Isolation and Characterization of a Fluoride Resistant Microbial Strain for Bioremediation of Polluted Water

Thesis Submitted in the partial fulfillment of the requirement of the Degree of

MASTER OF TECHNOLOGY IN FOOD AND BIOCHEMICAL ENGINEERING

Submitted By

Soocheta Jha

Examination Roll No. M4FTB19001

Registration No. 140861 of 2017 – 2018

Under The Guidance Of

Dr. Sunita Adhikari(Nee Pramanik)

Assistant Professor

Department of Food Technology & Biochemical Engineering

Faculty of engineering & technology

Jadavpur University

Kolkata – 700032

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Date:

Place:

_____

Soocheta Jha

M.Tech 2nd Year Student of Dept. of FTBE, JU

CERTIFICATE OF MERIT

DATE:

This is to certify that the work recorded in the report entitled "Isolation and Characterization of a Fluoride Resistant Microbial Strain for Bioremediation of polluted water" submitted by Soocheta Jha (M.Tech, 2nd Year, Department of Food Technology and Biochemical Engineering, Jadavpur University) is the faithful and bonafide research work carried out under my personal supervision and guidance. The result of investigation recorded in the report has not so far been submitted to any other project. The assistance and help received during the course of the process has been fully acknowledged.

Dr.Sunita Adhikari (NEE Pramanik)
Assistant Professor
Department of Food Technology and
Biochemical Engineering
Jadavpur University

Dr. Dipankar Haldar
HOD, Department of Food Technology
and Biochemical Engineering
Jadavpur University

Dean, Faculty Council of Engg. & Tech,

Jadavpur University, Kolkata - 700032

FACULTY OF ENGINEERING AND TECHNOLOGY

Jadavpur University

Kolkata - 700032

The foregoing thesis is hereby approved as a credible study in **Master of Technology in Food Technology and Biochemical Engineering** and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion therein but approve this thesis only for the purpose for which it is submitted.

Committee of Final examination for evaluation of thesis:

_____

(Signature of Examiner/s)

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(Signature of Supervisor)

Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research work by me as part of my Master of Technology in Food Technology and Biochemical Engineering studies.

All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

NAME: SOOCHETA JHA

EXAMINATION ROLL NO. : M4FTB19001

THESIS TITLE: Isolation and Characterization of a Fluoride Resistant Microbial Strain for the Bioremediation of Polluted water

SIGNATURE: _____

DATE :

ABSTRACT

In the present study the screening of potential bacterial strain from soil sample was performed. Although the selected bacterial strain showed an efficiency of removal of fluoride by 45 percent but when the same experiment was repeated for confirmation of its potential the results varied. Since the experiment with such bacterial strain proved unreliable, the study was shifted to the isolation and efficiency of fungal strain. The highly Fluoride resistant fungal strain *Aspergillus niger* and *Aspergillus flavus* were studied for their fluoride removal efficiency out of which *A.niger* having the maximal removal efficiency has been further studied by optimizing process parameters such as pH, time (days) and temperature. It has been observed, the maximal Fluoride removal efficiency exhibited by *A.niger* was of 54 percent at 30°C, pH 4 and the corresponding optimum time was 7 days. In this case, the same result was obtained while repeating the same experimental set up. In addition, the experiment includes isolation of potential fungal strain from rotten orange sample and the study of their maximum Fluoride removal efficiency by optimizing the same process parameters. In this case the maximal removal by the selected strain was 42 percent at pH 4, 30°C for 6 days. It was observed that at lower concentration of Fluoride (20 ppm) the removal efficiency of microbial strain was not much effective whereas it exhibited removal efficiency at higher concentration of 50ppm.

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1. INTRODUCTION

Trace elements can be considered to be potentially toxic when it exceed its safe and adequate exposure. Fluoride (F) ion which when exceeds its permissible limit becomes poison to the protoplasmic content of the cell and thus affects by leading to several biochemical alteration [1].

Toxic effects of fluoride are a major public health problem worldwide due to long term consumption of water with high F levels [2]. Due to rapid industrialisation and consequent increased urbanization has led to great extent of anthropogenic presuure in every component of the environment. The xenobiotics which as a result, being introduced in the environment is leading to a disturbance in ecological balance. The removal of such xenobiotics most of the time is not possible due to their foreign nature and their constant introduction to the ecology, hence creating an imbalance in nature dynamics. The increase of fluoride concentration in ground water resources is now one of the most important toxicological and geo environmental issues in India [4, 5]. The development of various kinds of industries has led to application of innovative and effective method of treatment of fluoride containing waste water. The decontamination of water is subjected to various physical and chemical techniques.

Fluorine (F) being the most electronegative among all the chemical elements and has unparallely violent reactivity property, thus is never found as elemental fluoride in nature but in its ionic form as fluoride. It has high oxidising and radical formation property, unique physicochemical properties and diverse application and such peculiar properties has contributed to its acknowledgement as "*small atom with a big ego*" .Since the toxicity of fluoride ion is inherent in its atomic structure, it cannot be further transmuted or mineralized to a totally innocuous form. Though, its oxidation state, solubility and association with other inorganic and organic molecules varies; microorganism play a bioremidiative role by transforming and concentrating these anions so that they are less available and less dangerous.The bioremediation of xenobiotics using microorganisms has achieved attention in recent years not only for its scientific novelity but also for its potential in industry application. Ionophores, the high affinity anion binding compounds are secreated from many plants and animals which binds specific chemical form of anions [6,7]. This anion ionosphore complex is then absorbed back into the organism for utilization reducing its availability and lethality.

Microorganisms have acquired the mechanism of adaptation to the presence of toxic elements through various mechanisms among which are metal sorption, mineralization, uptake and accumulation, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form, and efflux of xenobiotic from the cell as reported and these mechanisms are sometime encoded in plasmid genes, thereby facilitating the transfer of toxic metal resistance from one cell to another.

Thus the detoxifying ability of these resistant microorganisms can be manipulated and lead to bioremediation of toxic elements in wastewater.

1.1 BIOREMEDIATION AND BIODEGRADATION

Biodegradation is a biological process invoving catabolic activity of microbial enzymes leading the microorganisms to simplify the molecular arrangement of a compound. Bioremediation refers to strategic application of microorganisms capable to enervate contamination, either by neutralizing or by removing the target contaminants [8]. The concepts of both bioremediation and biodegradation are close knitted since the adaptation of bioremediation strategy depends upon the potential biodegradation capability of microorganisms ensuring restoration of healthy environment.

The bioremediation process employment has long been started from 19^{th} century for the bioremediation of oil spills and other hazardous products [9]. The bioremediation process since then has been developing and employment is being considered at varied contexts of contamination, the most important targets being the micropollutants and the emerging pollutants [10].

Bioremediation is an innovative, efficient and emerging tool as an alternative to the conventional treatment processes for the abatement of environmental pollution through contamination. Several physicochemical processes such as coagulation, biosorption, precipitation and adsorption may be applied for the removal of contaminats from the environment [9,10]. However, comparing these such processes it may be concluded that bioremediation provides following advantages over the convention processes; the advantages of bioremediation are as follows : (i) bioremediation technique is more cost effective than that of conventional physicochemical remediation techniques [11]. (ii) the bioremediation process generates less secondary wastes than that of conventional physicochemical remediation technique having high invasion property yielding secondary impacton the environment. (iii) rate of transfer is high than that of general attenuation tehniques. (iv) bioremediation technique involves minerialization of contaminants i.e to convert them into natural constituent elements.
(v) bioremediation technique while being applied for remediation of contaminants doesnot involve the transfer of contaminated soil or wastes for ex situ treatment as is common in various physicochemical remediation techniques[11]. (vi) bioremediation employment accelerates degradation process and causes conversion of phytovolatized contaminants into less toxic form. Moreover, bioremediation technique is an insitu, passive remediation technique and has high public acceptance.
Inspite of such advantages of bioremediation it too has limitations.The bioremediation techniques relies on the potential metabolically competent microorganisms which may not be active or may not be present at the contaminated site. The metabolic reactions which are highly dependent on microbial viability in turn influenced by variables which cannot be deliberately controlled such as convenient environmental conditions or appropriate amount of nutrients required. Moreover, to carry out the bioremediation technique byappropriate expression of key enzymes performing the remediation technique several following factors must be satisfied : adequate concentration of target contaminats and also their bioavailability, suitable temperature, pH and redox conditions. In addition, the bioremediation technique may at times disturb the ecological balance of the contaminated site by disturbing the native microbiota population dynamics [12].

1.2 TYPES OF BIOREMEDIATION : INTRINSIC AND ENGINEERED

The key concern to determine the compatibility of contaminated site to that of bioremediation is largely dependent on the type of type of bioremediation considered to be implemented. Bioremediation can be divided into two main groups : intrinsic and engineered.

Intrinsic bioremediation invoves retaining the innate capabilities of naturally occurring microbiota and employing them to abate the environmental contaminants without any help of engineered mechanism to augment the process. It involves thorough documentation of the role of microbiota in elimation of contaminants via field tests or tests on samples of soil, sediment or water derived from contaminated sites. Moreover, the effictivity of the intrinsic boremediation technique must be demonstrated by site monitoring regime routinely analysing the contaminant concentrations. The terms “natural”, “passive”, “spontaneous”, “bioattenuation” are the most referred terms for the intrinsic bioremediation.

Engineered bioremediation involves accelerated microbial activities with the help of engineered site modification procedures such as installation of wells to circulate fluid and maintaining appropriate concentration of microbial nutrients to stimulate microbial growth. The main strategy of engineered bioremediation involves isolation of the contaminated site and control over the contaminated field acting as in situ bioreactors. The most common terms being used for such engineered bioremediation is “ biorestoration” and “ enhanced bioremediation”.

1.3 FACTORS INFLUENCING BIOREMEDIATION AND BIODEGRADATION

The effective role in bioremediation is carried out by microorganisms especially bacteria which is virtually present everywhere. Bioremediation is the application of living organisms in degrading the environmental contaminants in innocuous form. Microbial metabolism plays the central role in biodegradation and bioremediation. Microorganisms is best suited in this task since the enzyme present in them allows them to take up these contaminants as food for growth in addition to mineralization and detoxification and moreover due to their reduced size they are capable of attaching with these contaminants easily. In situ bioremediation can be considered as one of the main purpose served by microorganisms for billions of years, if not this world would have been dumped with wastes and the necessary nutrients for continuation of life on the earth would have been inaccessible. The possibility of annihilation of man made contaminants in the subsurface depends on three factors : the type of organisms, the type of contaminants and the geological and chemical conditions of the contaminated site.

The common soil bacteria and fungi can degrade the majority of compounds. The most important soil factors that influence biodegradation are moisture, temperature, absence or presence of oxygen, organic matter and clay component. Now a days many of the man made pesticides applied are microbial degraded, mostly by enzymes developed in response to natural substrate [13-15]. Usually, microorganisms carrying out degradation require carbon and energy. Usually a increased size of pesticide degrading population leads to a faster rate of degradation. The enhanced degradation occurs due to repeated application of pestiscides which is favourable for enhanced degradation of toxic residues. The microbial is degradation is indeed the best method of detoxification of pesticides [16-21].

1.4 DESIGN OF BIOREMEDIATION APPLICATION

Bioremediation technique is gradually being adopted for remediation of an array of contaminants which are not easily degraded and for such efficient application intense research is undergoing.
The preliminary aim of any remediation process is to hinder the spread of contaminants. As for the ground water, with advancement of the ground water soluble component from the contaminated pool dissolves and enters the groundwater stream thereby contaminating the plume. Advancement or migration of contaminated plume is a threat to the environment and health. Though irrespective of their advancement or fixed deposition the remediation technology must ensure elimation from its source.

The selection and employement of the type of bioremediation technique depends on several factors. The first factor being the management of the contaminated site which may vary from simple contamination to meeting the specific regulatory standards of the contaminant concentration in the soil or ground water. The second factor includes the consideration of the type of contaminant, its location, extent of contamionation and its concentration based on which the designing of insitu bioremediation may be employed. The third factor is the stimulation of specific potential microorganism capable to attenuate that specific contamination through its potential metabolic activities thereby providing the requirements for the appropriate designing of insitu bioremediation process. The final factor that must be taken into consideration is of the contimant capability and pattern of spreading and influencing the site.

Once the above mentioned factors are perceived satisfactorily then we must look into the type of bioremediation to be employed depending on the site availibilty of appropriate nutrients and environmental conditions for the efficient performance of the microorganisms. If such parameters are well satisfied then we may progress with intrinsic bioremediation, or else, if we need to provide artificial suitable environmental condition and nutrient supply for efficient metabolic activity of the target microorganism, engineered bioremediation has to be considered. Overall, considering all the mentioned factors the most cost effective bioremediation technique to be employed can be designed.

1.5 ROLE OF MICROBES IN BIOREMEDIATION

The main aim of bioremediation is to stimulate potential microbiota and accelerate the rate of abatement of array of contaminants through proper support of supply of optimum nutrients and other chemicals and environmental conditions. Today's bioremediation is limited to the remediation activities of native microbiota though several undergoing researches has shown promising potential to enhance the remediation process of contaminated site through the introduction of suitable foreign potential microrganisms. The process bioaugmentation in near future may amplify the bioremediation techniques.

Although, native and non native microorganism is of latter concern, rather first we must have a clear conception about the method of annihilation of contaminants by the microrganisms. The type of bioremediation process to be employed in turn demands the appropriate nutritional supplements the bioremediation process have to cater. Moreover, the byproducts of such microbial processes indicates the extent of success of bioremediation method.

MICROBIAL METABOLISM:

Microorganisms degrade the contaminants to serve two of their purposes : a source of carbon essential for its growth and reproduction and as a source of electrons which helps to extract energy through breaking chemical bonds and transfer of electrons. The type of chemical reaction is the oxidation reduction type of reaction, contaminant being oxidized by donating electron and thus accepting the electron and with that of carbon new cells are produced and cell growth is maintained. These electron donor and electron acceptor are known as primary substrate.

The process of destruction of contaminant is accompanied by aerobic or anaerobic respiration by microbes. In case of aerobic respiration part of the carbon is converted to CO_2 whereas the remaining carbon is used for cell growth, oxygen get reduced forming water. The byproducts being carbon dioxide, water and increased microbial population.In case of anaerobic respiration, electrons from degraded contaminants can be accepted by inorganic chemicals, the byproducts being hydrogen sulphide, nitrogen gas, reduced form of metals and methane depending on the type of electron acceptor.

The fermentation process play an important role in oxygen free environment since it requires no external electron acceptor or an electron donor. In this process the fermentation products include the non toxic form of the contaminant produced by the microorgnisms through a series of internal electron transfer mechanism. Fermentation products include acetate, ethanol, hydrogen, propionate. Fermentation products can then be degraded my species of bacteria converting them to carbon dioxide, methane and water.

In some cases, non beneficial transformations of contaminants by microrganisms is also a type of microbial metabolism better referred as co-metabolism. It is an incidental reaction catalysed by enzymes involved in normal cell metabolism or detoxification reactions.

Another type of microbial metabolism include reductive dehalogenation which is a potential method of detoxification of halogenated organic contaminants. In such process, a halogen atom of the contaminant is replaced with a hydrogen atom by a microbial catalysed reaction. This reaction adds two electron to the contaminant molecule thereby reducing the contaminant.

DEMOBILIZATION OF CONTAMINANTS

In addition to transformation of contaminants to innocuous form, microorganisms also involve in demobilization of contaminants through three basic ways :

(i) Microbial biomes are capable of absorbing hydrpophobic organic molecules. The excess amount of biomass growth in the path of contamination migration could stop or slow contaminant movement. This process often referred to as biocurtain.

(ii) Microorganisms may produce reduced or oxidized species that lead to metal precipitation.

(iii) Microorganisms biodegrade organic compounds bound with metals and retain the metal in solution whereas the unbound metals gets precipitated and immobilized.

The fundamental principles of method of degradation by microrganisms are usually uncomplicated but every details of microbial metabolism is still not deciphered thus use of microbes successfully is not yet fully recognised. Some of the main complicating factors being inaccessible contaminant, toxic effects of contaminant on microorganisms, microorganism's preference of other chemicals or contaminants over targeted contaminants, degradation of contaminants partially leading to hazardous byproducts, inability to moderate the contaminant concentration and aquifer clogging due to excessive growth.

1.6 PERMISSIBLE LIMIT OF FLUORIDE INDRINKING WATER

The presence of Fluoride in drinking water is considered beneficial or detrimental depending on its concentration and the amount ingested.
The permissible limit of fluoride in drinking water is [26]:

1) World Health Organization (WHO) : 1.5 mg/l

2) Indian Standard (IS) : 1.0 mg/l

3) US Environment Protection Agency (USEPA) : 4 mg/l

4) European Union (EU) : 1.5 mg/ L

The target should be between 0.8 and 1.2 g/L to maximize benefits and minimize adverse effects. (WHO 2006; Edmunds and Smedley2013).

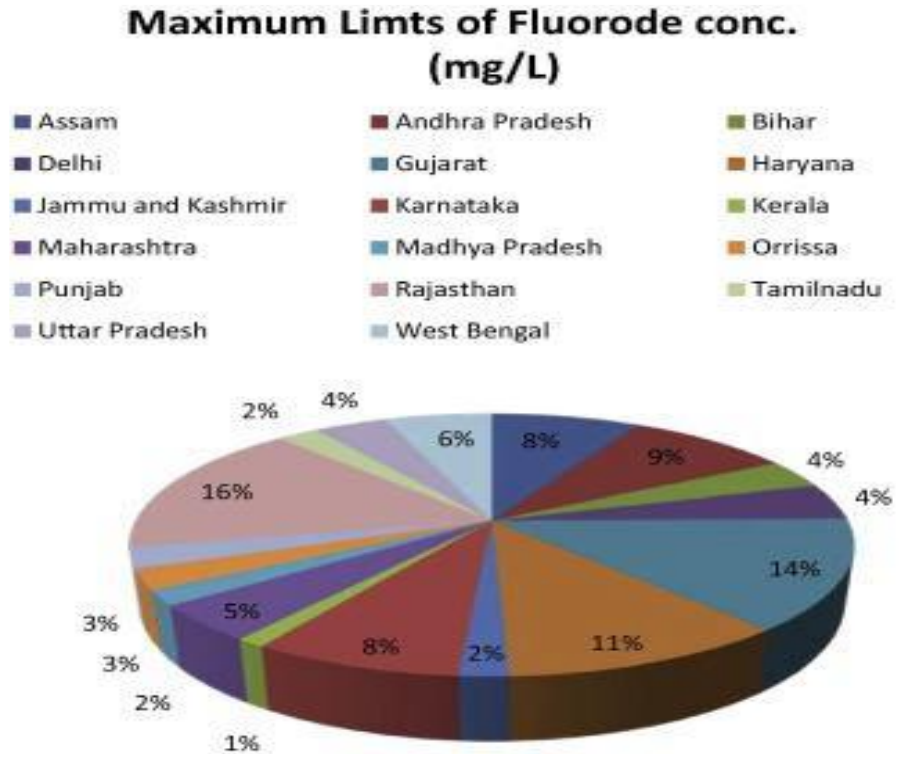
1.7 OCCURRENCE AND SOURCES

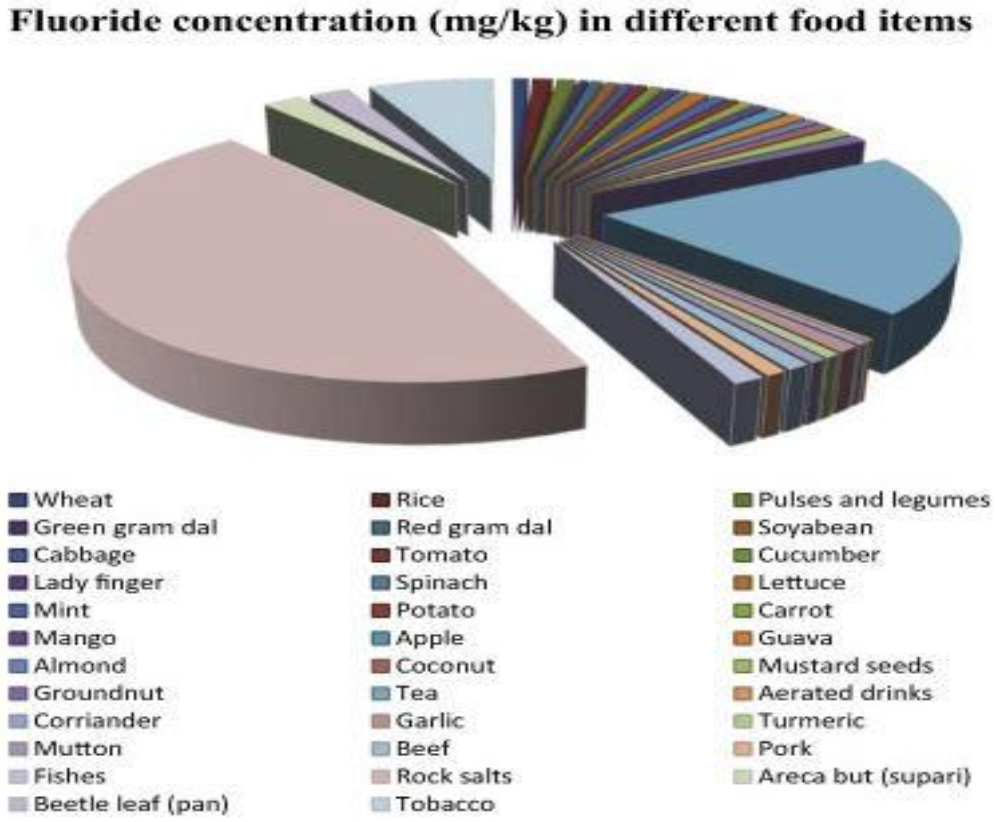
REGIONS OF AVAILABILITY OF FLUORIDE

According to literature report fluoride even in minute quantity plays an extremely crucial part for the normal mineralization of bones and formation of dental enamel. Its high intake might lead to slow, progressive crippling scourge as referred to as pathology.

According to square measure many developed and developing nations are endemic region. The square measure includes Argentina, U.S.A., Morocco, Algeria, Libya, Egypt, Jordan, Turkey, Iran, Iraq, Kenya, Tanzania, S. Africa, China, Australia, New Zealand, Japan, Thailand, Canada, Saudi Arabia and gulf, Sri Lanka, Syria and India etc.

According to the literature available on India, 1st it was detected in Nellore district of Andhra Pradesh in 1937, since then, research has been exhaustively carried out on different parts of Asian nations to explore halide laden water sources and their impacts on humans in addition to animals. Presently, pathology is rife in seventeen states of India as calculated.





The intensity of pathology is not only obsessed with the halide content in water, but conjointly on the halide from different sources, physical activity and dietary habits.

A brief review of fluoride in drinking water in different regions of India has been presented. The results of the Research and development review on this subject provided the basis of conclusion that primary cause of endemic fluorosis is indubitably the consumption of fluoride contaminated drinking water. Thus, its high time to look into this increasing trend of fluoride level in drinking water supported by conspicuous evidence of the endemic fluorosis in India.

1.8 SOURCES OF FLUORIDE CONTAMINATION

1. Background levels of fluoride in food

According to Waldbott et al, every food contains at least some fluoride virtually. The source of fluoride in case of plants are soil and air. The fluoride is transmitted from the soil through fine hair rootlets into the stems, and some reaches the leaves. It was observed plants absorb more fluoride from sandy than from clay soil and more from wet and acid soils than from dry and alkaline ones [22].

2. Cooking with fluoridated water

According to ATSDR, food cooked in fluorinated water results in increased dietary fluoride levels [23].

3. Processed food and beverages

According to DHSS, the contribution of natural food content is too insignificant to compare with the amount of fluoride produced through cooking and processing food in fluoridated water [24].

4. Pesticides

Among many such available herbicides, three most widely used are : Trifluralin, Fluometuron and Benefin (Befluralin). The category "Fluorine Insecticides" include Cryolite, Barium hexafluorosilicate, Sodium hexafluorosilicate, Sodium fluoride, and Sulfluramid.

5. Sodium Fluoride (NaF)

The use of sodium fluoride varies at a wide range acting as a rodenticide and insecticide (mainly for roaches and ants), as a disinfectant for fermentation apparatus in breweries and distilleries, in wood preservation, and in rimmed steel manufacture.

6. Superphosphate Fertilizer

The contents of phosphate rock include fluoride (up to 5%) thus the superphosphate industry is considered to be a key player in fluoride pollution and exposure of people to fluoride for over a century.

7. Bone meal

Bone meal, commonly used in organic gardening and farming consists of bones of farm animals consisting of more than 1000ppm of fluoride concentration.

8. Industrial air pollution

The amount of fluoride ranges to toxic level in many of the industrial effluents. Such industries include aluminum smelters, zinc smelters, brickworks, ceramic works, steel mills, uranium enrichment facilities, coal fired power plants, and oil refineries.

1.9 EFFECTS OF FLUORIDE CONTAMINATION

ADVERSE HEALTH EFFECTS OF FLUORIDE

Based on the level of exposure of fluoride, it has two effects. The effects are acute effects (i.e., short term effects) and chronic effects (i.e., Long term effects) on human body.

SHORT-TERM (ACUTE) EFFECTS

Toxicity of fluoride may be acute in case of accidental excess fluoride poisonings since acute effects of fluoride is rare [25]. The symptoms of acute toxicity include:

- Severe nausea
- Vomiting
- Excess saliva production
- Abdominal pain
- Diarrhoea
- Convulsions
- Irregular heartbeat
- Coma

LONG-TERM (CHRONIC) EFFECTS

The chronic effects of fluoride contamination are the following:

- Fluoride contamination has found to induce oxidative stress andDNA damage, leading to apoptosis and cell cycle changes,fluoride exposure result in covalent binding to DNA, which maylead to DNA damage and initiate chemical carcinogenesis [27].
- Fluoride is a chemically active ionized element which can effectoxygen metabolism and induce the production of oxygen freeradicals modulating intracellular redox homeostasis [28].
- Fluoride intake in excess leads to dental, skeletal and nonskeletalfluorosis [29].

1.10 REVIEW LITERATURE

CASE STUDY: 1

Doble M. Kumar studied two highly fluoride ion (F) tolerant bacterial strains, *Bacillus cereus* FT1 and *Bacillus marisflavi* FT2 which were isolated from soil samples collected from F endemic areas of Birbhum district (Rampurhat block II) of West Bengal, India. The F tolerance limit and absorption efficiency exhibited by these two bacterial isolates when monitored for 72 hr at 24 hr intervals at F concentration (730 ppm) the absorption efficiency was significantly increased compared to the control strain (*B.licheniformis* ONF2). F toxicity was monitored by preparing growth curves at two different concentrations, 1500 ppm and 3000 ppm of NaF [30]. In both cases, the lag phases in the growth curves were extended. However, the bacterial growth was not completely inhibited. The F tolerance efficiency exhibited by these two bacterial isolates was again confirmed by cell morphology study using a scanning electron microscope.Since both the isolates showed high F tolerance level, this property might be useful for investigating the F related expression of membrane channel protein, which will be important for developing F removing engineered bacteria in the future.

CASE STUDY: 2

Rani M.et.al in their study observed that the industrial effluents and the nearby sites of the contaminated areas consists of microorganisms that can indicate pollution and detoxify the contaminated water resources and with the emergence of xenobiotic resistant bacteria among them they might be used as a potential application in bioremediation. This investigation lead them to isolate and characterize screening of fluoride resistant bacteria from soil and water samples of different regions of India. Five isolates recovered from different samples were found to be fluoride resistant, two of which effectively reduced the fluoride from their media probably because of selective pressure development which lead to fluoride resistant among bacterial populant, may be through the mechanism involving high affinity anion binding compounds called ionophores [31]. It was concluded that F resistant microbes may play a bioremediative role by transforming and concentrating these anions into a less available and less dangerous form.

CASE STUDY: 3

The study of Baker JL.et.al showed that F contaminated groundwater collected from Asanjola and Madhabpur, West Bengal, India consist of fluoride-resistant bacteria having the defuoridation capability. 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 were observed to be exhibiting significant amount of reduction in fluoride. Isolate that latter identified as *Acinetobacter* sp. achieved a maximum fluoride removal of 25.7 % from the media at 30°C and pH 7 after 8 days of incubation..Growth of such strain was analysed at a diverse pH range, and it could thrive at pH 5–10. The investigation revealed that the selective pressure of fluoride resulted in growth of fluoride- resistant bacteria capable of secreting high-affinity anion-binding compounds [32]. The F resistant microbial strain concentrates the anions so that they become less available thus bioremediating. Hence, the fluoride-resistant bacteria, the particular strain of Acinetobacter sp. could be used as a promising strain for application in water defluoridation from contaminated sites.

CASE STUDY: 4

The study of Tope.et.al listed the ability of non-viable form of Trichoderma harzianum, isolated from F rich groundwater were subjected to laboratory scale experiment of F removal by this microbial strain [33] which depends on several significant process parameters which includes the fluoride concentration (2-8 mg L^{-1}), groundwater pH (5–9), temperature (20–45°C), bio sorbent dose (0.4-1.6 g/100ml) and biosorption time (30-120 min). It was concluded from the results obtained that the fluoride biosorption increased significantly with increase in groundwater pH, biosorbent dose, temperature and biosorption time though an increase in initial fluoride concentration reduced fluoride removal. The optimum condition of fluoride sorption was attained with 1.6g/100ml of biosorbent within 60 min, optimum pH 10 and temperature being 50°C.

2. OBJECTIVE :

1. Isolation of suitable microbial strain from environment capable of reducing the Fluoride concentration in water.

2. Optimization of some process parameters for the maximal removal of Fluoride using the isolated microbial strain.

CHAPTER 1

Isolation and Characterisation of Fluoride Resistant Bacterial Strain

1.1 METHODS AND MATERIALS

1.1.1 Estimation of Fluoride by Ion Selective Electrode Method

PRINCIPLE

Total solubilized fluoride is determined potentiometrically using a fluoride ion-selective electrode (ISE) in conjunction with a standard single-junction reference electrode, or a fluoride combination ISE, and a pH meter with an expanded millivolt scale or an ISE meter capable of being calibrated directly in terms of fluoride concentration.

Standards and samples are mixed 1:1 with a total ionic strength adjustment buffer (TISAB). TISAB adjusts ionic strength, buffers pH to 5-5.5, and contains a chelating agent to break up metal-fluoride complexes. Calibration is performed by calibrating the ion meter directly in terms of fluoride concentration.

Polyvalent cations (*e.g.*, Fe+3 and Al+3) interfere by forming complexes with fluoride which are not measured by the fluoride ISE. The addition of TISAB, which contains a strong chelating agent, eliminates this interference by complexing polyvalent cations. Again, adding TISAB prevents the interference by buffering the pH.

Temperature changes affect electrode potentials. Using an ISE calibrated at 25ºC.
Therefore, standards and samples must be equilibrated at the same temperature.
The presence of potential of interferences from colloidal substances can be avoided by filtering the sample when needed.

1.1.2. Preparation of medium for plate and slant culture

The medium composition of slant and plate culture was (g/L) – Peptone : 5.0 ; Beef extract : 3.0 ; Agar : 30 ; pH : 6.8 - 7.2. All the constituents except agar were dissolved in water and pH was adjusted to 6.8 – 7.2 using 1.0 (N) hydrochloric acid and 1.0 (N) sodium hydroxide. Then the agar was added and the whole solution was kept in boiling water bath until the whole agar melted. 5ml of the hot medium was transferred to each of the test tubes and were cotton plugged, wrapped with brown paper and sterilized at 121 °C for 15 mins. 15ml of molten medium was taken in each of the several test tubes to use in plate dilution technique and sterilized as usual.

1.1.3.Preparation of medium for inoculum and fermentation of the selected strain

Medium composition for preparation of inoculum as well as fermentation was – peptone: 0.5 %; Yeast extract: 0.2 %; beef extract: 0.1%; pH: 6.8-7.2. The medium was prepared by dissolving the weighed amount of those constituents in water. pH was adjusted to 6.8-7.2 using 1.0 (N) hydrochloric acid and 1.0 (N) sodium hydroxide. Then 50 ml medium was distributed in each of the 250 ml Erlenmeyer flasks. However all the flasks were plugged with cotton and wrapped with brown paper then sterilized at 121° C.

1.1.4. Isolation of organisms from natural sources

The medium composition for isolation and maintenance of the culture was as follows:

Peptone: 0.5%
Beef extract: 0.3%
Agar: 3.0 %
pH: 6.8-7.2

1.1.5. Selection of the most potent bacterial strain

Soil enrichment technique was employed for isolation of microorganism from soil using plate and dilution technique. Approximately 1 g of soil sample was taken in 100 ml sterile water in 250 ml Erlenmeyer flask and shaken well for 1 hour using a rotary shaker at 120 rpm. The sample was diluted up to 10^7 fold with sterile distilled water.

Petridishes containing different dilution of samples were incubated at 37° C for 24 hours to get the bacterial colonies. The bacterial colonies were aseptically transferred to the slant medium by using a wire loop. Then the slants were incubated at 37° C for 24 hours. After incubation one loopful of each slant culture was transferred into 50 ml medium containing 50 mg /Land 20 mg/L of NaF. Fluoride in 250 ml Erlenmeyer flask and incubated at 37° C for 24 hours with shaking speed 120 rpm. After fermentation was over, the fermented broth was centrifuged at 5500 rpm for 10 minutes. Clear supernatant was tested for residual Fluoride concentration.

1.1.6 Result and Discussion

Isolation of a bacteria strain capable of removing fluoride

A total of 26 isolates having apparently different colony morphology were collected and screened based on their fluoride removal capability. The result was shown in Table- 1.

TABLE -1

Intial Fluoride Concentration	Microrganisms (Strain No.)	Fluoride removed (%)
20 ppm	FR1	10
	FR2	12
	FR3	12
	FR4	10
	FR5	11
	FR6	15
	FR7	12
	FR8	11
	FR9	8
	FR10	16
	FR11	12
	FR12	12
	FR13	13

Intial Fluoride Concentration	Microrganisms (Strain No.)	Fluoride removed (%)
50 ppm	FR14	28
	FR15	33
	FR16	29
	FR17	**45**
	FR18	38
	FR19	42
	FR20	33
	FR21	41
	FR22	44
	FR23	41
	FR24	36
	FR25	37
	FR26	35

It can be concluded from the above listed results that **FR_{17}** has the maximum ability to remove the fluoride. But on repetition of the experiment using the screened **FR_{17}** the same result was not obtained. Thus this experiment using bacterial strain could not be considered to serve our purpose, thus the study was shifted to the isolation of fungal strain and its efficiency in Fluoride removal.

CONCLUSION :

The selected bacterial strain FR_{17} when subjected to repeated testing at the same experimental setup to confirm its potential, the results obtained varied. In such case the strain couldnot be considered for further study. Thus the study was then focused in isolation and characterization of fungal strain and its Fluoride removal efficiency.

CHAPTER 2

Isolation and Characterisation of Fluoride Resistant Fungal Strain and Optimization of Process Parameters

2.1 METHODS AND MATERIALS

2.1.1 Estimation of Fluoride by Ion Selective Electrode Method

The Fluoride reducing efficiency of three individual known fungal strain were studied using the Ion Selective Electrode, the principle of which can be referred at Chapter 1.

2.1.2 Preparation of Medium for Mold Culture (Czapek Dox Agar Media)

TABLE – 2

Name of the Compounds	Unit (g/L)
Glucose	**50**
Potassium Dihydrogen Phosphate	**1**
Potassium Chloride	**0.5**
Sodium Nitrate	**2**
Magnesium Sulfate heptahydrate	**0.5**
Iron (II) Sulfate Heptahydrate	**0.001**
Agar	**30**
pH	**4 – 4.2**

2.1.3 Preparation of medium for plate and slant culture

The Czapek Dox media except agar were dissolved in water and pH was adjusted to 4.2 using 1.0 (N) hydrochloric acid and 1.0 (N) sodium hydroxide. Then the agar was added and the whole solution was kept in boiling water bath until the whole agar melted. 5ml of the hot medium was transferred to each of the test tubes and were cotton plugged, wrapped with brown paper and sterilized at 121 °C for 15 mins. 15ml of molten medium was taken in each of the several test tubes to use in plate dilution technique and sterilized as usual

2.1.4 Preparation of medium for inoculum and fermentation of the selected strain

The liquid Czapek Dox medium was prepared in water with all the weighted above mentioned constituents except agar at pH: 4.0-4.2. The pH was adjusted using 1.0 (N) hydrochloric acid and 1.0 (N) sodium hydroxide. Then 50 ml medium was distributed in each of the 250 ml Erlenmeyer flasks. Next, all the flasks were plugged with cotton and wrapped with brown paper then sterilized at 121° C.

2.1.5 Measurement of Fluoride concentration

The mold colonies of one loopful was transferred aseptically from slant culture to the 50 ml sterilised medium containing 50mg/L of NaF in 250 ml Erlenmeyer flask and incubated at 30ºC for 7 days with shaking speed 120 rpm. After fermentation was over, the fermented broth was centrifuged at 5500 rpm for 10 minutes. Clear supernatant was tested for residual Fluoride concentration using the ion selective electrode of Thermo Scientific™ Orion™ Fluoride Electrodes (900100) at 25°C for 50ppm of initial Fluoride concentration. The result obtained determined the efficiency of the microbial strain in reducing the Fluoride concentration.

2.2 Optimization of Process Parameters

2.2.1 Optimization of Temperature:

The known strains of the organisms was grown on nutrient broth medium in presence of 50 ppm Fluoride broth and incubated at different temperatures i.e20,25, 30 ,35 , 40 and 45 ºC for 7 days. After 7 days the fermented broth was centrifuged and the clear supernatant was used for assay of the residual fluoride concentration.

2.2.2 Optimization of pH :

The known strains of the organisms was grown on Czapek Dox growth medium at different pH 3.0, 4.0, 5.0, 6.0, 7.0 and incubated at 30 ºC for 7 days. After 7 days the fermented broth was centrifuged and the clear supernatant was used for assay of the residual fluoride concentration.

2.2.3 Optimization of time (days) :

The known strains of the organisms was grown on Czapek Dox growth medium for varied time, 1, 2, 3, 4, 5, 6, 7, 8 days and incubated at 30 ºC for 7 days. After 7 days the fermented broth was centrifuged and the clear supernatant was used for assay of the residual fluoride concentration.

2.3 RESULTS AND DISCUSSION

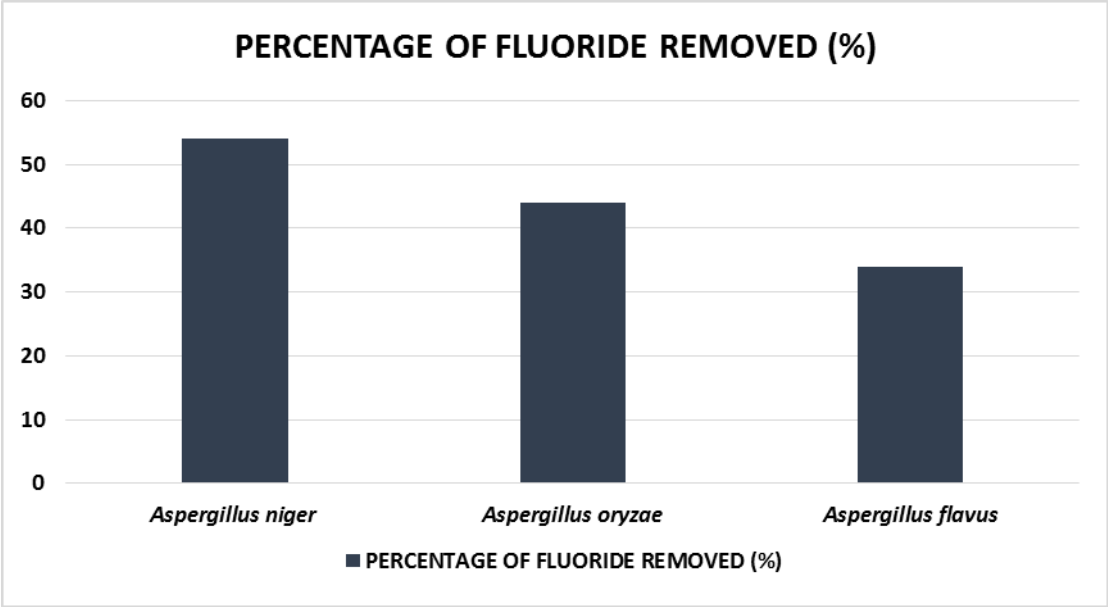
Inoculum volume of 2% is added to 50ml of Czapeck Dox media each for *Aspergillus niger*, *Aspergillus oryzae* and *Aspergillus flavus* and their fluoride removal efficiency is noted from which *A.niger* is selected for the next parameter optimization. The study of efficiency of removal of fluoride of *A.niger* was listed by optimization of parameters such as temperature (25°C, 30°C, 35°C, 40°C and 45°C), time (day – 1,2,3,4,5,6,7,8) variation and pH(3,4,5,6,7) . The optimum parameter concluded from the study was that of *A.niger* showing the maximum fluoride removal efficiencyof 54 percent at pH 4 and day 7 at 30°C.

2.3.1 Fluoride Removal Efficiency of *A.niger*, *A.oryzae* and *A.flavus*

TABLE - 3

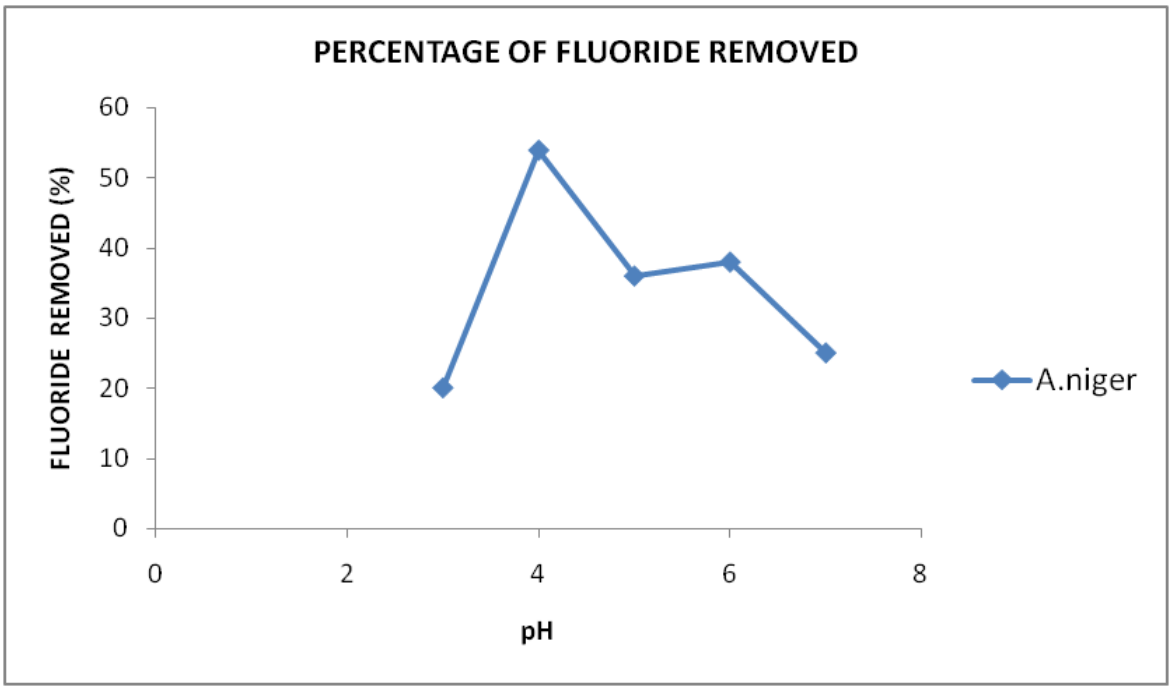
INITIAL CONCENTRATION : 50 ppm

MICROORGANISMS	PERCENTAGE OF FLUORIDE REMOVED
A.niger	**54**
A.flavus	34
A.oryzae	44



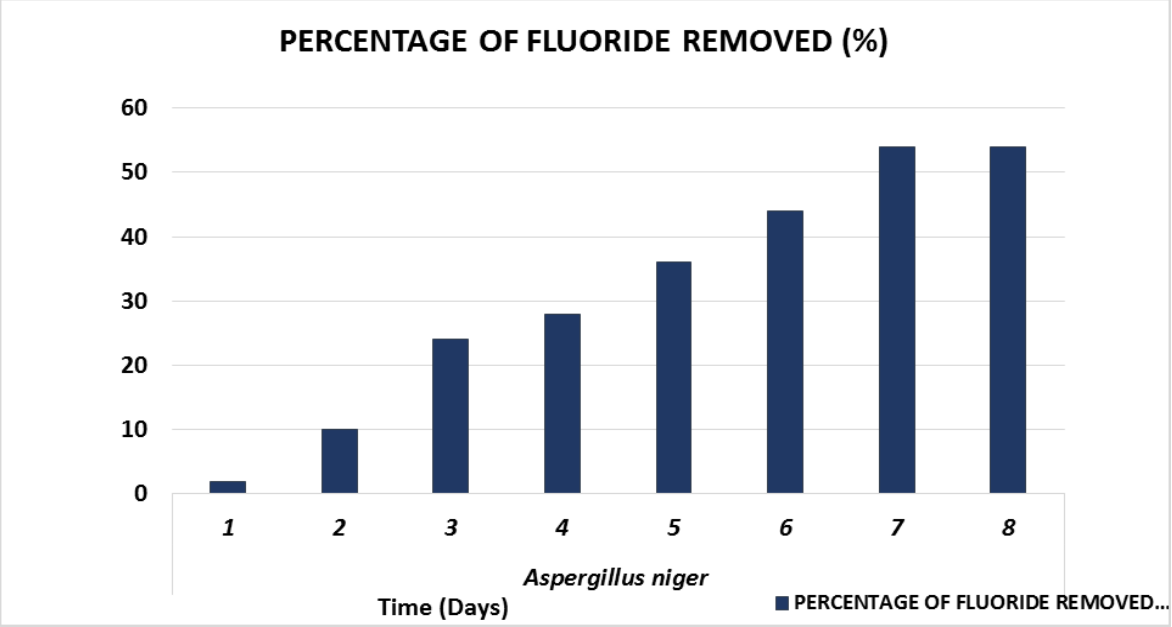
2.3.2 Fluoride Removal Efficiency of *A.niger* for varied pH

INITIAL CONCENTRATION : 50 ppm



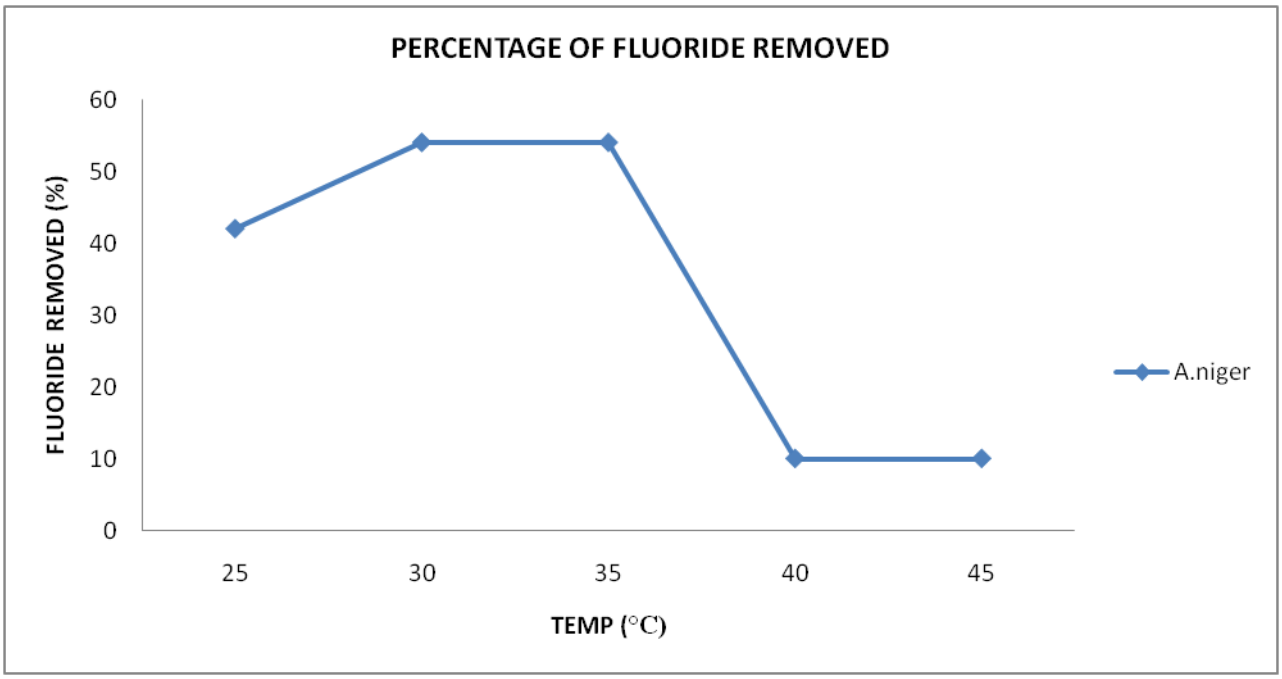
2.3.3 Fluoride Removal Efficiency of *A.niger* for varied time (days)

INITIAL CONCENTRATION : 50 ppm



2.3.4 Fluoride Removal Efficiency of *A.niger* for varied temperature

INITIAL CONCENTRATION : 50 ppm



CONCLUSION

In this study all the three individual fungal strains *A.niger*, *A.flavus* and *A.oryzae* were tested for their Fluoride removal efficiency out of which the *A.niger* turned out to be the most effective in reducing the fluoride concentration by 54 percent, thus further optimization of process parameters involving pH, time (days) and temperature(°C) were performed to obtain the optimum parameters for its maximal efficiency of removal of Fluoride. The repeated study of the selected strain showed the same result of removal efficiency of 54 percent at pH 4, 30°C and 7 days. Thus, we further carried out the same experiment with fungal strain from food sample.

CHAPTER 3

Isolation and characterization of fungal strain from food sample and Optimization of Process Parameters

3.1 METHODS AND MATERIALS

3.1.1 Isolation of fungal strain and optimization of process parameters

In this study, fungal strain was obtained from a citrus fruit which is one of the best source of mold growth. The isolation of suitable fungal strain involved serial dilution plate count method and the isolation and growth of mold strains from food sample was done using the following Czapek Dox medium composition from which the most potent fungal strain was screened. The potential of the screened fungal strain was measured using the Ion Selective Electrode, the principle of which can be referred to referred to Chapter 1. Next, the process parameters were optimized to conclude the optimim process parameters for themaximal removal of Fluoride by the isolated fungal strain.

3.1.2 Preparation of Medium for Mold Culture (Czapek Dox Agar Media)

TABLE – 4

Name of the Compounds	Unit (g/L)
Glucose	**50**
Potassium Dihydrogen Phosphate	**1**
Potassium Chloride	**0.5**
Sodium Nitrate	**2**
Magnesium Sulfate heptahydrate	**0.5**
Iron (II) Sulfate Heptahydrate	**0.001**
Agar	**30**
pH	**4 – 4.2**

3.1.3 Preparation of medium for plate and slant culture

The Czapex Dox media except agar were dissolved in water and pH was adjusted to 4.2 using 1.0 (N) hydrochloric acid and 1.0 (N) sodium hydroxide. Then the agar was added and the whole solution was kept in boiling water bath until the whole agar melted. 5ml of the hot medium was transferred to each of the test tubes and were cotton plugged, wrapped with brown paper and sterilized at 121 °C for 15 mins. 15ml of molten medium was taken in each of the several test tubes to use in plate dilution technique and sterilized as usual.

3.1.4 Preparation of medium for inoculum and fermentation of the selected strain

The Czapek Dox liquid media was prepared and pH was adjusted to 4.2 and then 50 ml medium was distributed in each of the 250 ml Erlenmeyer flasks. All of the flasks were plugged with cotton and wrapped with brown paper then sterilized at 121° C.After sterilisation the medium was set to cool to tolerant temperature, then, one loopful of each slant culture of *Aspergillus niger*, *Aspergillus oryzae* and *Aspergillus flavus* were transferred into 50 ml medium containing 50 mg /L Fluoride in 250 ml Erlenmeyer flask and incubated at 30° C for 7 days with shaking speed of 120 rpm. After fermentation was over, the fermented broth was centrifuged at 5500 rpm for 10 minutes. Clear supernatant was tested for residual Fluoride concentration.

3.1.5 Selection of most potent fungal strain

In this case, rotten orange was taken as the sample on which mould growth was sufficiently visible when allowed to rot in open laboratory atmosphere. Approximately 1 g of this sample was taken in 100 ml sterile water in 250 ml Erlenmeyer flask and shaken well for 1 hour using a rotary shaker at 120 rpm. The sample was diluted up to 10^7 fold with sterile distilled water.
Petridishes containing different dilution of samples were incubated at 30° C for 7 days to get the mold colonies. The mold colonies were aseptically transferred to the slant medium by using a wire loop. Then the slants were incubated at 30° C for 7 days. After incubation one loopful of each slant culture was transferred into 50 ml medium containing 50 mg /L Fluoride in 250 ml Erlenmeyer flask and incubated at 30° C for 7 days with shaking speed 120 rpm. After fermentation was over, the fermented broth was centrifuged at 5500 rpm for 10 minutes. Clear supernatant was tested for residual Fluoride concentration.

3.1.6 Measurement of Fluoride concentration

The mold colonies of one loopful was transferred aseptically from slant culture to the 50 ml sterilised medium containing 50mg/L of NaF in 250 ml Erlenmeyer flask and incubated at 30ºC for 7 days with shaking speed 120 rpm. After fermentation was over, the fermented broth was centrifuged at 5500 rpm for 10 minutes. Clear supernatant was tested for residual Fluoride concentration using the ion selective electrode of Thermo Scientific™ Orion™ Fluoride Electrodes (900100) at 25°C for 50ppm of initial Fluoride concentration. The result obtained determined the efficiency of the microbial strain in reducing the Fluoride concentration.

3.2 Optimization of Process Parameters

3.2.1 Optimization of Temperature:

The known strains of the organisms was grown on nutrient broth medium in presence of 50 ppm Fluoride broth and incubated at different temperatures i.e20,25, 30 ,35 , 40 and 45 ºC for 7 days. After 7 days the fermented broth was centrifuged and the clear supernatant was used for assay of the residual fluoride concentration.

3.2.2 Optimization of pH :

The known strains of the organisms was grown on Czapek Dox growth medium at different pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and incubated at 30 ºC for 7 days. After 7 days the fermented broth was centrifuged and the clear supernatant was used for assay of the residual fluoride concentration.

3.2.3 Optimization of time (days) :

The known strains of the organisms was grown on Czapek Dox growth medium for varied time, 1, 2, 3, 4, 5, 6, 7, 8 days and incubated at 30 ºC for 7 days. After 7 days the fermented broth was centrifuged and the clear supernatant was used for assay of the residual fluoride concentration.

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation of most potent fungal strain

A total of 9 isolates having apparently different colony morphology were collected and screened based on their fluoride removal capability. The result is as following :

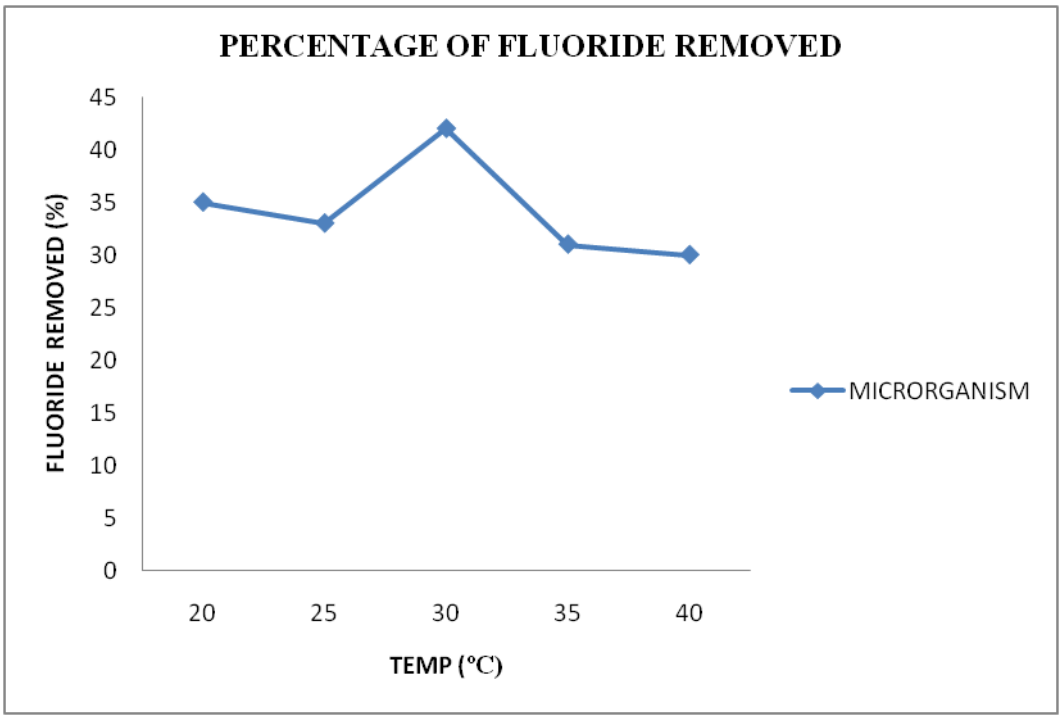
TABLE ----- 5

MICROORGANISM (STRAIN NO.)	INITIAL FLUORIDE CONCENTRATION	PERCENTAGE OF FLUORIDE REMOVED
F1	50	28
F2	50	20
F3	50	31
F4	50	20
F5	**50**	**42**
F6	50	18
F7	50	21
F8	50	16
F9	50	33

The most potent strain **F5** was selected for further study and was subjected to following process parameter optimization.

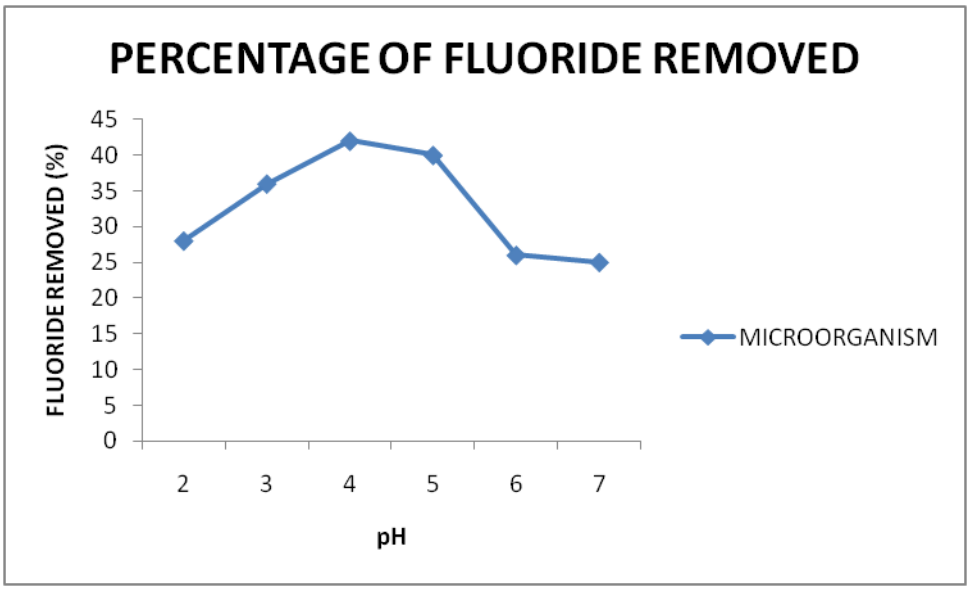
3.3.2 Optimization of temperature

INITIAL CONCENTRATION : 50 ppm



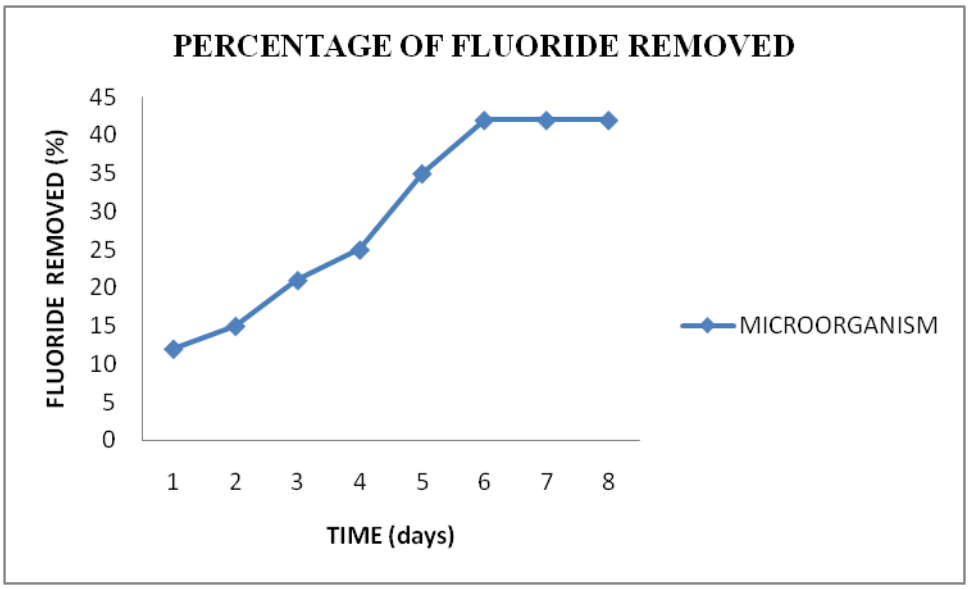
3.3.3 Optimisation of pH

INITIAL CONCENTRATION : 50 ppm



3.3.4 Optimization of time (days)

INITIAL CONCENTRATION : 50 ppm



CONCLUSION

It can be concluded that the isolated strain shows maximum of 42 percentage of fluoride removal efficiency at pH 4, temperature of 30°C and minimum incubation of 6 days.

6. DISCUSSIONS

Fluoride is more likely to exist in the form of hydrogen fluoride (HF), which is capable of crossing the cell membrane and act as a proton conductor.Thus, microorganisms capable of tolerating and reducing fluoride can be employed for the detoxification of fluoride contaminated environment. Hence such extensive research is being carried out to augment the suitable microbiota for target bioremediation of fluoride contaminant. Here, the study presents isolation, characterisation and process parameter optimization to achieve the maximum efficiency of microorganisms (bacterial and fungal strains) in attenuation of fluoride toxicity in fluoride contaminated water and soil through which the environment and human population is worst affected.

In this present study we screened bacterial and fungal strains from soil and food samples respectively and studied the efficiency of *A. niger* and other bacterial and fungal strain at optimized process parameters. The bacterial strain on repeated study was found unreliable to consider for further study due to instability of results obtained for its Fluoride removal efficiency. The study was then carried out with fungal strain of known species which provided a reliable result in its effective Fluoride removal efficiency.The study provides us with a fluoride removal percentage of around 54 percent at optimum pH of 4 and temperature of 30°C for around 7 days with *A.niger*. Hence, on such stable results obtained from known fungal strain further study was considered with unknown fungal strain from citrus fruit sample in search of fungal strain with better Fluoride removal capacity. But, in this case the most potent strain screened had the maximal fluoride removal efficiency of 42 percent at pH 4, 30ºC and 6 days. Further, we must continue the study and the integral part of the future study must consider its commercial application.

7. REFERENCES

1. **Eren, E., Ozturk, M., Mumcu, E.F., and Canatan, D.,Toxicol. Ind. Hlth. 2005, vol. 21, no. 10, pp. 255–258.**

2. **Hanaa, A., Mokhtar, I., and Yousef, B., Fd. Chem. Toxicol, 2009, vol. 47, no. 9, pp. 2332–2337**

3. **Toxicological Profile for Fluorides, Hydrogen Fluoride, and Fluorine (F),Agency for Toxic Substances and Disease Registry, U.S. Georgia: U.S. Department of Health and Human Services, 1993**

4. **Susheela, A.K., Bhatnagar, M., Vig, K., and Mondal, N.K., Fluoride, 2005, vol. 38, no. 2, pp. 98–108.**

5. **Misra, U.K., Neurol. India, 2010, vol. 58, no. 2, pp. 338–507.**

6. **Dolowy, K., Cell. Biol. Mol. Lett., 2001, vol. 6, no. 2A, pp. 343–347**

7. **Kim, K.S., Cui, C., and Cho, S. J., J. Phys. Chem. B, 1998, vol. 102, no. 2, pp. 461–463**

8. **Crawford, R.L, Bioremediation: Principles and Applications, Cambridge UniversityPress, 1998.**

9. **Das, S., and Dash, H.R, "Microbial Bioremediation: A Potential Tool for Restoration of Contaminated Areas," in Microbial Biodegradation and Bioremediation, (Oxford: Elsevier), 2018, vol. 1, pp. 1-21.**

10. **US-EPA "Treatment Technologies for Mercury in Soil, Waste and Water", Office of Superfund Remediation and Technology Innovation, 2007.**

11. **Kumar, A., Bisht, B.S., Joshi, V.D., and Dhewa, T. Review on Bioremediation of Polluted Environment: A Management Tool. International Journal of Environmental Sciences, 2011, vol 1, no. 6, pp. 1079-1093.**

12. **Thompson, I.P., Van Der Gast, C.J., Ciric, L., and Singer, A.C.Bioaugmentation for bioremediation: the challenge of strain selection. Environmental Microbiology, 2005, vol. 7, no.7, pp. 909-915**

13. **Balba M, Al-Awadhi N, Al-Daher R , Bioremediation of oil contaminated soil: microbiological methods for feasibility assessment and field evaluation.Journal of Microbiological methods,1998, vol 1, no.32, pp.155-164.**

14. **Büyüksönmez F, Rynk R, Hess TF, Bechinski E. The Occurrence, Fate, and Degradation of Pesticides during Composting. Part II. Occurrence and Fate of Pesticides in Compost and Composting Systems. Compost Science and Utilization, 2000, vol 8, pp.61-82.**

15. **Bampus J, Aust S. Bioremediation of environmental pollutants by the white rot fungus Phanerochaete chrysosporium: involvement of the lignin degrading system. Bioassays, 1987, vol 6, pp.166-170**

16. **Bhatanagar L, Li SP, Jain MJ, Zeikus JG. Growth of methanogenic and acidogenic bacteria with PCP as co-substance, Biotechnology Applications in Hazardous Waste Treatment, 1989, pp. 383-393.**

17. **Canet R, Birnstingl JG, Malcolm DG, Lopez-Real JM, Beck AJ. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. Bioresour Technol, 2001, vol 76, pp. 113-117.**

18. **Chen ST, Kang SY. Pentachlorophenol and crystal violet degradation in water and soil-using heme and hydrogen peroxide. Water Research, 1999, vol 33, pp. 3657-3665.**

19. **Davis MW, Glaser JA, Evans JW, Lamar RT. Field evaluation of the lignin degrading fungus Phanerochaete sordida to treat creosote contaminated soil. Environmental Sciences and technology, 1993, vol 27, pp.2572-2576.**

20. **Edgehill RU, Finn RK, Microbial treatment of soil to remove pentachlorophenol. Appl Environ Microbiol, 1983, vol 45, pp.1122-1125.**

21. **Hestbjerg H, Willumsen P, Christensen M, Andersen O, Jacobsen C. Bioaugmentation of tar-contaminated soils under field conditions using Pleurotusostreatus refuse from commercial mushroom production. Environmental Toxicology and chemistry, 2003, vol 22, pp. 692-698**

22. **Waldbot GL, Burgstahler AW, McKinney, HL. Fluoridation: The Great Dilemma, Coronado Press, Inc. Lawrence, Kansas, 1978.**

23. **Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Fluorides, Hydrogen Fluoride, and Fluorine (F). U.S. Department of Health & Human Services, Public Health Service, 1993, ATSDR/TP-91/17**

24. **DHHS, Review of Fluoride: Benefits and Risks, Report of the Ad Hoc Committee on Fluoride of the Committee to Coordinate Environmental Health and Related Programs. Department of Health and Human Services, USA, 1991.**

25. **Leland, D.E., Powell, K.E. and Anderson, R.S. A fluoride overfeed incident at Harbour Springs, Mich., Journal of the American Water Works Association, 1998, vol 4, no. 72, pp. 238–243.**

26. **Toxicological Profile for Fluorides, Hydrogen Fluoride, and Fluorine (F),Agency for Toxic Substances and Disease Registry, U.S. Georgia: U.S. Department of Health and Human Services, 1993**
27. **Zhang, M., Wang, A., Xia, T, and He, P., Toxicol. Lett., 2008, vol. 179, no. 1,pp. 1–5.**
28. **Chouhan, S., Lomash, V., and Flora, S.J.S., J. Appl. Toxicol, 2010, vol. 30, no. 1, pp. 63–73.**
29. **Jolly SS, Prasad S, and Sharma R. Review of 25 years research on fluoride. Journal Association of Physicians of India; 1970, vol.18, pp. 459-71.**
30. **Doble M, Kumar A, Biotreatment of industrial effluents. Elsevier, New York, 2005**
31. **Johncy-Rani M, Hemambika B, Hemapriya J, Rajeshkannan V. Comparative assessment of heavy metal removal by immobilized and dead bacterial cells: a biosorption approach.Global J Environ Res, 2010, vol 4, pp. 23-30.**
32. **Baker JL, Sudarsan N, Weinberg Z, Roth A, Stockbridge RB, Breaker RR. Widespread genetic switches and toxicity resistance proteins for fluoride. Science 2012;335:233-5.33 Praveen Kumar V, Hari Priya VR. Molecular characterization of fluorine degrading bacteria from soil samples for its industrial exploitation. Int J Adv Life Sci, 2013, vol 6, pp. 351-5.**
33. **Joshi_Tope, G.A. and Francis, J., J. Bacteriol., 1995, vol. 177, no. 8, pp. 1989–1993.**
34. **Bellack, E. and Schouboe, P.J. Rapid photometric determination of fluoride in water. Anal. Chem, 1958, vol 30, pp. 2032-2034.**