experiment and it influences the removal of fluoride. Using 1.5% of cellulose in this study, there was no significant removal of fluoride. In which both 0.5 and 1% of cellulose were strictly influenced the fluoride removal as well as the growth rate of Bacillus licheniformis PPR8.

4.3.5. Removal of fluoride in MSM with various concentration of starch

The effect of various concentrations (0.5, 1.0 and 1.5%) of starch on the removal of fluoride by Bacillus licheniformis PPR8 was presented in Fig.4.5a. In this study, the MSM was used with containing 15 ppm of fluoride and 1% inoculum. In which, using 0.5% of starch there was no significant fluoride removal was observed from 1 to 5th day of the experiment. In the study after the 6th to 8th, there was a significant fluoride removal was observed which was 82%. By using
starch at 1%, the maximum fluoride removal was observed (90%) during 5 to 9th day of the experiment. By using 1.5% of starch, there was a significant fluoride removal (91%) was observed from 6 to 9th day of the experiment. In the optimization of starch at different concentrations, the starch at 1.5% was shown a very significant removal of fluoride. The Fig.4.5b was showed the growth rate of *Bacillus licheniformis* PPR8 at various concentrations (0.5, 1.0 and 1.5%) of starch in the MSM medium containing 15 ppm of fluoride. Various concentrations of starch were severely influenced the removal of fluoride as well as the growth rate of *Bacillus licheniformis* PPR8.

**4.3.6. Removal of fluoride in MSM with various concentrations of beef extracts**

The effect of various concentrations (0.5, 1.0 and 1.5%) of beef extracts on the removal of fluoride by *Bacillus licheniformis* PPR8 was presented in Fig.4.6a. In this study, the MSM broth containing 15 ppm of fluoride and 1% inoculum was used. Using 0.5% of beef extract, there was no significant fluoride removal (30%). By using 1% of beef extract, the fluoride removal was maximum (72%) from 2 to 9th day of the experiment. By using 1.5% of beef extract, there was a significant fluoride removal (78%) from 2 to 9th day of experiment. In the optimization of beef extract at different concentration, 1.5% of beef extract was shown a very significant fluoride removal. The Fig.4.6b was showed the growth rate of *Bacillus licheniformis* PPR8 at various concentrations of beef extract (0.5, 1 and 1.5%) in the MSM medium containing 15 ppm of fluoride. The beef extract at all concentrations does not show a significant influence on the removal of fluoride as well as growth rate of *Bacillus licheniformis* PPR8 in MSM.

**4.3.7. Removal of fluoride in MSM with various concentrations of yeast extracts**

The effect of various concentrations (0.5, 1.0 and 1.5%) of yeast extract on the removal of fluoride by *Bacillus licheniformis* PPR8 was presented in Fig.4.7a. In this study, the MSM containing 15 ppm of fluoride and 1% inoculum showed a very good efficiency on fluoride removal. In which, by using 0.5% of yeast extract,
there was a significant fluoride removal (93%) from 1 to 10th day of experiment. By using 1% of yeast extract the maximum fluoride removal was observed (90%) from 1 to 8th day of the experiment. By using 1.5% of yeast extract, there was less fluoride removal (56%) from 1 to 10th day of the experiment. In this optimization study, 1.5% of yeast extract was achieved very significant fluoride removal. The Fig.4.7b was showed the growth rate of *Bacillus licheniformis* PPR8 under various concentrations of yeast extract (0.5, 1 and 1.5%) in the MSM medium containing 15 ppm of fluoride. The yeast extract at various concentrations were severely influenced the removal of fluoride as well as the growth rate of *Bacillus licheniformis* PPR8 in MSM.

### 4.3.8. Removal of fluoride in MSM with various concentrations of peptone

The effect of various concentrations (0.5, 1.0 and 1.5%) of peptone on the removal of fluoride by *Bacillus licheniformis* PPR8 was presented in Fig.4.8a. In this study, the MSM containing 15 ppm of fluoride and 1% inoculum was used. In which, using 0.5% of peptone there was no significant fluoride removal (44%) was observed. The maximum fluoride removal was observed (58%) using 1% of peptone as a nitrogen source. In the case of peptone at 1.5%, there was a significant fluoride removal (90%). In the overall optimization with different concentration of peptone, the 1.5% of peptone was showing a very significant removal of fluoride. The Fig.4.8b was showed the growth rate of *Bacillus licheniformis* PPR8 at various concentrations of peptone (0.5, 1 and 1.5%) in the MSM medium containing 15 ppm of fluoride. The peptone at all concentrations showed a significant influence on the fluoride removal as well as the growth rate of *Bacillus licheniformis* PPR8.

### 4.3.9. Removal of fluoride in MSM at various pH

The effect of various pH (5, 6, 7, 8 and 9) on the removal of fluoride by *Bacillus licheniformis* PPR8 was presented in Fig.4.9a. In the present study, the effects of different pH (5, 6, 7, 8 and 9) on the removal of fluoride were studied. The varying pH was severely influenced the removal of fluoride. The operation
conditions; 15 ppm of fluoride/100 ml medium, temperature 35°C and 1ml of one OD inoculums was used in this study. All the pH from 5 to 9 was very significantly acted for removing the fluoride from the aqueous medium. In which, the maximum removal of fluoride was observed at pH7. Hence, the further studies were carried out using MSM with pH7. The figure 4.9b was showed the growth rate of *Bacillus licheniformis* PPR8 at various pH conditions. All pH parameters were significantly influenced the growth rate and also removal of fluoride.

**4.3.10. Removal of fluoride in MSM at various temperatures**

The effect of various temperatures (25, 30, 35, 40 and 45°C) on the removal of fluoride by *Bacillus licheniformis* PPR8 was presented in Fig.4.10a. Temperature is an important factor which has effects on microbial removal of fluoride. In the present study, the effect of *Bacillus licheniformis* PPR8 at different temperature and incubation time on fluoride removal was studied. The operation conditions were 15 ppm of fluoride/100 ml MSM medium, pH7 and inoculums volume of 1% of one OD. The Fig. 4.10b was showed the growth rate of *Bacillus licheniformis* PPR8 at various temperatures. In this study, all the temperatures were influenced the removal of fluoride. However, the 35°C was showed maximum removal of fluoride when compared to other temperatures.

**4.3.11. FTIR analysis of bacterial cell before and after treatment of fluoride**

FTIR spectrum of *Bacillus licheniformis* PPR8 cells in the presence and absence of fluoride was shown the adsorption. The FTIR analysis of the potential isolate is required to investigate chemical bonds that played a role in the absorption of fluoride. The FTIR spectrum of PPR8 grown in the nutrient broth obtained and the results are presented in Fig. 4.11a. The band 3448.11 cm\(^{-1}\) was represented the N-H symmetric amide groups and 1642.13 cm\(^{-1}\) shown that the C=O stretching. The band 1445.48 cm\(^{-1}\) represent the N-H band and 701.10 cm\(^{-1}\) represent the C-H band. The band 1121.34 to 1082.24 cm\(^{-1}\) was very clearly represented the C-F stretching due to the accumulation of fluoride on the bacterial
cells with and it was presented in (Fig. 4.11b). It was clearly shown that all band positions have small shifts after adsorption of fluoride. The previous study reported that porous zirconium alginate beads adsorbent for fluoride sorption from aqueous solution and in that the analysis of fluoride treated zirconium alginate beads by FTIR showed the bands at 1384.1 and 1036.6 cm$^{-1}$ moved towards higher wavelength indicating interactions among adsorbent and fluoride (Qiusheng et al., 2014).

4.3.12. Analysis of bacterial cells by SEM and TEM-EDAX before and after treatment of fluoride

The SEM analysis of potential bacterial strain after 72 h of incubation of with and without fluoride exposure was studied to determine the morphological characteristics. The Fig.4.12a was shown that the structure of the bacterial strain. The bacterial cell was short, rod shaped and has a smooth surface. But when the bacterial cells exposed to fluoride at 15 ppm, the nature of the bacterial cell wall was occurring as small ridges and changes in morphology (Fig. 4.12b). The assessment of fluoride accumulation inside the bacterial cell was observed by TEM-EDAX for the identification of fluoride accumulation inside the cells of Bacillus licheniformis PPR8 was performed in control (Fig. 4.13 a & c) and treated cells (Fig. 4.13b & d). The TEM images revealed that intracellular changes in PPR8 strain due to the exposure to fluoride showed the black coloured cells indicated that fluoride was accumulated inside the bacterial cells. The analyses of untreated and treated bacterial cells were investigated by EDAX. As shown in Fig. 4.13e, there was no fluoride peak observed in untreated bacterial cells, but when compared to a treated bacterial cell with fluoride, the fluoride peak was observed due to the interaction between the cell with fluoride (Fig. 4.13f).

4.3.13. Optimized conditions in batch mode studies

In batch mode studies, the effect of various concentrations of carbon sources, nitrogen sources, pH and temperature on fluoride removal was studied in
mineral salt medium (MSM) amended with 15 ppm of fluoride. The efficient fluoride removal was achieved in MSM amended with 1.0 % of cellulose and 0.5 % of yeast extract at with an optimum pH 7 and temperature at 35°C. Hence, 1.0 % cellulose and 0.5% yeast extract were selected as optimum carbon and nitrogen sources respectively for further ground water treatment. The results for optimized conditions were presented in Table 4.3.

**Table 4.3. Optimized conditions in the batch mode study**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cellulose (Carbon source)</td>
<td>1%</td>
</tr>
<tr>
<td>2</td>
<td>Yeast extract (Nitrogen source)</td>
<td>0.5%</td>
</tr>
<tr>
<td>3</td>
<td>pH</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Temperature</td>
<td>35°C</td>
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**4.3.14. Removal of fluoride from fluoride contaminated water through lab scale bioreactor**

Bioreactor study was carried out on the removal of fluoride in ground water sample collected from Alamelupuram areas of the Pappiredypatti block in Dharmapuri District since it contains maximum levels (13 ppm) of fluoride. Water sample containing 13 ppm of fluoride was treated with the fluoride tolerant bacterial strain *Bacillus licheniformis* PPR8. About 90% (from 13 to 1.3 ppm) of fluoride was removed by PPR8 in water samples containing 13 ppm of fluoride supplemented with 1% cellulose from 3 to 8th day of experiment (Fig. 4.14). Followed by 97% (from 13 to 0.4 ppm) of fluoride was removed by PPR8 in water sample containing 13 ppm of fluoride supplemented by 0.5% yeast extract from 4 to 10th day of the experiment. Finally, the strain PPR8 removed the fluoride below the permissible limits. During fluoride removal, the bacterial populations were also increased to higher numbers.
4.3.15. Physico-chemical parameters of fluoride contaminated water

The raw fluoride contaminated water sample was collected from the Alamelupuram area of the Pappireddypatti block in Dharmapuri District in Tamil Nadu. The before and after the treatment using aerobic bacterium, the physico-chemical parameters of water samples were checked and the results are presented in Table 4.4. The collected fluoride contaminated water sample has more salinity and the other parameters were noted above the permissible limits. After the treatment of fluoride contaminated water supplemented with 1% of cellulose using *Bacillus licheniformis* PPR8 was showing a significant removal of salinity and other physico-chemical parameters below the permissible limits as fixed by BIS and other organizations. After the treatment by *Bacillus licheniformis* PPR8 in the fluoride contaminated water with 0.5% of yeast extract, the most of the salts and other parameters were also reduced below the permissible limits as fixed by the BIS. In the treated water samples supplemented with 0.5% yeast extract, the physic chemical parameters were significantly reduced when compared to water treated with 1% cellulose.

4.3.16. Mechanism of intracellular accumulation of fluoride in bacterial cells

The mechanism of fluoride sorption was clearly presented in the diagrammatic representation (Fig. 4.15). Biosorption of fluoride by *Bacillus licheniformis* PPR8 was proved by determining with various instrumental analyses. The export of fluoride anion through fluoride specific ion channels of the Fluc family. The plasma membrane of many bacteria was carried fluoride exporter proteins to maintain cytoplasmic fluoride below inhibitory concentrations (Stockbridge *et al.*, 2015). In the absence of potential proteins, those microorganisms were lead to hypersensitivity to fluoride. So those microorganisms produced F-/H+ antiporters of the CLC anion transporters that were called as “Fluc” family of fluoride specific ion channel. The phenomenon of export of fluoride ions into the bacterial cell was by stimulus molecules bind to the protein channel.
of fluc and stimulated to open the channel, through the pore anionic fluoride enter the bacterial cell.

4.4. Discussion

In general, the fluoride removal by microorganisms is depends upon the nature and its chemistry of the fluoride, characteristic features and its physiology of the microorganisms and physicochemical influences from the environment e.g. pH, temperature and media composition. For that, these responsible factors for fluoride removal was considered in the given bioremediation process. In particular, the effect of carbon and nitrogen source, pH and temperature on the fluoride removal was carried out. The carbon sources, nitrogen sources and various environmental parameters were significantly influenced the fluoride removal and bacterial growth since they are closely essential factors for bacterial growth and removal of fluoride. The optimum growth rate of bacterial strain, *Bacillus licheniformis* PPR8 in nutrient and MSM medium without any additional carbon source on fluoride removal showed that no major difference, it was revealed that MSM medium holds up the better the growth rate at neutral pH. In the presence of fluoride amended MSM, the growth rate of *Bacillus licheniformis* PPR8, revealed that the ability of utilization fluoride. The Oltmanns et al. (1989) reported that the effects of various 4-fluorobenzoate concentrations on the growth of the organisms and on the release of fluoride were determined in liquid culture. The highest optical density was reached not proportional to the amount of 4-fluorobenzoate amended to the culture medium. Turbidity greater than before at concentrations of up to 5 and 4 mM 4-fluorobenzoate for *Aureobacterium* sp. strain RH025 and *Alcaligenes* sp. strain RH022.

4.4.1. Removal of fluoride in MSM with various carbon and nitrogen source

In this study, the effect of various concentrations of carbon and nitrogen sources (0.5, 1.0 and 1.5%) on the removal of fluoride by bacteria was investigated. In which, 0.5% glucose was showed maximum fluoride removal which was 87%. In
the case of 1% cellulose showed 94% of fluoride removal and 1.5% showed the 98% of fluoride removal. In overall carbon sources, 1% cellulose was showing a very significant fluoride removal throughout the experimental period, when compared to other carbon sources. An effect of various concentrations of nitrogen sources (0.5, 1.0 and 1.5%) on the removal of fluoride by bacteria was investigated. In which, 1.5% of beef extract showed 78% of fluoride removal, 0.5% of yeast extract showed 98% of fluoride removal and 1.5% of peptone showed 90% of fluoride removal. As similar to our study, Mukherjee et al. (2017) was reported that effect of various carbon and nitrogen sources on the growth of Providencia vermicola (KX926492) and removal of fluoride was studied. The isolate KX926492 could utilize all the three carbon sources with dextrose showed the highest efficiency on fluoride removal. And use of nitrogen sources other than nutrient broth could not show the much effect on bacterial growth as well as fluoride removal.

4.4.2. Effect of various pH on the removal of fluoride

The pH plays a very significant role during the biological fluoride removal. So that a lot of researchers intense that the pH of the system is the most essential working parameter influencing the fluoride removal. In the present study, the effect of incubation time on removal of fluoride at different pH (5, 6, 7, 8 and 9) was studied. In which, all pH from 5 to 9 were very significantly removing the fluoride from the aqueous medium. In which the maximum removal of fluoride was observed at pH7 which is similar to previous studies reported by Mukherjee et al. (2017) and fluoride removal was decreased with increasing pH of the medium. This is due to the attributed to the fact that at higher pH. The presence of hydroxyl ions (OH-) is more and fluoride being strongly electronegative reacts repelled by the negatively charged hydroxyl ions (Mukherjee and Halder, 2016). The Mukherjee et al. (2018) was reported that 91.8% of fluoride removal was observed by using immobilized bacterial cells at neutral pH and least fluoride removal was observed at alkaline pH9. The pH7 was one of the superior conditions for the maximum fluoride
removal. This pH condition provided to be higher than the surrounding medium, it helps to provide free and bound intracellular fluoride (Whitford et al., 1977).

### 4.4.3. Effect of various temperatures on the removal of fluoride

In the present study, the effect of incubation time on removal of fluoride by *Bacillus licheniformis* PPR8 at different temperatures (25, 30, 35, 40 and 45°C) was studied. In which, all temperatures were influenced the removal of fluoride. The temperature at 35°C was showed maximum removal of fluoride when compared to other temperatures. Temperature is considered to be one of the vital parameters, particularly in case of energy dependent mechanisms in fluoride removal by bacteria. On certain environmental condition, temperature might affect the most stable conformation of the bacterial cell wall often ionizing the chemical entities that constitute the cell wall of bacteria (Subhashini et al., 2011).

### 4.4.4. Characterization of bacterial cells in before and after treatment of fluoride

In FTIR spectrum, the band 3448.11 cm\(^{-1}\) was represented the N-H symmetric amide groups and 1642.13 cm\(^{-1}\) shown that the C=O stretching. The band 1445.48 cm\(^{-1}\) represent the N-H band and 701.10 cm\(^{-1}\) represent the C-H band. The band 1121.34 to 1082.24 cm\(^{-1}\) was very clearly represented the C-F stretching due to the interaction between the bacterial cell with fluoride. The SEM image revealed that the bacterial cell was short, rod shaped and has a smooth surface. But when the bacterial cells exposed to fluoride at 15 ppm, the nature of the bacterial cell wall was small ridges and changes in morphology. As the previous reports, certain bacterial strains produced high-affinity anion-binding compounds called ionophores. These anionic ionophores bind to some forms of anionic and form a complex that can be utilized by the bacteria (Doble and Kumar 2005).

### 4.5. Conclusion

In the present study, the fluoride tolerant bacterial strain *Bacillus licheniformis* (PPR8) was used for removal of fluoride in aqueous medium. The 1%
of cellulose and 0.5% of yeast extract was showing very significantly influences on the defluoridation in aqueous media. The optimal pH for *Bacillus licheniformis* (PPR8) was 7.0 and temperature was 35°C. In this study, the bacterium PPR8 achieved a significant defluoridation in aqueous medium. The accumulation of fluoride inside the bacterial cells was characterized by FTIR, SEM and TEM-EDAX. All the studies carried out with instrumentation showed a strong support on the accumulation of fluoride inside the bacteria cells. The results in the study suggesting that the *Bacillus licheniformis* (PPR8) bacterium could be a potential strain for defluorination of drinking water and it may provide a good opportunity to develop a new bioremediation method.
Table 4.1. Analytical methods followed for estimation of physico-chemical parameters of water samples

<table>
<thead>
<tr>
<th>S.No</th>
<th>Test parameters</th>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>pH value</td>
<td>pH meter (Elico)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>2.</td>
<td>EC µS.cm⁻¹</td>
<td>Conductivity meter</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>3.</td>
<td>Total dissolved solid</td>
<td>Evaporation and drying (105°C)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>4.</td>
<td>Total suspended solid</td>
<td>Filtration and drying (105°C)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>5.</td>
<td>Total Hardness</td>
<td>Versenate method (Titration)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>6.</td>
<td>Total Alkalinity</td>
<td>Titration</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>7.</td>
<td>Dissolved oxygen</td>
<td>Dissolved oxygen meter</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>8.</td>
<td>BOD</td>
<td>Winkler's method (Titration)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>9.</td>
<td>COD</td>
<td>Reflex method (Titration)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>10.</td>
<td>Calcium</td>
<td>Versenate method (Titration)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>11.</td>
<td>Magnesium</td>
<td>Versenate method (Titration)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>12.</td>
<td>Chloride</td>
<td>Argentometric method (Titration)</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>13.</td>
<td>Sulphate</td>
<td>Turbidity method</td>
<td>APHA, 2005</td>
</tr>
<tr>
<td>14.</td>
<td>Fluoride</td>
<td>Spadns reagent method</td>
<td>APHA, 2005</td>
</tr>
</tbody>
</table>
Table 4.2. Physiological and biochemical characteristics of *Bacillus licheniformis* PPR8

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Originated from</td>
<td>Soil</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Finely wrinkled, opaque adherent colonies</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Average 0.5 to 1.2 μm, rod-shaped</td>
</tr>
<tr>
<td>Optimum growth temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Optimum pH for growth &amp; fluoride removal</td>
<td>7.0</td>
</tr>
<tr>
<td>Gram staining, spore staining, oxidase, starch hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>Indole production</td>
<td>Negative</td>
</tr>
<tr>
<td>Voges Proskauer and citrate utilization test</td>
<td>Positive</td>
</tr>
<tr>
<td>Glucose, starch and cellulose</td>
<td>Positive</td>
</tr>
<tr>
<td>16S rRNA gene identify</td>
<td><em>Bacillus licheniformis</em> (95% identify)</td>
</tr>
<tr>
<td>Accession number</td>
<td>KX646393</td>
</tr>
</tbody>
</table>
Table 4.4. Physico-chemical parameters of bacterial treated water samples collected from Alamelupuram village of pappireddypatti block

<table>
<thead>
<tr>
<th>S.No</th>
<th>Test parameters</th>
<th>Before treatment</th>
<th>After bacterial treatment</th>
<th>1.0% Cellulose</th>
<th>0.5% Yeast Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Colour</td>
<td>Colourless</td>
<td>Colourless</td>
<td>Light yellow</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>pH value</td>
<td>7.82</td>
<td>7.15</td>
<td>7.38</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>EC µS.cm⁻¹</td>
<td>14</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Total dissolved solid</td>
<td>912</td>
<td>625</td>
<td>813</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Total suspended solid</td>
<td>846</td>
<td>1012</td>
<td>1169</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Total Hardness</td>
<td>620</td>
<td>457</td>
<td>542</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Total Alkalinity</td>
<td>284</td>
<td>195</td>
<td>217</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Dissolved oxygen</td>
<td>7.4</td>
<td>2.6</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>BOD</td>
<td>46</td>
<td>163</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>COD</td>
<td>284</td>
<td>193</td>
<td>218</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Calcium</td>
<td>22</td>
<td>3.2</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Magnesium</td>
<td>57</td>
<td>21</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Chloride</td>
<td>238</td>
<td>165</td>
<td>199</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Sulphate</td>
<td>247</td>
<td>79</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Fluoride</td>
<td>13</td>
<td>1.3</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

All the values are expressed in mg/L except pH and EC
Fig. 4.1. Standard curve for fluoride analysis using SPADNS reagent

\[
y = -39.89x + 44.01 \\
R^2 = 0.943
\]

OD

Fig. 4.2. Potential strain of *Bacillus licheniformis* PPR8 for fluoride removal
Fig. 4.3. Removal of fluoride in MSM with various concentration of glucose

Fig. 4.4. Removal of fluoride in MSM with various concentration of cellulose
Fig. 4.5. Removal of fluoride in MSM with various concentrations of starch

Fig. 4.6. Removal of fluoride in MSM with various concentrations of beef extracts
Fig. 4.7. Removal of fluoride in MSM with various conc. of yeast extracts

Fig. 4.8. Removal of fluoride in MSM with various concentrations of peptone
Fig. 4.9. Removal of fluoride in MSM at various pH

Fig. 4.10. Removal of fluoride in MSM at various temperatures
Fig. 4.11. FTIR analysis of bacterial cell before and after treatment of fluoride.
Fig. 4.12. SEM analysis of bacterial cells before and after treatment of fluoride
Fig.4.13. TEM-EDAX analysis of bacterial cells before and after treatment of fluoride
Fig. 4.14. Removal of fluoride from fluoride contaminated water through lab scale bioreactor

![Graph showing fluoride removal over time with different conditions.]

Fig. 4.15. The mechanism of intracellular accumulation of fluoride in bacterial cells

**Outside of cells**

1. Stimulus molecule
2. Binding site
3. Fluoride ions
4. Protein channel
5. Plasmic membrane
6. Cytoplasm
7. Chromosomal DNA
8. Cell wall
9. The polar substances can diffuse across the membrane
Plate 4.1. Effect of various concentration of glucose (0.5, 1 and 1.5%) on the removal of fluoride

Plate 4.2. Effect of various concentration of cellulose (0.5, 1 and 1.5%) on the removal of fluoride
Plate 4.3. Effect of various concentration of starch (0.5, 1 and 1.5%) on the removal of fluoride

Plate 4.4. Effect of various pH (6, 7, 8 and 9) with 1% of cellulose and 0.5% glucose on the removal of fluoride
Plate 4.5. Removal of fluoride from fluoride contaminated ground water through lab scale bioreactor study
Plate 4.6. Filtration of fluoride contaminated water after treatment by sand filtration