In situ bioremediation of Chromium contaminated soil using a novel cationic lipopeptide biosurfactant from *Alcaligenes aquatilis sp.*

A Dissertation

Submitted in partial fulfillment of the requirement

For the award of degree of

Master of Technology in Environmental Science and Technology

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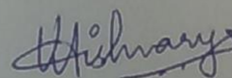
DECLARATION

I hereby declare that the work presented in the thesis entitled "***In situ bioremediation of Chromium contaminated soil using a novel cationic lipopeptide biosurfactant from Alcaligenes aquatilis sp.***" in partial fulfillment of the requirement for the award of the degree of Master of Sciences in Environmental Sciences and Technology, Thapar University, Patiala is an authentic record of my own work carried out under the supervision of Mr. K.S. Babu, Assistant Professor, School of Energy and Environment, Thapar University, Patiala and Dr. G. Sekaran, Chief Scientist, Environmental Technology Division, CLRI. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.



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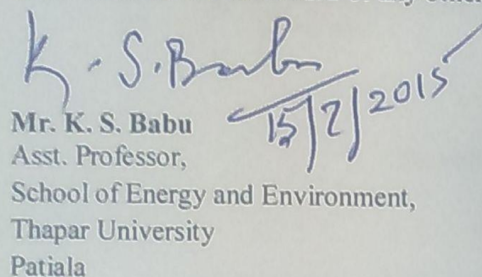

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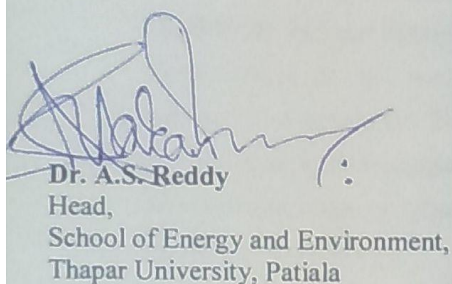

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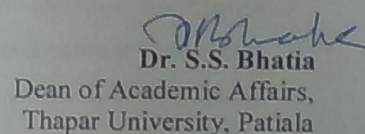

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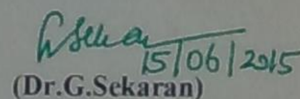

(Dr.G.Sekaran)

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ABSTRACT

Biosurfactants are surface-active compounds derived from various microbial sources including bacteria and fungi. They have a wide range of properties and applications in various fields. *Alcaligenesaquatilis sp.* bacterium was isolated from soil. The cationic nature of the biosurfactant was confirmed by tests such as Blue plate agar test and by determining the amino acid composition of the biosurfactant. The optimized conditions for the production of cationic lipopeptide were found to be 96 h, pH 4 and temperature 37^0C.The biochemical characteristics of the cationic lipopeptide show that the protein concentration is 26.06 mg/g and lipid concentration is 60 mg/g respectively. The SDS-PAGE revealed that the molecular weight of the biosurfactant is 100.2 KDa. Chromium removal studies were performed to determine the effect of time,pH and temperature on the removal of Cr by the cationic biosurfactant as well as anionic biosurfactant. Adsorption isotherm studies were performed using Langmuir and Freundlich isotherms to determine the extent of adsorption of the biosurfactant onto the soil. The characterization of the Immobilized Cationic Biosurfactant (ICB) was carried out using FTIR, TGA, DSC and XRD. This ICB along with anionic biosurfactant was used for Insitu Bioremediation of Cr from contaminated soil. The initial concentration of Cr in the soil sample collected was found to be 19.5mg/g of soil using standard Cr estimation method.The removal of Cr from the soil was confirmed after estimating the Cr concentration in the soil treated with ICB. Finally seed germination studies were carried out for Cr sensitive seeds such as *Zea mays* (Maize), *Luffa* (Ridge Gourd), *Trigonellafoenum-graecum* (Fenugreek) using the treated and untreated soil and comparisons were made.

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CHAPTER 1: INTRODUCTION

1.1 Leather Industry

Tanning is one of the oldest industries in the world. During ancient times, tanning activities were organized to meet the local demands of leather footwear, drums and musical instruments. With the increase in number of population, the increasing requirement of leather and its products led to the establishment of large commercial tanneries (Durai and Rajasimman, 2011). Leather tanning is the process of converting raw hides or skins into leather. Hides and skins have the ability to absorb tannic acid and other chemical substances that prevent them from decaying and make them resistant to wetting, and keep them supple and durable. Tanneries are typically characterized as pollution intensive industrial complexes which generate widely varying, high-strength wastewaters. Variability of tannery wastewaters are not only from the fill and draw type operation associated with tanning processes, but also from the different procedures used for hide preparation, tanning and finishing. Tanning industry also has one of the highest toxic intensity per unit of output (Khan et al., 1999).

During tanning process at least about 300 kg chemicals are added per ton of hides (Verheijen et al., 1996). Tannery effluent is among one of the hazardous pollutants of industry. Major problems are due to wastewater containing heavy metals, toxic chemicals, chloride, lime with dissolved and suspended salts and other pollutants (Uberoi, 2003).

Disposal of industrial and urban wastes into soil and water has led to disastrous consequences to the ecosystems. Due to excessive loading of these wastes beyond their self cleaning capacities, the ecosystems have resulted in decreased availability of clean water to drink and normal soils for crop production. Many stringent regulations regarding the disposal of organic and inorganic wastes have been enacted in many countries including India. Compared to the organic wastes, inorganic wastes such as heavy metals pose a great threat as they cannot be completely removed/degraded from the ecosystem (Sara Parwin Banu, 2003).

More than 1,70,000 tons of Cr waste are discharged to the environment annually as a consequence of industrial and manufacturing activities. Chromium is one such heavy metal, whose accumulation in soil is posing a great threat to plant and human life in several ways. Chromium is released into the environment by a large number of industrial operations such a

electro plating, chromate manufacturing, leather tanning, textile industry, copy machine toner and wood preservation. Soil, contains on an average 400 parts per million total chromium, but this depends on the balance of oxidizing and reducing agents in the soil. Chromium can change its valence state in soil and sediments depending upon the local physical, chemical, and biological conditions.

Though chromium exists in nine valence states ranging from −2 to +6, Cr(III) and Cr(VI) are of major environmental significance. Trivalent Cr is weakly soluble in acidic and alkaline soils where as hexavalent Cr is highly soluble in both acidic and alkaline soils. Cr(VI) is toxic, carcinogenic, and mutagenic to animals as well as humans. Like other heavy metals, chromium influences the enzymatic activity of soil and affects plant morphology causing a decrease in its growth. In contrast, trivalent chromium Cr(III) is relatively less toxic and less mobile.

Tanneries are one of the most prominent sources of chromium pollution to the environment. If not adequately treated, wastewater from tanneries contaminate surface water and sediments to an unacceptable level. The tanning process is carried out with the use of chromium tanning agents, principally basic chromium sulphates (Pawlikoski et al, 2006). Chromium sulphate is applied to the hides to protect them from decay and aging. Chromium from tanneries is thus discharged mainly as Cr(III) bound to organic and inorganic ligands . At neutral or slightly basic pH, chromium is relatively immobile in tannery sludge.

Cr(III) is considered toxic and dangerous due to various reasons .It enters many types of cells. It can be absorbed by the lung, gastro-intestinal tract and by the skin. Under physiological conditions it can be reduced to reactive intermediates such as Cr(V) and Cr(IV) and any of these species could attack the DNA , proteins, membrane lipids, thereby disrupting cell integrity and functions. Hence there is a need to treat chromium contaminated environments (mainly soils and aquifers).

Chromium (III) is a cationic species and it is immobile due to its low solubility, high adsorption and complexation. Chromium (VI) is more toxic and mobile. There are various factors that determine the reactions that take place with chromium in the soil. Some components such as Natural Organic Matter (NOM), sulphide components, manganese dioxide, Fe (II) bearing minerals present in the soil cause significant reactions with chromium. NOM plays a very crucial role in the interaction between chromium and soil.

NOM can absorb Cr(III) on its surface thus reducing the amount of mobile Cr(III) susceptible to oxidation.

Metal contaminants in the environment are usually tightly bound to colloidal particles and organic matter. This represents a major constraint to their removal using currently available insitu remediation technologies. The promising new remedial strategy for treating the soil bound metal contamination in soil is the use of biosurfactants. Bioremediation using biosurfactants have several advantages over the conventional bioremediation methods. Bioremediation is a waste management process which uses naturally occurring microorganisms to breakdown hazardous substances into less toxic substances.

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. They are used for soil washing or flushing due to their ability to mobilize contaminants. They contain a hydrophobic portion with little affinity for the bulk medium and a hydrophilic group that is attracted to the bulk medium. Surfactants have been used industrially as adhesives, flocculating, wetting and foaming agents, de-emulsifiers and penetrants (Mulligan and Gibbs, 1993).They also reduce the surface tension between two liquids which are immiscible.(eg: water and oil)

Biosurfactants are compounds which are produced on the surfaces of living microorganisms or are secreted extracellularly. These microorganisms could be either yeast, bacteria or fungi. They are surface active agents which are capable of reducing the interfacial tension between liquids, solids or gases. The hydrophobic portion can be a lipid structure, an isoprenoid structure such as cholesterol, or a hydrophobic amino acid or peptide and the hydrophilic portion can be an amino acid, phosphate, or a carbohydrate group. The structure of a biosurfactant is predominantly determined by the producing organism, but can to a certain extent be influenced by the culture conditions. Biosurfactants are classified based on their charge principally into 3 types: (1) Anionic (2) Cationic (3) Non-ionic. Biosurfactants can be classified into four groups based on their chemical composition: (1) Glycolipids (2) Oligopeptides and Lipopeptides (3) Phospholipids, fatty acids and neutral lipids (4) Polymeric biosurfactants Biosurfactants have several advantages over synthetic surfactants such as: high biodegradability, lower toxicity, good compatibility with eukaryotic organisms, effectiveness at a wide range of temperatures, pH values, and salinities, synthesis under user friendly conditions (low temperatues and pressures) (Sineriz.F, 2012). The substrates used for the production of biosurfactants such as vegetable oils and dairy wastes are economical and

are renewable resources. Biosurfactants have been tested for various environmental applications such as bioremediation of oil spills and enhanced oil recovery and transfer of crude oils and are thought to be potential candidates to replace chemical surfactants in the future. (Banat et al, 2000)

The main objective of this present study is to provide a novel strategy for the removal of chromium from chromium contaminated soils using lipopeptide biosurfactant.

1.2 Aim & Objective

The goal of the study is to remove Chromium present in contaminated soil in tannery clusters and reduction of toxicity using cationic biosurfactant.

Objectives:

- To isolate biosurfactant producing bacteria.
- To isolate and extract the biosurfactant produced from bacteria.
- To characterize the nature of soil and chromium toxicity level.
- To characterize the cationic biosurfactant.
- To remove the Cr from contaminated soil using biosurfactant in batch study.
- To optimize the conditions for the removal of Cr.
- To study adsorption isotherm kinetics for the biosurfactant adsorption onto soil.
- To study the Insitu bioremediation of chromium removal from soil using cationic and anionic biosurfactant.
- To study the toxicity of soil by seed germination index.

CHAPTER 2: REVIEW OF LITERATURE

Annual global leather production is about 6.8 million tonnes, around 80% of which is chrome-tanned. Tanning is the process of treating skins of animals to produce leather, which is more durable and less susceptible to decomposition. Chrome tanning uses a solution of chemicals, acids and salts, mainly chromium sulphates to tan the hide. It is estimated that in India alone, about 2000–3000 tons of Cr escape into environment annually from the tanning industries. Although chromium can exist in several redox states, the trivalent and hexavalent forms are the most environmentally significant.

2.1 Chromium

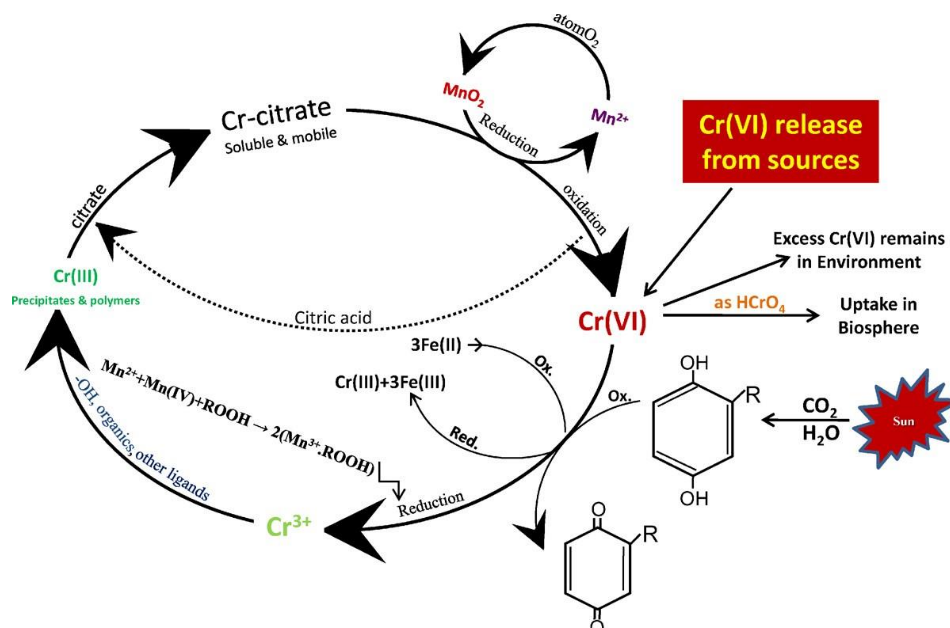


Fig 2.1: Chromium cycle

Chromium is one of the most frequently used metal contaminants and is considered to be one of the top 20 contaminants on the Superfund priority list of hazardous substances for the past 15 years. The biogeochemical properties of Cr and the associated soil matrix can affect the removal efficiency of many treatment strategies, an understanding of these properties is essential for choosing an effective treatment method (Elisabeth L. Hawley et al., 2004).

The properties of Cr are highly dependent on the molecular structure of the Cr compound, particularly on the oxidation state (or oxidation number) of the Cr. Cr is an element that exists primarily in two different oxidation states, hexavalent and trivalent. These oxidation states are symbolized as Cr(VI) and Cr(III), respectively. Except for the rarely, naturally-

found, elemental Cr with an oxidation number of zero, Cr(0), other oxidation states of Cr are unstable and therefore, are not found in the natural environment. But in soils the most stable and common forms are trivalent, Cr(III) and hexavalent, Cr(VI) species, which display quite different chemical properties and affect organisms in different ways. Hexavalent chromium is water soluble in the full pH range, while trivalent chromium tends to get adsorbed on soil surface or precipitate as chromium hydroxide in a slightly acidic and alkaline environment. Therefore, Cr(III) has the high potential for environmental contamination, especially of aquifers and surface water. In its hexavalent form, the U.S. Environmental Protection Agency (EPA) has classified chromium as a Group 'A' human carcinogen and is one of the main pollutants (Elisabeth L. Hawley et al., 2004).

2.2 Soil

Knowledge of physical and chemical properties of soil is most important for designing the parameters of remediation process. The mechanisms of interaction between the soil and contaminants are also important to know. Soil can be defined as loose material composed of weathered rock, other minerals, and also partly decayed organic matter, that covers large parts of the land surface. The soil is composed of three phases: a solid, liquid, and gas phase. The soil components include about 50% by volume mineral particles, 25% water, 20% air and 5% organic matter. With the exception of a few organic soils, the bulk of soil material is mineral in character, and has been derived from solid geological deposits and the addition of organic debris soils contain minerals and organic matter. The clay minerals and humus usually bears a negative charge because of their structure and chemical composition. The occurrence of negative charge is due to the dissociation of protons from the surfaces and edges of the clay minerals (alumino silicates and hydrous (hydrated) oxides of iron and aluminium) and from acidic groups in humus, which increases as pH is raised. The mineral constituents of the soil are represented by the particles of widely varying size, shape and chemical composition. Three groupings of soil particles are in common use, namely sand, sit, and clay. The groups are subdivided according to requirements.

2.3 Extraction and Transport of Metals in the soil

2.3.1 Extraction mechanism

It has been shown that surfactants could be used potentially for environmental remediation of heavy metals from soil, through research in this area has been limited. It is possible that

surfactants can be used as extractants for transferring metal ions from an aqueous to an organic phase (Roundhill, 2011). The possible mechanisms for the extractions of heavy metals by surfactants are ions exchange, precipitation-dissolution and counter ion binding (Rosan, 1979).

The first mechanism for enhanced heavy metal removal from soil surface is ion- exchange. This process takes place when a negatively charged ion adsorbed on to a subsurface is replaced by negatively charged surfactant ion from solutions. Since miscelles are not directly involved in ion exchange, exchangeable ions will increase linearly below the CMC and remain relatively constant above CMC (Nivas *et al.*, 1996; Doong et al., 1998).

The second mechanism for enhanced heavy metal removal from soil surface is counter ion binding. For ionic micelles, the interfacial region between the aqueous region and micelle contain the ionic head group , the stern layer of the electrical double layer pertaining to these group, more than one half of the counter ions associated with the micelle and water . Nivas et al. (1996) suspected that precipitating cations (eg Ca^{2+},Ba^{2+}) would be attracted to the surfactant micelles to promote dissolution of precipitated heavy metals when the concentration of surfactant exceeds the CMC , thereby enhancing the removal of chromium from soil.

2.3.2. Fate and Transport Mechanism

The fate and transport of heavy metal in soils depend significantly on the chemical form and specification of the metal (Allen and Torres, 1991). While the various metals undergo similar reactions in the number of aspects, the extent and nature of these reactions varies under particular conditions. Generally, heavy metal in soil can be grouped in to five fractions: exchangeable, carbonate, Fe-Mn oxide, organic and residual fractions. The presence of hydrous metal oxides of Fe, Al, Mn can strongly influence the metal concentration because these minerals can remove cations and anions from solutions by ion exchange, specific adsorption and surface precipitation. The most important chemical processes affecting the behavior and bio availability of metals in soils are those concerned with the adsorption of metals from the liquid phase and from the solid phase. Several different mechanisms can be involved in the adsorption of metal ion, including cation exchange, specific adsorption, organic complication and co-precipitation.

2.3.2.1 Cation Exchange

Most heavy metals exist as cations in the soil solutions, and their adsorption therefore, depends on the density of negative charges on the surfaces of the soil colloids. All other factor being equal, surfaces with a greater number of charged sites per unit surface area will be able to bind greater quantities of dissolved ions. Surfaces with a high density of negatively charged sites (cation exchanged) will selectively bind positively charged ions while those with a high density of positively charged sites will be selective for anions. Cation exchange capacity (CEC) refers to the concentration of readily exchangeable cations on the mineral surface and is often used to indicate the affinity of soils for uptake cation such as metals. The CEC of minerals soils can range from the few to 60 meq/ 100 g, but in organic soils it may exceed 200 mg/100g (Alloway, 1990) .Anion exchange capacity (AEC) indicate the affinity of soils for uptake of anions, and is significantly lower than the CEC of the soils, owing to the greater number of negative charges on the colloid surfaces.

2.3.2.2 Specific Adsorption

Specific Adsorption involves the exchange of heavy metal cations and most anions with surface ligands to form partly covalent bonds with lattice ions. Specific adsorption is strongly pH dependant and is related to the hydrolysis of the heavy metal ions. The metal most able to form hydroxyl complexes are specifically adsorbed to the greatest extent. In addition to being adsorbed on mineral surfaces, heavy metal ions also can diffuse in to mineral such as goethite, Mn oxides, and some other minerals. Adsorption comprises of three different steps, First, Surface adsorption, Second diffusion in to mineral particles and third adsorption and fixation at position with in mineral particles (Alloway, 1990).

2.4 Few Methodologies for the decontamination of chromium contaminatedsoil

According to (Castelo-Grande et al., 2008)there were advantages as well as disadvantages for the various techniques used for de-contamination of chromium contaminated soil.

2.4.1 Soil washing

Soil washing can be used to remove metals from the soil by chemical or physical treatment methods in aqueous suspension. Soil samples are subjected to chelant extraction using a solution of disodium salt of ethylene diamine tetracetic acid, sodium metabisulfite solution, solution of 2 reagents. Batch and column washing with de-ionized water revealed that 20-

25% chromium was removed. Hence this method proved to show less efficiency in chromium removal.

Table 2.1 Advantages and disadvantages of Soil washing

ADVANTAGE	DISADVANTAGE
Reduces the volume of contaminant, therefore, further treatment or disposal is less problematic;	Contaminant toxicity is unchanged, although volume is reduced; Cost of disposal of waste streams must be considered.
Commercially available	Less effective when soil contains a high percentage of silt and clay;

2.4.2 Vitrification

It is a waste treatment process that involves combustion of contaminant substances.

Table 2.2 Advantages and Disadvantages of Vitrification

ADVANTAGE	DISADVANTAGE
Mobility of metals is reduced or eliminated. The vitrified mass resists leaching for geologic periods of time.	The process requires intensive energy and high temperatures up to near 2000 K. Water in soil affects operation and increases the total costs of the process.
Widely used and available commercially	Off gases must be collected and treated before release. Certain types of soils such as clay soils or soils containing rocks may need.

2.4.3 Electrokinetic Treatment

Electrokinetic remediation technologies apply a low density current to contaminated soil in order to mobilize contaminants in the form of charged species. The current is applied by inserting electrodes into the subsurface and relying on the natural conductivity of the soil (due to water and salts) to effect movement of water, ions and particulates through the soil.

Table 2.3 Advantages and Disadvantages of Electrokinetic Remediation

ADVANTAGE	DISADVANTAGE
In situ technology that has small impact on environment (soil removal is not required). Metals are actually removed from soil unlike stabilization, which leaves the metals in the soil.	Alkaline soils reduce the effectiveness of the process. Requires soil moisture.

The conventional physicochemical methodologies such as ion exchange, chemical precipitation, reverse osmosis and evaporative recovery for soils contaminated with Cr(VI) require high energy and plenty of chemical reagents when applied to large scale. There are many more biological and non-biological methodologies such as bio-venting, bio-sparging, phytoremediation, land farming, land filling, soil flushing, and stabilization/solidification. But none of these methods are considered to be the best treatment options because either they offer a temporary solution or are costly when applied to larger areas. These methods will become inefficient when Cr(VI) is in low concentrations and it could not remove Cr(VI) completely. Therefore these methods are not economically feasible. Consequently, economical and environmentally friendly remediation methods of Cr(VI) contaminated sites is in urgent need. Bioremediation is one of the promising methods to clean up the Cr contaminated site.

2.5 Bioremediation

Bioremediation is a waste management technique to remove or neutralize the pollutants at a particular site using microorganisms. It is based on the idea that organisms are capable to take in pollutants from the environment and use them to enhance their growth and metabolism or

convert them from a toxic to a nontoxic form. Bacteria and fungi are well known for degrading complex molecules and contaminants.

- The microbial reduction of hexavalent chromium into less soluble trivalent chromium seems to be a potential method for the remediation of Cr(VI) contamination.
- This reduction can take place in either aerobic or anaerobic conditions depending on the oxidation reduction potential of the environment.

Chromium is known to undergo various chemical and biological reactions in the natural systems that govern speciation of the metal and in turn its environmental behavior. Important reactions include oxidation/reduction, precipitation/dissolution, and absorption/desorption. Both oxidation of Cr(III) and reduction of Cr(VI) can occur in both geological and aquatic environments (Sara Parwin et al.,2003).

Recently Barrera-Diaz et al. reviewed the chemical, electrochemical and biological methods for aqueous Cr(VI) reduction. Most of these approaches are economically expensive and have disadvantages like incomplete metal removal, high reagent consumption and energy requirements, while contaminating the ground water because of generation/disposal of toxic sludge/secondary wastes. Some of the treatment methods/processes have major limitations such as its economic viability only at high or moderate concentrations of metals and not at low concentrations (1 to 100 mg L^{-1}). On the other hand bioremediation appears to have wider implications to tide over the problem of low concentration of heavy metal in detoxification.

2.6 Surfactants

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. They are used for soil washing or flushing due to their ability to mobilize contaminants. They contain a hydrophobic portion with little affinity for the bulk medium and a hydrophilic group that is attracted to the bulk medium. Surfactants have been used industrially as adhesives, flocculating, wetting and foaming agents, de-emulsifiers and penetrants (Mulligan and Gibbs, 1993).

The industrial need for surfactants is constantly growing. It has become evident that the surfactants have become commercially important from the increasing trends in their production and the number of industrial applications (Luna et al., 2011). Their environmental

uses are related principally to the bioremediation of petroleum hydrocarbons in groundwater and soil and in the degradation of hazardous compounds (Coimbra et al., 2009). In the oil industry, they are used in microbial-enhanced oil recovery, in the cleaning of contaminated vessels and to facilitate transportation of heavy crude oil by pipeline (Muthusamy et al., 2008).

Surfactants are a more cost effective tool for desorbing contamination. Surfactant mixtures can be used to desorb and solubilize a range of organic contaminants from soil including even highly hydrophobic compounds like PAHs and some pesticides. Once the contamination has been released it can be addressed by several remediation methods including bioremediation, recovery or chemical oxidation. Selecting the right surfactants for desorption is important because the hydrophobic moiety of the surfactant must be matched to the organic contaminant of concern and the surfactant must be evaluated with site soil to ensure it will not sorb to soil and increase oxidant demand (Bethany McAvoy et al., 2013).

2.7 Classification of surfactants

From the commercial point of view surfactants are often classified according to their use. However, this is not very useful because many surfactants have several uses, and confusions may arise from that. The most accepted and scientifically sound classification of surfactants is based on their dissociation in water.

Anionic Surfactants are dissociated in water in an amphiphilic anion, and a cation, which is in general an alkaline metal ($Na+$, $K+$) or a quaternary ammonium. They are the most commonly used surfactants. They include alkylbenzene sulfonates (detergents), (fatty acid) soaps, lauryl sulfate (foaming agent), di-alkyl sulfosuccinate (wetting agent), lignosulfonates (dispersants). Anionic surfactants account for about 50 % of the world production.

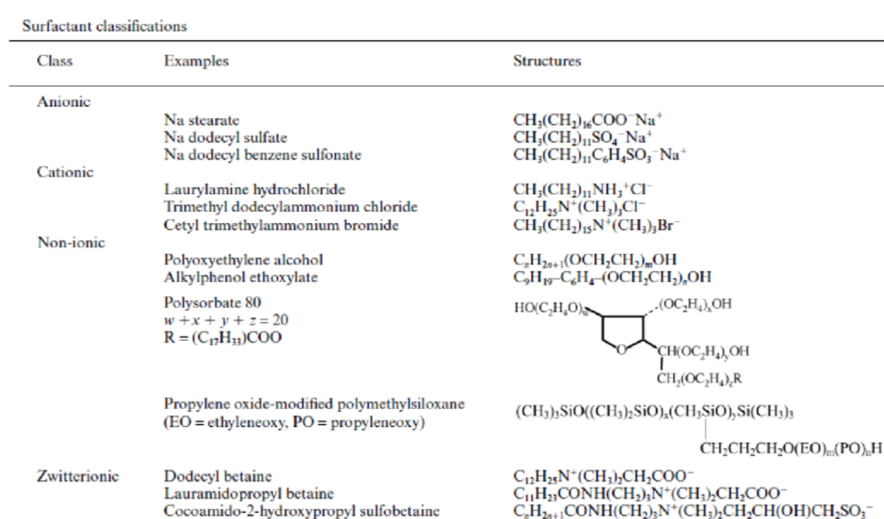
Nonionic Surfactants is the next with about 45% of the overall industrial production. They do not ionize in aqueous solution, because their hydrophilic group is of a non-dissociable type, such as alcohol, phenol, ether, ester, or amide. A large proportion of these nonionic surfactants are made hydrophilic by the presence of a polyethylene glycol chain, obtained by the polycondensation of ethylene oxide. They are called polyethoxylated nonionics.

Cationic Surfactants dissociate in water into an amphiphilic cation and an anion, most often of the halogen type. A very large proportion of this class corresponds to nitrogen compounds

such as fatty amine salts and quaternary ammoniums, with one or several long chain of the alkyl type, often coming from natural fatty acids. These surfactants are in general more expensive than anionics, because of a high pressure hydrogenation reaction to be carried out during their synthesis.

When a single surfactant molecule exhibit both anionic and cationic dissociations it is called **amphoteric**or**zwitter ionic**. This is the case of synthetic products like betaines or sulfobetaines and natural substances such as amino acids and phospholipids.

Table.2.4 Classification of synthetic surfactants based on their charge



Surfactant classifications

Class	Examples	Structures
Anionic		
	Na stearate	$CH_3(CH_2)_{16}COO^- Na^+$
	Na dodecyl sulfate	$CH_3(CH_2)_{11}SO_4^- Na^+$
	Na dodecyl benzene sulfonate	$CH_3(CH_2)_{11}C_6H_4SO_3^- Na^+$
Cationic		
	Laurylamine hydrochloride	$CH_3(CH_2)_{11}NH_3^+Cl^-$
	Trimethyl dodecylammonium chloride	$C_{12}H_{25}N^+(CH_3)_3Cl^-$
	Cetyl trimethylammonium bromide	$CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$
Non-ionic		
	Polyoxyethylene alcohol	$C_nH_{2n+1}(OCH_2CH_2)_mOH$
	Alkylphenol ethoxylate	$C_9H_{19}–C_6H_4–(OCH_2CH_2)_nOH$
	Polysorbate 80 $w + x + y + z = 20$ $R = (C_{17}H_{33})COO$	$HO(C_2H_4O)_w$, $(OC_2H_4)_xOH$, $CH(OC_2H_4)_yOH$, $CH_2(OC_2H_4)_zR$
	Propylene oxide-modified polymethylsiloxane (EO = ethyleneoxy, PO = propyleneoxy)	$(CH_3)_3SiO((CH_3)_2SiO)_x(CH_3SiO)_ySi(CH_3)_3$ $CH_2CH_2CH_2O(EO)_m(PO)_nH$
Zwitterionic	Dodecyl betaine	$C_{12}H_{25}N^+(CH_3)_2CH_2COO^-$
	Lauramidopropyl betaine	$C_{11}H_{23}CONH(CH_2)_3N^+(CH_3)_2CH_2COO^-$
	Cocoamido-2-hydroxypropyl sulfobetaine	$C_nH_{2n+1}CONH(CH_2)_3N^+(CH_3)_2CH_2CH(OH)CH_2SO_3^-$

2.8Biosurfactants

Biosurfactants are compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly. They are produced by bacteria, yeast or fungi. They have the capacity to increase the surface area of hydrocarbons and increase their solubility thereby increasing their biodegradability as well. Biosurfactants as in comparison to synthetic surfactants have many chemical forms such that they can be selected based on their high specificity for different metals and thus metal removal efficiency. Biosurfactants are present in various pools inside cells: as intracellular molecules, extracellularly secreted products or as compounds located at the cell surface (Prabhu and Phale, 2003). Together they have been known to enhance degradation by alteration in cell hydrophobicity and enhancement of dispersion of water immiscible compounds (Zang and Miller, 1992; Zang and Miller, 1994; Patricia and Jean-Claude, 1999). Pseudomonas aeruginosa strains are known to produce rhamnolipid type of biosurfactant which is a mixture of mainly mono and dirhamnolipids.

Application of rhamnolipids to pure cultures or in soil systems has been shown to enhance biodegradation of a number of hydrocarbons including hexadecane (Herman et al., 1997). Thus a clear correlation exists between surface active agent production and alkane utilization by the degrading organism.

Table.2.5 Classification of Biosurfactants and their Microbial origin(Adapted from Mulligan &Gibbs, 1993; Banat, 1995 &Mulligan et al, 2001c)

Rhamnolipids	Pseudomonas aeruginosa
Lipopeptides	Arthobacter sp.
	Bacillus pumilis
	Bacillus subtilis
	Bacillus licheniformis
	Pseudomonas fluorescens
Lipopolysaccharides	Acinetobcter calcoaceticus
	Pseudomonas sp.
	Candida lipolytica
	Thiobacillus thio oxidans
	Corynebacterium alkanolyticum
Fatty acids	Penicillium spiculisporum
	Corynebacterium lepus
	Arthobacter parfinieus
	Talaranyces trachyspermus

In bioremediation, biosurfactants effectively enable the transport of the more hydrophobic compounds, while the less hydrophobic ones are retarded due to the sorption of surfactant micelles to the soil surface. Biosurfactants are coming into limelight and are preferred more than synthetic surfactants because they have lesser impacts on the environment. They have higher biodegradability, lower toxicity, good biocompatibility with eukaryotic organisms and effectiveness at a wide range of pH, temperatures and salinities.

2.9 Biosurfactants and its use in the removal of heavy metals

Heavy metals are not biodegradable; they can only be transferred from one chemical state to another, which changes their mobility and toxicity. Microorganisms can influence metals in several ways. Some forms of metals can be transformed either by redox processes or by alkylation. Metals can also be accumulated by microorganisms by metabolism-independent (passive) or by intracellular, metabolism dependent (active) uptake. Microorganisms can influence metal mobility indirectly by affecting pH or by producing or releasing substances which change mobility of the metals. Two following methods, soil washing or soil flushing, are involved in remediation of metal contaminated soil. The first technique used is ex situ where contaminated soil is excavated, put into the glass column and washed with biosurfactant solution. In turn, soil flushing of insitu technologies involves use of drain pipes and trenches for introducing and collecting biosurfactant solution to and from the soil. Interestingly, biosurfactants can be used for metal removal from the soil. Biosurfactants can be applied to a small part of contaminated soil in which soil is put in a huge cement mixer, biosurfactant-metal complex is flushed out, soil deposited back, and biosurfactant-metal complex treated to precipitate out biosurfactant, leaving behind the metal. The bond formed between the positively charged metal and the negatively charged surfactant is so strong that flushing water through soil removes the surfactant metal complex from the soil matrix. This method can also be carried out for deeper subsurface contamination only with more pumping activities. The usefulness of biosurfactants for bioremediation of heavy metal contaminated soil is mainly based on their ability to form complexes with metals. The anionic biosurfactants create complexes with metals in a nonionic form by ionic bonds. These bonds are stronger than the metal’s bonds with the soil and metal-biosurfactant complexes are desorbed from the soil matrix to the soil solution due to the lowering of the interfacial tension. The cationic biosurfactants can replace the same charged metal ions by competition

for some but not all negatively charged surfaces (ion exchange). Metal ions can be removed from soil surfaces also by the biosurfactant micelles. (Pacwa-Płociniczak et al., 2011)

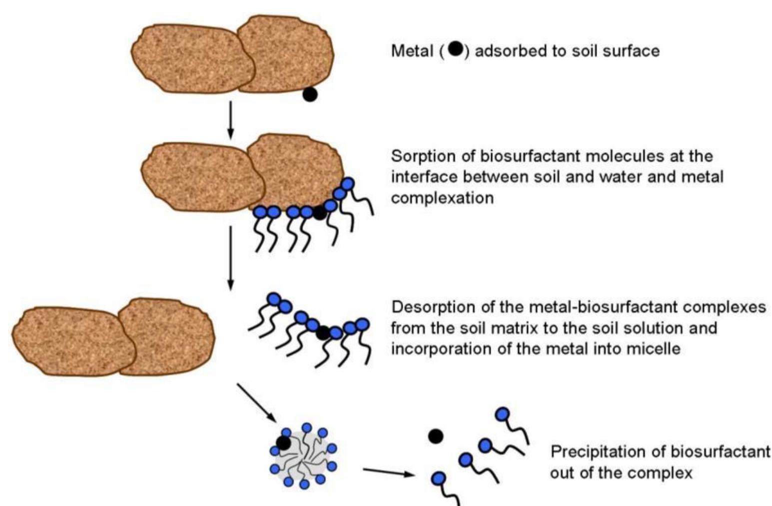


Fig. 2.2 Mechanism of attachment of biosurfactant to the metal contaminant in soil

Biosurfactants which are used in bioremediation of metal-contaminated soils have been proposed for use in metal removal in recent years. High potential of biosurfactants in mobilization and decontamination of heavy metal contaminated soil was confirmed by Juwarkar et al., who used di-rhamnolipid biosurfactant produced by Pseudomonas aeruginosa BS2 for mobilization of metals from multi-metal contaminated soil. To study the feasibility of di-rhamnolipid to remove chromium, lead, cadmium and copper from soil, a column study was conducted. Heavy metal spiked soil into a glass column was washed with 0.1% di-rhamnolipid biosurfactant solution. The results indicated that di-rhamnolipid selectively removed heavy metals from soil in the order of Cd = Cr > Pb = Cu > Ni. Besides the mobilization, biosurfactants can be involved in other processes connected with remediation of heavy metals. They are used, for example, in entrapping of trivalent chromium in micelles which provides bacterial tolerance and resistance towards high concentration of Cr(III). Gnanamani et al. studied the bioremediation of chromium (VI) by biosurfactant producing, marine isolate Bacillus sp. MTCC 5514. The remediation carried out by this strain proceeded via two processes: reduction of Cr(VI) to Cr(III) by extracellular chromium reductase and entrapment of Cr(III) by the biosurfactants. The first process transforms the toxic state of chromium into less-toxic state and the second process prevents the bacterial cells from the

exposure of Cr(III). Both reactions keep bacterial cells active all the time and provide tolerance and resistance toward high hexavalent and trivalent chromium concentrations.

2.10 Importance of surfactant adsorption in remediation

Surfactants are used as potential agents for enhanced solubilisation and removal of contaminants from soil and sediments. In a surfactant-enhanced remediation process surfactant adsorption on soil and/or sediment is an important parameter. The effectiveness of surfactant is decreased when a significant amount is adsorbed by the soil, since amount of surfactant available for solubilizing the contaminants decreases, and its mobility through the medium to which it is applied is reduced. In addition to that, adsorption of surfactant increase the hydrophobicity of the soil, as a result, removed solubilized organic will be re-adsorbed on soil surface. To facilitate the application of surfactant-based technologies in soil and groundwater remediation as well as water purification, it is important to develop a better understanding of surfactant adsorption and desorption on soil. Another reason for investigating surfactant adsorption onto soil and its component is to understand the transport of surfactants and organic compounds inside soil matrix.

2.11 Cationic surfactant adsorption

Adsorption of cationic surfactants at solid/liquid interfaces has a wide range of applications, such as detergency, fabric softeners, wetting, ore flotation, and corrosion inhibition etc. Moreover, cationic surfactants have also been suggested for potential use in the remediation of contaminated soils and aquifers. Cationic surfactants are adsorbing strongly onto soil and sediments because of favorable electrostatic interactions with the predominately negatively charged soil mineral surfaces Thus, the surface of the clay may be greatly modified to become strongly hydrophobic after adsorption of cationic surfactants.

2.12 Effect of chromium on seed germination

Chromium affects seed germination. 1-5 ppm Cr present in the available form in the soil solution, either as Cr(III) or Cr(IV) is the critical level for a number of plant species. (E.A. Irfan et al., 2010). A Range of high Cr concentration in plant tissues toxicity symptoms which was observed was from 5 ppm for barley, corn,oats and citrus to 175 ppm for tobacco (Sinha et al.,2005).

Cr interferes with several metabolic processes, causing toxicity to plants as exhibited by reduced seed germination or early seedling development, root growth and biomass, chlorosis, photosynthetic impairing and finally plant death. (Scoccianti et al., 2006) Following a Cr(VI) treatment at concentrations of 5 and 10 mg l^{-1} on the maize species, the percentage of germination did not differ significantly between treated material and controls in the species examined. However, Cr(VI) supply caused a decrease in the growth rate of the primary root and also a strong inhibition in the shoot growth. (Sanita. L et al, 2006) Oxidative stress induced by chromium initiates degradation of photosynthetic pigments causing decline in growth. Chromium phytotoxicity can result in inhibition of seed germination; degrade pigment status, antioxidative enzymes, nutrient balance and induced oxidative stress in plants. Chromium (VI) is more phytotoxic than Chromium (III). In this research, the effect of aqueous chromium (Cr VI) solution on seed germination and seedling growth of wheat is studied and found that the Cr (VI) might have interfered with metabolic process and caused toxicity to the plant by reducing its root growth. As the chromium concentration increases from 2 mg/L to 10 mg/L, the seed germination decreases from 100% to 40% and seedling vigor indices decrease from 557 to 64. The increasing chromium concentrations increase the phytotoxicity of shoot and root and decrease the tolerance indices.

2.13 Economic Factors concerning Biosurfactants

According to (BS Saharan et al., 2012) the production of biosurfactants is highly expensive due to the use of synthetic culture media. Therefore, greater emphasis is being laid on procurement of various cheap agro-industrial substrates including vegetable oils, distillery and dairy wastes, soya molasses, animal fat, waste and starchy waste as raw materials. These wastes can be used as substrates for large scale production of biosurfactants with advanced technology. To overcome the expensive cost constraints associated with biosurfactant production, two basic strategies are generally adopted worldwide to make it cost effective: (i) the use of inexpensive and waste substrates for the formulation of fermentation media which lower the initial raw material costs involved in the process; (ii) development of efficient and successfully optimized bioprocesses, including optimization of the culture conditions and cost-effective recovery processes for maximum biosurfactant production and recovery. As millions of tons of hazardous and non-hazardous wastes are generated each year throughout the world, a great need exists for their proper management and utilization.

2.14 Advantages of Biosurfactant

- They are Biodegradable.
- They are less toxic.
- They have good biocompatibility and digestibility properties.
- The raw material required for the production of biosurfactants is easily available.
- They are more specific and effective against the contaminant.
- They have the capacity to increase the surface area of hydrocarbons and increase their solubility thereby increasing their biodegradability as well.
- They are effective in the solubilisation of low solubility compounds.
- They can withstand high temperature, pH and salinity.
- If the substrates used are by-products or agro-industrial wastes then the production of biosurfactants is inexpensive.

2.15 Limitations of Biosurfactants

- The production cost of the synthetic media used for biosurfactants is expensive.
- Since it is a biological process it can be time consuming.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Substrate

The substrate used for this study for the production of biosurfactant is palm oil from a local store.

3.1.2 List of Chemicals

Table.3.1: List of chemicals

Chemicals	**Company**
1,5 Diphenyl carbazide	Merck
Sulphuric acid	Rankem
Nitric acid	Rankem
Perchloric acid	Merck
SDS-Sodium dodecyl sulphate	Hi Media
CTAB- Cetyl trimethylammonium bromide	Hi Media

3.2 Acclimatization and isolation of microorganism

The soil sample was collected from the required tannery site. 500ml of nutrient media was prepared and 1% of each lipid substrate (palm oil, fish oil, and chicken tallow) was added. The media was then sonicated. The pH was adjusted to 4 and the media was autoclaved. The soil sample collected from the tannery was then inoculated into the media and acclimatized for 1 week at 37˚C. Serial dilutions of the soil upto 10^{-9} collected dilutions. 1ml of each dilution was poured into sterile petriplate on top which 20 ml of nutrient agar was poured.

Plates were inverted and incubated for 48hrs.After incubation morphologically distinct colonies were selected for further studies.

3.3 Production of biosurfactant

To 500ml nutrient media 1% palm oil was added as lipid substrate. Media was sonicated and the pH was adjusted to acidic conditions (4-6). The media was autoclaved and then 5% of the subculture of the isolated bacteria was inoculated into the media. The media was incubated 37˚C for 96 hrs. It was centrifuged at 6000rpm for 20 minutes. The supernatant was collected and 6N HCL was added to maintain the pH at 2 and left it to precipitate overnight at 4˚C. The next day again, centrifugation at 8000rpm for 15 min was carried out and pellet was collected. The pellet was dispersed in acetone for purification and kept overnight at 4˚C. The next day the pellet was dispersed in suitable amount of phosphate buffer.

3.4 Screening of biosurfactant producing organisms

The isolated colonies were obtained in pure cultures and tested for their biosurfactant production through quantitative and qualitative analysis by the following methods.

3.4.1 Oil spreading technique

- In oil spreading assay, 10 µl of crude oil was added to the surface of 40 ml of distilled water in a petridish to form a thin oil layer.
- Then, place 10 µl of culture or culture supernatant on the centre of the oil layer.
- The biosurfactant displaced the oil and a clear zone was formed, which correlates to the surfactant activity.

3.4.2 Blue agar plate (BAP) method

- Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) was used for the detection of anionic biosurfactant. Mineral salt agar medium supplemented with glucose as carbon source (2%) and Sodium Dodecyl sulfate (SDS: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) was used for the detection of cationic biosurfactant.
- 30 µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate. The plate was incubated at 37°C for 48-72 h.

- A dark blue halo zone around the culture in the plate containing CTAB was considered positive for anionic biosurfactant production and a dark blue halo zone around the culture in the plate containing SDS was considered positive for anionic biosurfactant.

3.4.3 Drop collapsing test

- Screening of biosurfactant production was performed using the qualitative drop-collapse test described.
- 2 μL of palm oil was applied to the well regions delimited on the covers of 96 well micro plates and these were left to equilibrate for 24 h.
- 5 μL of the 48 h culture was transferred, before and after centrifugation at 12,000 rpm for 5 min to remove cells, to the oil-coated well regions and the drop size was observed after 1 min with the aid of a magnifying glass.
- The result was considered positive for biosurfactant production if the drop is flat and those cultures that gave rounded drops were scored as negative, indicating the lack of biosurfactant production.

3.4.4 Emulsification test (EI24)

- Several colonies of pure culture were suspended in test tubes containing 2 mL of cell free broth medium. After 48 h of incubation, 2 mL palm oil was added to each tube.
- The mixture was vortexed at high speed for 1 min and allowed to stand for 24 h. The emulsion index (EI24) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100.

$$\textbf{Emulsification Index (EI24) (\%)} = \frac{\textbf{Height of emulsified layer (mm)}}{\textbf{Total height of the liquid column (mm)}}$$

Based on the screening test results, the positive isolates were inoculated into the mineral salt medium for the biosurfactant production. Based on the quantification of biosurfactant produced, the best strain is selected, and then identification of the bacterium is done.

3.5 Optimization studies on the production of cationic lipopeptide from Alcaligenes aquatilis sp.

3.5.1 Effects of time, temperature, pH on the biosurfactant production

In this study the effects of the main parameters such as time (24, 48, 72, 96, 120 hrs), temperature (at 20, 30, 40, 50, 60 and 70◦C) and pH (at 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) using 6 N HC1/1 N NaOH, on the growth and biosurfactant production of the *Alcaligenes aquatilis sp.* was studied. All cultivations were carried out in 500 ml flasks containing 100 ml of nutrient broth with palm oil as a substrate. The flasks were inoculated with the bacterial culture and incubated at 150 rpm. The bacterial cell growth, dry cell weight, biosurfactant production and emulsification index were determined.

3.6 Fermenter conditions

3.6.1 Biosurfactant Production conditions

Biosurfactant production kinetics was studied in a 3-L fermenter (LARK) with 3 L working volume at 30 °C and 130 rpm. The aeration was controlled at a rate of 1.0 vvm. The pH was maintained at about 4.0. Cellular growth was expressed in terms of dry cell weight which was calculated from the equation of a calibration curve constructed between optical density and cell dry weight of the *Alcaligenes aquatilis sp.* (gl^{-1})=0.34×OD 600. Optical density was measured at 600 nm by UV visible spectrophotometer (PerkinElmer, model Lambda25, USA) during different time intervals up to 96 h.

3.7 Preparation of functionalized nanoporous activated carbon (FNAC)

Nanoporous activated carbon (NAC) was prepared from rice husk and the functionalization of the NAC was carried out as explained by Ramani et al. (2012). The washed FNAC material was dried at 110°C for 6 h to obtain the final product and it was used for the immobilization of the biosurfactant.

3.8 Characterization of the biosurfactant

3.8.1 Protein Estimation

Procedure: To 1.0 mL of the sample 5.0 mL of the mixed reagent is added. The mixture is incubated for 10 min.To this 0.5mL of Folin's Reagent is added. The mixture is then

incubated for 30 min. The absorbance of the biosurfactant is then measured at 650nm. A standard is plot with varying concentrations of Bovine Albumin Serum.

Calculation:

$$\textbf{Protein (mg/L)} = \textbf{(OD /slope)} \times \textbf{(1000/Volume of the sample)}$$

3.8.2Lipid Estimation

Procedure: To 1ml of the sample, 5mL of sulphuric acid was added to it. The mixture was then incubated in a boiling water bath for 10 min and cooled to room temperature. From this 0.5mL of sample was taken and 6mL of phosphovanilin reagent was added. This mixture was incubated in dark for 45min.Absorbance was measured at 533nm.

3.8.3 SDS PAGE

The molecular weight of the biosurfactant was determined by using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) according to the method followed by Lammeli (Laemmli, 1970) on a 5 % stacking gel and 12 % resolving gel. The protein marker ranging from 3.75KDa to 205 KDa was used as a standard marker for the determination of molecular weight.

Procedure:

1. The stacking and separating gel were cast and the comb paced to create wells for the sample loading.
2. The samples were mixed with the sample buffer in equal proportions and loaded in the wells.
3. The gel was run at 50v until the loading dye reached the bottom of the gel.
4. Coomassive brilliant blue staining was used to view the bands.

Table 3.2: Composition of 12% separating gel and stacking gel for SDS-PAGE

Components		**Separating gel**	**Stacking gel**
Tris HCL buffer	pH 8.8	4.5ml	_
	pH 6.8	_	2.25ml

Acrylamide	6ml	1.5ml
APS	60µl	45µl
SDS	180µl	90µl
Glass distilled water	7.2ml	5.25ml
TEMED	30µl	15µl

3.8.4 Amino acid composition analysis by HPLC

The biosurfactant (both anionic and cationic) were hydrolyzed at 100 ℃ for 24 h with 6 N HCl and neutralized with 1N NaOH. The amino acid composition was analyzed using Agilent 1100 HPLC amino acid analyzer, and the data analysis was performed by using HP chem station.

3.9 Instrumental analysis

The surface morphology of the immobilized biosurfactant and the immobilized biosurfactant exposed to chromium was determined using Scanning Electron Microscope (SEM, Anna University) combined with energy-dispersive Xray spectroscopy (EDX) for element mapping, TGA and DSC analysis of purified biosurfactant, ICB before and after exposure to soil Cr were carried out with TGA Q50. About 5–8 mg of lyophilized sample was loaded on a platinum pan and its energy level was scanned in the ranges of 30–480◦C and 30–450◦C for TGA and DSC analysis respectively, under a nitrogen atmosphere with a temperature gradient of 10◦C/min. Both analyses were performed under gradual increase in temperature by plotting the weight percentage and heat flow against temperature (Jain et al., 2012; Mishra et al., 2011). An infrared spectrophotometer was used for the investigation of the surface functional groups (Perkin-Elmer). The samples with KBr (spectroscopic grade) pellets were prepared in the size of diameter, 10–13 mm and thickness, 1mm. The free biosurfactant, immobilized biosurfactant and immobilized biosurfactant exposed to chromium were scanned in the spectral range of 4000–400 cm^{-1}. UV-Vis spectroscopic measurements of the samples were performed on a Varian, CARY 100C double beam spectrophotometer using a 1 cm quartz cuvette and were scanned from l200 to 800 nm and Fluorescence spectroscopy also done. The XRD pattern of immobilized biosurfactant and immobilized biosurfacatnt exposed

to chromium were determined using XRD analysis with high resolution GUINER powder X-ray diffractometer (SEIFERT, Germany).

3.10 Chromium estimation

3.10.1 Digestion of the sample

500mg of soil sample was dissolved in 100mL distilled water. The organic matter was destroyed using a 10ml mixture of concentrated sulphuric acid,perchloric acid, and nitric acid (1:1:1). The solution was brought to a slow boil on a hot plate at 120˚C and concentrated to half its original volume. The heating was discontinued and 5ml conc.HNO_3 and 10ml conc.H_2SO_4. The solution evaporated on a hot plate at 120˚C until dense white fumes of SO 3 just appeared. If the solution was not clear, 10ml conc. HNO_3 was added and heated until excess HNO_3 was removed. The heating was discontinued when the solution was clear and no brown fumes were observed. 50ml of water was added to the solution and boiled to dissolve any soluble salts, filtered, if necessary and then Cr (III) was oxidized to Cr(VI) with 5% KMnO4 solution in an acidic medium.

3.10.2 Oxidation of Cr(III) to Cr(VI) by KMnO4

The digested sample was heated to boiling on a hot plate and 2 drops of 5% KMnO4 was added until a faint pink colour persisted, and the heating was continued on a steam bath for 20min.If the colour disappeared ,5% KMnO4 solution was added drop wise to maintain a slight pink colour and boiled for another 2 mins.1ml of 1% sodium azide solution was added drop wise to the boiling sample until the pink colour of KMnO4 just disappeared. The sample was boiled again for 5 min, cooled and finally made up to 100ml in a volumetric flask.

3.10.3 Colour development and measurement

About 2 ml of aliquot of the digested sample was neutralized with 5% NaOH solution and was pipetted into a 100ml volumetric flask. It was then acidified with 3 ml 6N H2SO4, 2ml of 0.5% 1, 5-Diphenyl carbazide (DPC) solution was added, and it was made up to 100ml. The solution was mixed thoroughly and kept for full colour development for 5 min. After the development of the colour, the solution was transferred to a 1-cm absorption cell and absorbance was measured at 540nm. Using distilled water as reference. Absorbance readings were corrected by subtracting absorbance of a blank carried through the method. From the corrected absorbance, the amount of chromium was determined from the calibration curve.

3.10.4 Preparation of the Calibration curve

Potassium di chromate (10µg of Cr 6+/ml) was used as standard and treated by the same method as that of the soil sample. Standard graph was plotted between concentration of Cr^{6+} and absorbance at 540nm.

Calculation:

Amount of total chromium present in soil sample (µg of Cr/g of soil) =

(OD/ Slope) × (Dilution factor/gms of soil taken

3.11 Cr III removal from contaminated soil by free biosurfactants (Batch studies)

3.11.1 Effect of time

The effect was investigated by varying the time of contact from 1hr to 8 hrs. To 1 gm of Chromium contaminated soil, 0.5 g of biosurfactant (anionic & cationic) was added. To this 50ml of deionised water was taken in Erlenmyer flasks of volume 100ml and they were kept in shaking incubator at 100 rpm for 1-8 hrs, samples were collected for every one hour and the amount of chromium removal was determined by standard method.

To determine the time required for maximum removal of chromium, 1 g of chromium contaminated soil was taken in a 100 mL conical flask containing 50 ml of deionized water with 250 mg of cationic lipopeptide. The contents of the flask were agitated in a rotary mechanical shaker at 37 °C, and the chromium content of the soil was measured at different time intervals .The total chromium content in the biosurfactant and soil was estimated by measuring absorbance of the purple complex of chromium with 1,5-diphenylcarbazide at 540 nm using a carry win UV-vis spectrophotometer. The uptake of chromium by the biosurfactant was calculated from the difference between the initial and final concentration as follows:

$$q= [(Co-C_f)V]/M$$

where q is the chromium uptake (mg/g), Co is the initial chromium concentration, C_f is the final chromium concentration (mg/L), M is the biosurfactant dosage (mg) and V is the

solution volume (L). Each experimental result was obtained by averaging the data from two parallel experiments.

3.11.2 Effect of pH

The effect of pH was studied by taking different pH buffers (4-9).50mL of each buffer was taken in Erlenmeyer flasks of volume 100mL. To each flask 1 g of Cr contaminated soil, 0.5g of biosurfactants (anionic & cationic) was added and they were kept in shaking incubator at 100 rpm at their optimum incubation time and amount of Cr removal was determined by standard method.

3.11.3 Effect of Temperature

Effect of temperature on biosurfactant for the Cr removal was determined at different temperature range (20, 30, 40, 50, 60, 70 & 80˚C).To 1 gm of Cr contaminated soil, 0.5 gms of biosurfactant (anionic &cationic) was added. To this 50mL of deionized water was taken in Erlenmeyer fasks of volume 100mL.They were kept in the shaking incubator at 100rpm at their optimum time. The amount of Cr removal was determined by standard method.

3.12 Adsorption Isotherms

3.12.1 Collection and Preparation of Adsorbent and Adsorbate

All chemicals used were of analytical grade. The amount of soil taken for the study was 10 g. The amount of lipopeptide biosurfactant was taken in different concentrations. The amount of biosurfactant was expressed in terms of protein content (mg/L) by using BSA (Bovine serum albumin) as standard. The samples were collected periodically and the analysis was carried out.

3.13 In situ bioremediation of Cr from contaminated soil using immobilized cationic biosurfactant (ICB)

The batch procedure used to investigate immobilized cationic biosurfactant facilitated desorption of soil-bound chromium has been done. A known amount of Cr contaminated soil was taken. To this known volume diluted anionic biosurfactant was added to the dried soil. And to this ICB was introduced into the soil. The soil was left in flood for seven days and the soil samples were collected at different spots and then dried for analysis. The samples were analyzed for Cr estimation using standard method. Finally, the added ICB was removed from

the soil and made to ash for estimation. This gave the removal capacity of ICB. All the experiments were conducted as triplicates.

3.14 Soil Characteristics

The subsurface soil used in this study was collected from a tannery cluster area Common Effluent Treatment Plant in Chennai, Tamil Nadu, India.

3.14.1Bulk Density

1. Known amount of soil (5-10gms) was taken and added into a weighing bottle.
2. The soil was placed in the bottle which was tapped 15-20 times on a table by letting it fall from a height of about 2-3 cm. This tapping is assumed to produce the same packing in the soil which occurs naturally in the field.
3. The volume of the packed soil was noted which is equal to the volume of the container.

$$\textbf{Bulk density} = (M_2 - M_1) / V \ \text{gm/cm}^3$$

where M1 is mass of empty weighing bottle, M2 is mass of empty bottle with soil, M2-M1 is mass of soil, V is volume of bottle

3.14.2 Porosity

1. Known amount of soil was taken and added it into the weighing bottle.
2. The soil was placed in the bottle which was tapped 15-20 times on a table by letting it fall from a height of about 2-3 cm. This tapping is assumed to produce the same packing in the soil which occurs naturally in the field.
3. Known volume of water was added to the soil till a level where the soil is just submerged.
4. The total volume occupied by the soil and water was noted.

Pore space = Volume of sand and water – Volume of water

Porosity = Pore space / Total volume of soil and water.

3.14.3 Soil moisture

1. The moist soil sample was placed in a moisture box and weighed immediately.

2. The box without the lid was placed in an oven (150˚C) and the soil was dried to a constant weight.
3. The sample was removed from the oven, replacing the lid and the box was placed in the dessicator until it cooled.
4. The sample was weighed and the mass of the empty moisture box was weighed. The mass of moisture was determined.

% Moisture content on dry mass basis (θg) = [(M2 – M3)/ (M3 – M1)] * 100

% Moisture content on volume basis (θv) = θg * (Db/ Dw)

where, Mass of empty moisture box = M1 gms, Mass of the box + moist soil = M2 gms, Mass of the box + oven dry soil = M3 gms, Mass of water in the soil = (M2 – M3) gms, Mass of oven dry soil = (M3 –M1) gms, D_b = Bulk density of soil and D_w = Bulk density of water

3.14.4 Soil pH

1. 20 gm of 2.0 mm air dry soil was weighed into a beaker. 50 ml of distilled water was added and stirred with a glass rod thoroughly for about 5 minutes and kept for half an hour.
2. In the mean time turn the pH meter was turned on, allowed to warm up for 15 minutes. The glass electrode using standard buffer of pH = 7 was standardized and calibrated with buffer pH =4 and pH =9.2.

The electrodes were dipped in the beakers containing the soil water suspension with constant stirring.

1. While recording pH, the pH meter was switched on to pH reading. After 30 seconds the pH value was recorded to the nearest 0.1 unit. The pH meter was put on standby mode immediately after recording.
2. The electrodes were removed from soil suspension and the electrodes were cleaned with distilled water.
3. The electrodes were rinsed after each determination and carefully blotted them dry with filter paper before the next determination. The glass electrodes were standardized after every 10 determinations.

4. The electrodes were dipped in distilled water, when not in use and made sure that the reference electrode always contained saturated potassium chloride solution in contact with solid potassium chloride crystals.
5. Three to four drops of toluene were added in standard buffer solutions to prevent growth of mould.

3.14.5 Determination of organic carbon in soil

A. Preparation of Standard Curve:

1. 1 gm sucrose was taken and added to 1000 ml distilled water.
2. From this solution 0, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50…till 250 ml were taken in 100 ml flask and 10 ml potassium dichromate and 20 ml sulphuric acid were added in each flask.
3. The mixture was shaken and allowed to cool on asbestos sheet.
4. The volume of each solution was made to 100 ml with distilled water and observes optical densities at 660 nm wavelength.
5. The standard curve was calculated and factor F was calculated.

Factor F = X/24

where, X is the sum total of carbon % for 1 OD.

B. Organic carbon in soil

1. The soil sample was sieved with 1 mm sieve and 1 gm of sieved soil sample was taken in 100 ml flask.
2. 10 ml potassium dichromate was taken and 20ml sulphuric acid was added and shaken well and allowed to cool on an asbestos sheet.
3. The volume was made to 100 ml with distilled water and kept overnight.
4. Optical density at 660 nm wavelength was measured on spectrophotometer.

Organic Carbon % = Optical density × Factor F

3.2.13.6 Determination of calcium carbonate (free lime) in soil

1. 5gms of soil was taken in a 250ml flask.
2. 10 ml of concentrated sulphuric acid, perchloric acid and nitric acid was added. Sample was digested.

3. After digestion the sample was diluted with 100 ml of water. 2ml from this was taken and made upto 50ml.
4. 2ml of 1 normal NaOH was added and 1 spatula of muroxide indicator was added.
5. This was titrated against 0.01N EDTA solution. Colour changed from pink to violet.

Calcium hardness (mg/ml) = Titre value × 0.01 × 1 × 100/ (volume of sample)

3.2.13.7 Determination of TKN

1. 10 g of sieved soil was taken in the kjeldal flask.
2. Distilled water was added with the help of jet in such a way that the particles of soil did not remain stuck to the sides of the flask.
3. 20 ml sulphuric acid was added in each of the flasks.
4. The soil sample was digested in the kjeldal apparatus for 2 hrs and then the distillate was collected in boric acid.
5. The sample was titrated against 0.03N H_2SO_4.
6. The end point reached when the colour changed from green to purple.

TKN (mg/g) = (Titre volume × 0.03 × 14)/ (volume of sample)

3.15 Phytotoxicity analysis for the Chromium contaminated soil before and after treatment by seed germination index

Zea mays (Maize), Luffa (Ridge Gourd), Trigonella foenum-graecum (Fenugreek) were selected as model plant systems. To prevent fungal contamination, seeds were soaked in 50% ethanol for 30 min and washed five times with double distilled water immediately before use. Prior to use, the petriplates were acid washed and used in the phytotoxicity trials. The seeds were then allowed to germinate in sterilized Petri dishes, on Whatmann filter-paper Seed germination rate was noted down every 24 hours up to eight days. The Petri dishes, covered with a net, were kept in laboratory under ambient conditions. The length of shoot and root were recorded by using a centimetre scale, % phytotoxicity for shoot and root of 8 day old seedlings were calculated by the formula given by Chou and Lin 16. All the Petri dishes with 10 seeds were kept under optimum agricultural farm conditions by their exposure to ambient temperature, air and light conditions in the laboratory. A control experiment was carried out using double-distilled water to irrigate red soil with seeds and the water is devoid of any metal concentration.

3.15.1 Germination parameters and their determination

Experiments were carried out to determine whether the untreated Cr contaminated soil and treated soil by ICB has influence on seed germination, seedling growth including shoot and root length, seedling indices, etc. The tests were performed on 10 seeds. Radicle and plumule lengths were noted down. All the analyses were made in triplicates.

3.15.2 Statistical analysis

Mean standard deviations, variance, minimum, maximum, standard error, and correlation coefficient were calculated from at least three replicates. Statistical significance was determined. After 8 days, 10 seedlings of each Petri dish were sampled to measure the root length and shoot height using a centimeter scale (against a black background).

- Vigor Indices were determined by the following formula given by Iqbal and Rahmati. The Vigor Index (VI) was calculated using the formula,

VI = (mean root length + mean hypocotyls/shoot length) × % germination

- Seedling vigor index (SVI) was determined as per formula given by Bewly and Black,

Seedling Vigor Index = % of Germination × Mean seedling length (cm)

- Tolerance indices (T.I.) were determined through using following formula given by Iqbal and Rahmati,

T.I = (Mean root length in metal solution / Mean root length in distilledwater)× 100

- The length of shoot and root were recorded by using a centimetre scale, % phytotoxicity for shoot and root of seedlings were calculated by the following formula given by Chou and Lin,

% Phytotoxicity of Shoot=(Shoot length of control - Shoot length of treatment) × 100

% Phytotoxicity of Root = (Root length of control - Root length of treatment) × 100

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Substrate

The substrate used for this study for the production of biosurfactant is palm oil from a local store.

3.1.2 List of Chemicals

Table.3.1: List of chemicals

Chemicals	**Company**
1,5 Diphenyl carbazide	Merck
Sulphuric acid	Rankem
Nitric acid	Rankem
Perchloric acid	Merck
SDS-Sodium dodecyl sulphate	Hi Media
CTAB- Cetyl trimethylammonium bromide	Hi Media

3.2 Acclimatization and isolation of microorganism

The soil sample was collected from the required tannery site. 500ml of nutrient media was prepared and 1% of each lipid substrate (palm oil, fish oil, and chicken tallow) was added. The media was then sonicated. The pH was adjusted to 4 and the media was autoclaved. The soil sample collected from the tannery was then inoculated into the media and acclimatized for 1 week at 37˚C. Serial dilutions of the soil upto 10^{-9} collected dilutions. 1ml of each dilution was poured into sterile petriplate on top which 20 ml of nutrient agar was poured.

Plates were inverted and incubated for 48hrs.After incubation morphologically distinct colonies were selected for further studies.

3.3 Production of biosurfactant

To 500ml nutrient media 1% palm oil was added as lipid substrate. Media was sonicated and the pH was adjusted to acidic conditions (4-6). The media was autoclaved and then 5% of the subculture of the isolated bacteria was inoculated into the media. The media was incubated 37˚C for 96 hrs. It was centrifuged at 6000rpm for 20 minutes. The supernatant was collected and 6N HCL was added to maintain the pH at 2 and left it to precipitate overnight at 4˚C. The next day again, centrifugation at 8000rpm for 15 min was carried out and pellet was collected. The pellet was dispersed in acetone for purification and kept overnight at 4˚C. The next day the pellet was dispersed in suitable amount of phosphate buffer.

3.4 Screening of biosurfactant producing organisms

The isolated colonies were obtained in pure cultures and tested for their biosurfactant production through quantitative and qualitative analysis by the following methods.

3.4.1 Oil spreading technique

- In oil spreading assay, 10 µl of crude oil was added to the surface of 40 ml of distilled water in a petridish to form a thin oil layer.
- Then, place 10 µl of culture or culture supernatant on the centre of the oil layer.
- The biosurfactant displaced the oil and a clear zone was formed, which correlates to the surfactant activity.

3.4.2 Blue agar plate (BAP) method

- Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) was used for the detection of anionic biosurfactant. Mineral salt agar medium supplemented with glucose as carbon source (2%) and Sodium Dodecyl sulfate (SDS: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) was used for the detection of cationic biosurfactant.
- 30 µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate. The plate was incubated at 37°C for 48-72 h.

- A dark blue halo zone around the culture in the plate containing CTAB was considered positive for anionic biosurfactant production and a dark blue halo zone around the culture in the plate containing SDS was considered positive for anionic biosurfactant.

3.4.3 Drop collapsing test

- Screening of biosurfactant production was performed using the qualitative drop-collapse test described.
- 2 μL of palm oil was applied to the well regions delimited on the covers of 96 well micro plates and these were left to equilibrate for 24 h.
- 5 μL of the 48 h culture was transferred, before and after centrifugation at 12,000 rpm for 5 min to remove cells, to the oil-coated well regions and the drop size was observed after 1 min with the aid of a magnifying glass.
- The result was considered positive for biosurfactant production if the drop is flat and those cultures that gave rounded drops were scored as negative, indicating the lack of biosurfactant production.

3.4.4 Emulsification test (EI24)

- Several colonies of pure culture were suspended in test tubes containing 2 mL of cell free broth medium. After 48 h of incubation, 2 mL palm oil was added to each tube.
- The mixture was vortexed at high speed for 1 min and allowed to stand for 24 h. The emulsion index (EI24) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100.

$$\textbf{Emulsification Index (EI24) (\%)} = \frac{\textbf{Height of emulsified layer (mm)}}{\textbf{Total height of the liquid column (mm)}}$$

Based on the screening test results, the positive isolates were inoculated into the mineral salt medium for the biosurfactant production. Based on the quantification of biosurfactant produced, the best strain is selected, and then identification of the bacterium is done.

3.5 Optimization studies on the production of cationic lipopeptide from Alcaligenes aquatilis sp.

3.5.1 Effects of time, temperature, pH on the biosurfactant production

In this study the effects of the main parameters such as time (24, 48, 72, 96, 120 hrs), temperature (at 20, 30, 40, 50, 60 and 70◦C) and pH (at 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) using 6 N HC1/1 N NaOH, on the growth and biosurfactant production of the *Alcaligenes aquatilis sp.* was studied. All cultivations were carried out in 500 ml flasks containing 100 ml of nutrient broth with palm oil as a substrate. The flasks were inoculated with the bacterial culture and incubated at 150 rpm. The bacterial cell growth, dry cell weight, biosurfactant production and emulsification index were determined.

3.6 Fermenter conditions

3.6.1 Biosurfactant Production conditions

Biosurfactant production kinetics was studied in a 3-L fermenter (LARK) with 3 L working volume at 30 °C and 130 rpm. The aeration was controlled at a rate of 1.0 vvm. The pH was maintained at about 4.0. Cellular growth was expressed in terms of dry cell weight which was calculated from the equation of a calibration curve constructed between optical density and cell dry weight of the *Alcaligenes aquatilis sp.* (gl^{-1})=0.34×OD 600. Optical density was measured at 600 nm by UV visible spectrophotometer (PerkinElmer, model Lambda25, USA) during different time intervals up to 96 h.

3.7 Preparation of functionalized nanoporous activated carbon (FNAC)

Nanoporous activated carbon (NAC) was prepared from rice husk and the functionalization of the NAC was carried out as explained by Ramani et al. (2012). The washed FNAC material was dried at 110°C for 6 h to obtain the final product and it was used for the immobilization of the biosurfactant.

3.8 Characterization of the biosurfactant

3.8.1 Protein Estimation

Procedure: To 1.0 mL of the sample 5.0 mL of the mixed reagent is added. The mixture is incubated for 10 min.To this 0.5mL of Folin’s Reagent is added. The mixture is then

incubated for 30 min. The absorbance of the biosurfactant is then measured at 650nm. A standard is plot with varying concentrations of Bovine Albumin Serum.

Calculation:

$$\textbf{Protein (mg/L)} = \textbf{(OD /slope)} \times \textbf{(1000/Volume of the sample)}$$

3.8.2Lipid Estimation

Procedure: To 1ml of the sample, 5mL of sulphuric acid was added to it. The mixture was then incubated in a boiling water bath for 10 min and cooled to room temperature. From this 0.5mL of sample was taken and 6mL of phosphovanilin reagent was added. This mixture was incubated in dark for 45min.Absorbance was measured at 533nm.

3.8.3 SDS PAGE

The molecular weight of the biosurfactant was determined by using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) according to the method followed by Lammeli (Laemmli, 1970) on a 5 % stacking gel and 12 % resolving gel. The protein marker ranging from 3.75KDa to 205 KDa was used as a standard marker for the determination of molecular weight.

Procedure:

5. The stacking and separating gel were cast and the comb paced to create wells for the sample loading.
6. The samples were mixed with the sample buffer in equal proportions and loaded in the wells.
7. The gel was run at 50v until the loading dye reached the bottom of the gel.
8. Coomassive brilliant blue staining was used to view the bands.

Table 3.2: Composition of 12% separating gel and stacking gel for SDS-PAGE

Components		**Separating gel**	**Stacking gel**
Tris HCL buffer	pH 8.8	4.5ml	_
	pH 6.8	_	2.25ml

Acrylamide	6ml	1.5ml
APS	60µl	45µl
SDS	180µl	90µl
Glass distilled water	7.2ml	5.25ml
TEMED	30µl	15µl

3.8.4 Amino acid composition analysis by HPLC

The biosurfactant (both anionic and cationic) were hydrolyzed at 100 ℃ for 24 h with 6 N HCl and neutralized with 1N NaOH. The amino acid composition was analyzed using Agilent 1100 HPLC amino acid analyzer, and the data analysis was performed by using HP chem station.

3.9 Instrumental analysis

The surface morphology of the immobilized biosurfactant and the immobilized biosurfactant exposed to chromium was determined using Scanning Electron Microscope (SEM, Anna University) combined with energy-dispersive Xray spectroscopy (EDX) for element mapping, TGA and DSC analysis of purified biosurfactant, ICB before and after exposure to soil Cr were carried out with TGA Q50. About 5–8 mg of lyophilized sample was loaded on a platinum pan and its energy level was scanned in the ranges of 30–480◦C and 30–450◦C for TGA and DSC analysis respectively, under a nitrogen atmosphere with a temperature gradient of 10◦C/min. Both analyses were performed under gradual increase in temperature by plotting the weight percentage and heat flow against temperature (Jain et al., 2012; Mishra et al., 2011). An infrared spectrophotometer was used for the investigation of the surface functional groups (Perkin-Elmer). The samples with KBr (spectroscopic grade) pellets were prepared in the size of diameter, 10–13 mm and thickness, 1mm. The free biosurfactant, immobilized biosurfactant and immobilized biosurfactant exposed to chromium were scanned in the spectral range of 4000–400 cm^{-1}. UV-Vis spectroscopic measurements of the samples were performed on a Varian, CARY 100C double beam spectrophotometer using a 1 cm quartz cuvette and were scanned from l200 to 800 nm and Fluorescence spectroscopy also done. The XRD pattern of immobilized biosurfactant and immobilized biosurfacatnt exposed

to chromium were determined using XRD analysis with high resolution GUINER powder X-ray diffractometer (SEIFERT, Germany).

3.10 Chromium estimation

3.10.1 Digestion of the sample

500mg of soil sample was dissolved in 100mL distilled water. The organic matter was destroyed using a 10ml mixture of concentrated sulphuric acid,perchloric acid, and nitric acid (1:1:1). The solution was brought to a slow boil on a hot plate at 120˚C and concentrated to half its original volume. The heating was discontinued and 5ml conc.HNO_3 and 10ml conc.H_2SO_4. The solution evaporated on a hot plate at 120˚C until dense white fumes of SO 3 just appeared. If the solution was not clear, 10ml conc. HNO_3 was added and heated until excess HNO_3 was removed. The heating was discontinued when the solution was clear and no brown fumes were observed. 50ml of water was added to the solution and boiled to dissolve any soluble salts, filtered, if necessary and then Cr (III) was oxidized to Cr(VI) with 5% KMnO4 solution in an acidic medium.

3.10.2 Oxidation of Cr(III) to Cr(VI) by KMnO4

The digested sample was heated to boiling on a hot plate and 2 drops of 5% KMnO4 was added until a faint pink colour persisted, and the heating was continued on a steam bath for 20min.If the colour disappeared ,5% KMnO4 solution was added drop wise to maintain a slight pink colour and boiled for another 2 mins.1ml of 1% sodium azide solution was added drop wise to the boiling sample until the pink colour of KMnO4 just disappeared. The sample was boiled again for 5 min, cooled and finally made up to 100ml in a volumetric flask.

3.10.3 Colour development and measurement

About 2 ml of aliquot of the digested sample was neutralized with 5% NaOH solution and was pipetted into a 100ml volumetric flask. It was then acidified with 3 ml 6N H2SO4, 2ml of 0.5% 1, 5-Diphenyl carbazide (DPC) solution was added, and it was made up to 100ml. The solution was mixed thoroughly and kept for full colour development for 5 min. After the development of the colour, the solution was transferred to a 1-cm absorption cell and absorbance was measured at 540nm. Using distilled water as reference. Absorbance readings were corrected by subtracting absorbance of a blank carried through the method. From the corrected absorbance, the amount of chromium was determined from the calibration curve.

3.10.4 Preparation of the Calibration curve

Potassium di chromate (10µg of Cr 6+/ml) was used as standard and treated by the same method as that of the soil sample. Standard graph was plotted between concentration of Cr^{6+} and absorbance at 540nm.

Calculation:

Amount of total chromium present in soil sample (µg of Cr/g of soil) =

(OD/ Slope) × (Dilution factor/gms of soil taken

3.11 Cr III removal from contaminated soil by free biosurfactants (Batch studies)

3.11.1 Effect of time

The effect was investigated by varying the time of contact from 1hr to 8 hrs. To 1 gm of Chromium contaminated soil, 0.5 g of biosurfactant (anionic & cationic) was added. To this 50ml of deionised water was taken in Erlenmyer flasks of volume 100ml and they were kept in shaking incubator at 100 rpm for 1-8 hrs, samples were collected for every one hour and the amount of chromium removal was determined by standard method.

To determine the time required for maximum removal of chromium, 1 g of chromium contaminated soil was taken in a 100 mL conical flask containing 50 ml of deionized water with 250 mg of cationic lipopeptide. The contents of the flask were agitated in a rotary mechanical shaker at 37 °C, and the chromium content of the soil was measured at different time intervals .The total chromium content in the biosurfactant and soil was estimated by measuring absorbance of the purple complex of chromium with 1,5-diphenylcarbazide at 540 nm using a carry win UV-vis spectrophotometer. The uptake of chromium by the biosurfactant was calculated from the difference between the initial and final concentration as follows:

$$q= [(Co-C_f)V]/M$$

where q is the chromium uptake (mg/g), Co is the initial chromium concentration, C_f is the final chromium concentration (mg/L), M is the biosurfactant dosage (mg) and V is the

solution volume (L). Each experimental result was obtained by averaging the data from two parallel experiments.

3.11.2 Effect of pH

The effect of pH was studied by taking different pH buffers (4-9).50mL of each buffer was taken in Erlenmeyer flasks of volume 100mL. To each flask 1 g of Cr contaminated soil, 0.5g of biosurfactants (anionic & cationic) was added and they were kept in shaking incubator at 100 rpm at their optimum incubation time and amount of Cr removal was determined by standard method.

3.11.3 Effect of Temperature

Effect of temperature on biosurfactant for the Cr removal was determined at different temperature range (20, 30, 40, 50, 60, 70 & 80˚C).To 1 gm of Cr contaminated soil, 0.5 gms of biosurfactant (anionic &cationic) was added. To this 50mL of deionized water was taken in Erlenmeyer fasks of volume 100mL.They were kept in the shaking incubator at 100rpm at their optimum time. The amount of Cr removal was determined by standard method.

3.12 Adsorption Isotherms

3.12.1 Collection and Preparation of Adsorbent and Adsorbate

All chemicals used were of analytical grade. The amount of soil taken for the study was 10 g. The amount of lipopeptide biosurfactant was taken in different concentrations. The amount of biosurfactant was expressed in terms of protein content (mg/L) by using BSA (Bovine serum albumin) as standard. The samples were collected periodically and the analysis was carried out.

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The batch procedure used to investigate immobilized cationic biosurfactant facilitated desorption of soil-bound chromium has been done. A known amount of Cr contaminated soil was taken. To this known volume diluted anionic biosurfactant was added to the dried soil. And to this ICB was introduced into the soil. The soil was left in flood for seven days and the soil samples were collected at different spots and then dried for analysis. The samples were analyzed for Cr estimation using standard method. Finally, the added ICB was removed from

the soil and made to ash for estimation. This gave the removal capacity of ICB. All the experiments were conducted as triplicates.

3.14 Soil Characteristics

The subsurface soil used in this study was collected from a tannery cluster area Common Effluent Treatment Plant in Chennai, Tamil Nadu, India.

3.14.1Bulk Density

4. Known amount of soil (5-10gms) was taken and added into a weighing bottle.
5. The soil was placed in the bottle which was tapped 15-20 times on a table by letting it fall from a height of about 2-3 cm. This tapping is assumed to produce the same packing in the soil which occurs naturally in the field.
6. The volume of the packed soil was noted which is equal to the volume of the container.

$$\textbf{Bulk density} = (M_2 - M_1) / V \ gm/cm^3$$

where M1 is mass of empty weighing bottle, M2 is mass of empty bottle with soil, M2-M1 is mass of soil, V is volume of bottle

3.14.2 Porosity

5. Known amount of soil was taken and added it into the weighing bottle.
6. The soil was placed in the bottle which was tapped 15-20 times on a table by letting it fall from a height of about 2-3 cm. This tapping is assumed to produce the same packing in the soil which occurs naturally in the field.
7. Known volume of water was added to the soil till a level where the soil is just submerged.
8. The total volume occupied by the soil and water was noted.

Pore space = Volume of sand and water – Volume of water

Porosity = Pore space / Total volume of soil and water.

3.14.3 Soil moisture

5. The moist soil sample was placed in a moisture box and weighed immediately.

6. The box without the lid was placed in an oven (150˚C) and the soil was dried to a constant weight.
7. The sample was removed from the oven, replacing the lid and the box was placed in the dessicator until it cooled.
8. The sample was weighed and the mass of the empty moisture box was weighed. The mass of moisture was determined.

% Moisture content on dry mass basis (θg) = [(M2 – M3)/ (M3 – M1)] * 100

% Moisture content on volume basis (θv) = θg * (Db/ Dw)

where, Mass of empty moisture box = M1 gms, Mass of the box + moist soil = M2 gms, Mass of the box + oven dry soil = M3 gms, Mass of water in the soil = (M2 – M3) gms, Mass of oven dry soil = (M3 –M1) gms, D_b = Bulk density of soil and D_w = Bulk density of water

3.14.4 Soil pH

3. 20 gm of 2.0 mm air dry soil was weighed into a beaker. 50 ml of distilled water was added and stirred with a glass rod thoroughly for about 5 minutes and kept for half an hour.
4. In the mean time turn the pH meter was turned on, allowed to warm up for 15 minutes. The glass electrode using standard buffer of pH = 7 was standardized and calibrated with buffer pH =4 and pH =9.2.

The electrodes were dipped in the beakers containing the soil water suspension with constant stirring.

6. While recording pH, the pH meter was switched on to pH reading. After 30 seconds the pH value was recorded to the nearest 0.1 unit. The pH meter was put on standby mode immediately after recording.
7. The electrodes were removed from soil suspension and the electrodes were cleaned with distilled water.
8. The electrodes were rinsed after each determination and carefully blotted them dry with filter paper before the next determination. The glass electrodes were standardized after every 10 determinations.

9. The electrodes were dipped in distilled water, when not in use and made sure that the reference electrode always contained saturated potassium chloride solution in contact with solid potassium chloride crystals.
10. Three to four drops of toluene were added in standard buffer solutions to prevent growth of mould.

3.14.5 Determination of organic carbon in soil

A. Preparation of Standard Curve:

6. 1 gm sucrose was taken and added to 1000 ml distilled water.
7. From this solution 0, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50…till 250 ml were taken in 100 ml flask and 10 ml potassium dichromate and 20 ml sulphuric acid were added in each flask.
8. The mixture was shaken and allowed to cool on asbestos sheet.
9. The volume of each solution was made to 100 ml with distilled water and observes optical densities at 660 nm wavelength.
10. The standard curve was calculated and factor F was calculated.

Factor F = X/24

where, X is the sum total of carbon % for 1 OD.

B. Organic carbon in soil

5. The soil sample was sieved with 1 mm sieve and 1 gm of sieved soil sample was taken in 100 ml flask.
6. 10 ml potassium dichromate was taken and 20ml sulphuric acid was added and shaken well and allowed to cool on an asbestos sheet.
7. The volume was made to 100 ml with distilled water and kept overnight.
8. Optical density at 660 nm wavelength was measured on spectrophotometer.

Organic Carbon % = Optical density × Factor F

3.2.13.6 Determination of calcium carbonate (free lime) in soil

6. 5gms of soil was taken in a 250ml flask.
7. 10 ml of concentrated sulphuric acid, perchloric acid and nitric acid was added. Sample was digested.

8. After digestion the sample was diluted with 100 ml of water. 2ml from this was taken and made upto 50ml.
9. 2ml of 1 normal NaOH was added and 1 spatula of muroxide indicator was added.
10. This was titrated against 0.01N EDTA solution. Colour changed from pink to violet.

Calcium hardness (mg/ml) = Titre value × 0.01 × 1 × 100/ (volume of sample)

3.2.13.7 Determination of TKN

7. 10 g of sieved soil was taken in the kjeldal flask.
8. Distilled water was added with the help of jet in such a way that the particles of soil did not remain stuck to the sides of the flask.
9. 20 ml sulphuric acid was added in each of the flasks.
10. The soil sample was digested in the kjeldal apparatus for 2 hrs and then the distillate was collected in boric acid.
11. The sample was titrated against 0.03N H_2SO_4.
12. The end point reached when the colour changed from green to purple.

TKN (mg/g) = (Titre volume × 0.03 × 14)/ (volume of sample)

3.15 Phytotoxicity analysis for the Chromium contaminated soil before and after treatment by seed germination index

Zea mays (Maize), Luffa (Ridge Gourd), Trigonella foenum-graecum (Fenugreek) were selected as model plant systems. To prevent fungal contamination, seeds were soaked in 50% ethanol for 30 min and washed five times with double distilled water immediately before use. Prior to use, the petriplates were acid washed and used in the phytotoxicity trials. The seeds were then allowed to germinate in sterilized Petri dishes, on Whatmann filter-paper Seed germination rate was noted down every 24 hours up to eight days. The Petri dishes, covered with a net, were kept in laboratory under ambient conditions. The length of shoot and root were recorded by using a centimetre scale, % phytotoxicity for shoot and root of 8 day old seedlings were calculated by the formula given by Chou and Lin 16. All the Petri dishes with 10 seeds were kept under optimum agricultural farm conditions by their exposure to ambient temperature, air and light conditions in the laboratory. A control experiment was carried out using double-distilled water to irrigate red soil with seeds and the water is devoid of any metal concentration.

3.15.1 Germination parameters and their determination

Experiments were carried out to determine whether the untreated Cr contaminated soil and treated soil by ICB has influence on seed germination, seedling growth including shoot and root length, seedling indices, etc. The tests were performed on 10 seeds. Radicle and plumule lengths were noted down. All the analyses were made in triplicates.

3.15.2 Statistical analysis

Mean standard deviations, variance, minimum, maximum, standard error, and correlation coefficient were calculated from at least three replicates. Statistical significance was determined. After 8 days, 10 seedlings of each Petri dish were sampled to measure the root length and shoot height using a centimeter scale (against a black background).

- Vigor Indices were determined by the following formula given by Iqbal and Rahmati. The Vigor Index (VI) was calculated using the formula,

VI = (mean root length + mean hypocotyls/shoot length) × % germination

- Seedling vigor index (SVI) was determined as per formula given by Bewly and Black,

Seedling Vigor Index = % of Germination × Mean seedling length (cm)

- Tolerance indices (T.I.) were determined through using following formula given by Iqbal and Rahmati,

T.I = (Mean root length in metal solution / Mean root length in distilledwater)× 100

- The length of shoot and root were recorded by using a centimetre scale, % phytotoxicity for shoot and root of seedlings were calculated by the following formula given by Chou and Lin,

% Phytotoxicity of Shoot=(Shoot length of control - Shoot length of treatment) × 100

% Phytotoxicity of Root = (Root length of control - Root length of treatment) × 100

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Isolation of the cationic lipopeptide producing bacteria

The strain was identified by both biochemical tests and 16S rRNA sequencing. Genomic DNA was isolated from the pure culture pellet. With consensus primers, the 1.5 kb 16S rRNA fragment was amplified using high fidelity PCR polymerase. The PCR product was cloned and plasmid DNA was bi-directionally sequence using forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologous for the microbe.

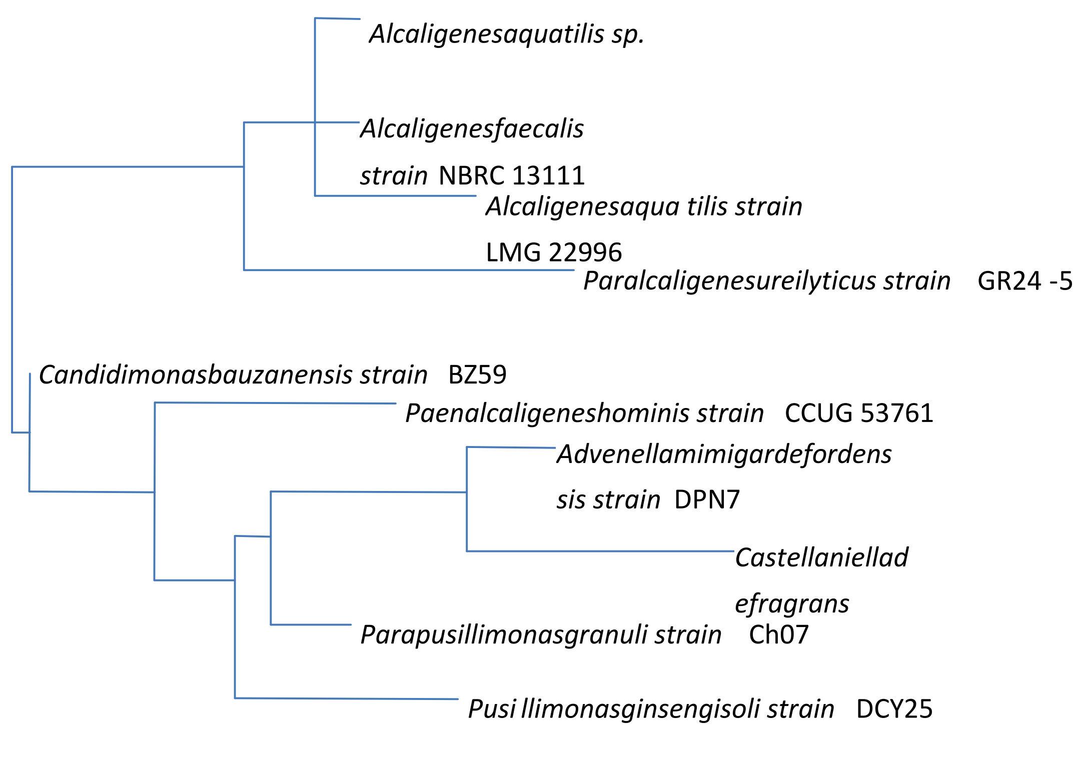


Fig. 4.1: Phylogenetic tree of *Alcaligenes aquatilis sp*. strain

The organism isolated was *Alcaligenes aquatilis sp*. It is a gram negative bacteria. It is a catalase and cytochrome oxidase positive bacteria.

4.2 Screening for biosurfactant producers

The two isolates were then subjected to screening for biosurfactant production by multiple methods like Oil spreading technique, drop collapse test and CTAB agar plate test. In the oil spreading technique, Morikawa et al. showed that the extent of oil displacement is directly

proportional to the concentration of the biosurfactant produced. All the two samples significantly displaced the oil layer and started to spread in the water, showing a zone of displacement. Jain et al. described the drop collapse test according to which the degree of collapse of the culture supernatant describes the surfactant concentration. I1 & I2 strains showed near-complete collapse, while for isolate 2 the drops turned absolutely flat. In the CTAB test designed by Siegmund and Wagner, isolate I1 showed bluish halos around the colonies on CTAB methylene blue agar medium, this confirms the lipopeptide biosurfactant corresponds to anionic and the isolate I2 showed a positive result for SDS blue plate agar which confirms the presence of cationic lipopeptide.. Emulsification index of both isolates are shown in the table.

Table 4.1: Screening of biosurfactant activity

TestTest	I1	I2
Oil spreading	++	++
Blue plate agar	-	+
Drop collapse	++	++

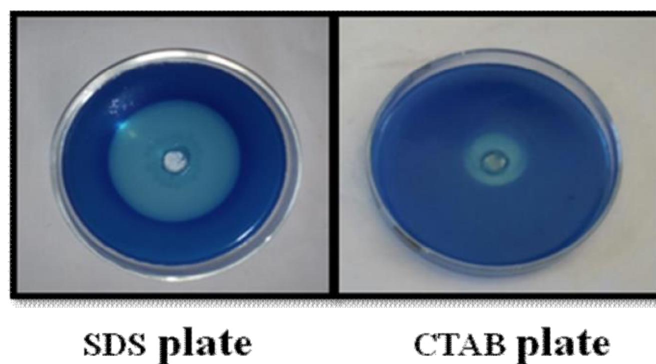
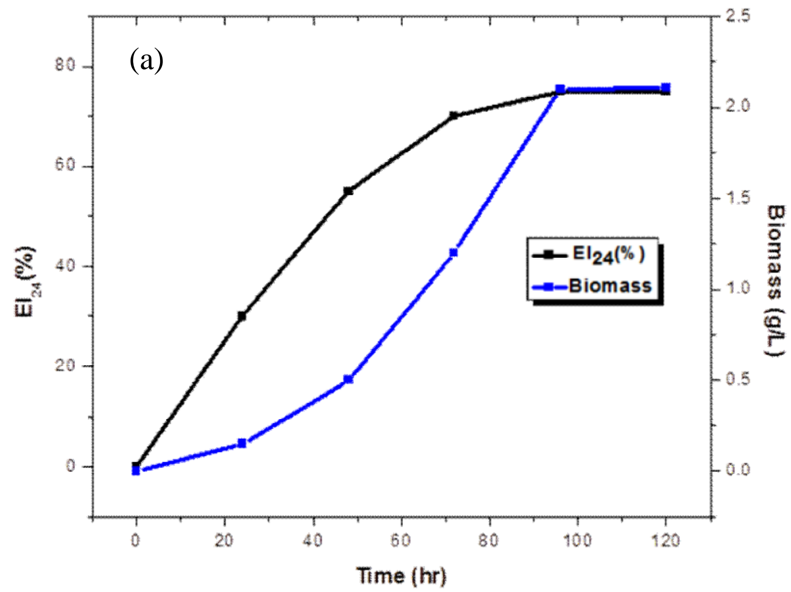
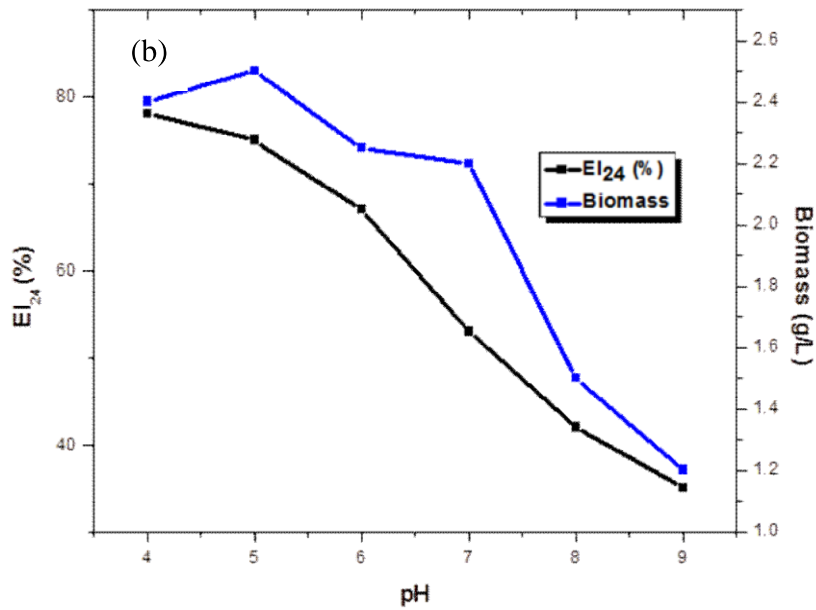


Fig.4.2: Blue agar plate a) Isolate 1- positiveand b) Isolate 2 – positive

4.3 Optimization studies on the production of cationic lipopeptide from *Alcaligenes aquatilis sp.*





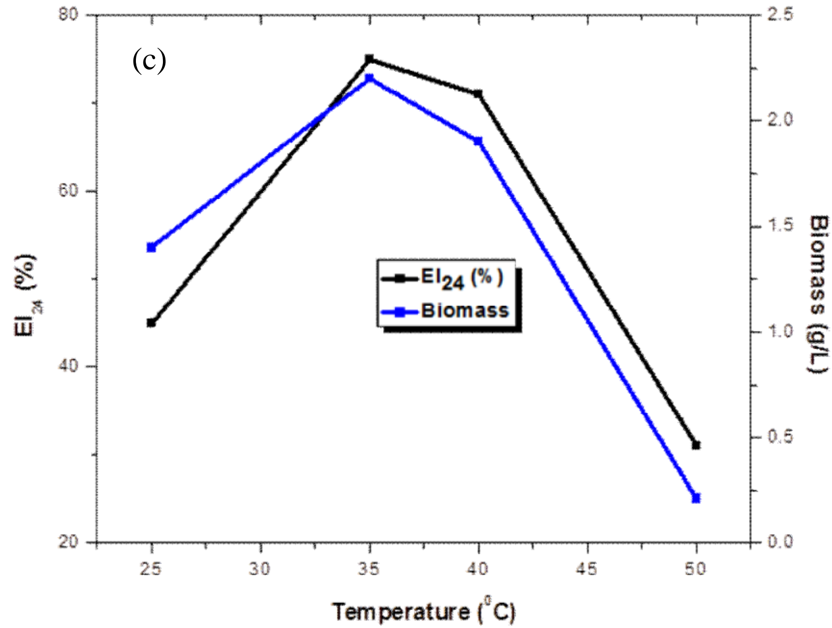


Fig 4.3: a) Effect of Time (0 – 8 h) b) Effect of pH (1-9) and c) Effect of temperature (25˚C-50˚C) on the production of biosurfactant

The effect of time, temperature and pH was the time required for the maximum yield of cationic lipopeptide was found to be at 96 h. In this hour the emulsification index was 75% and the biomass of 2.11 g/0.5 L was collected. After 96 hours there was no change in the emulsification activity. From this it is concluded that 96 h is the maximum time required for the production of efficient cationic lipopeptide. Maximum biosurfactant production was obtained at 37^0C and pH 4.0 after 96 h of incubation.

4.4 Fermenter conditions

Table.4.2: Fermenter conditions for the production of cationic biosurfactant from *Alcaligenes aquatilis sp.*

Time	Dissolved Oxygen	pH	Temp	ORP
Before inoculation	**75.3**	**4**	**26**	**-75**
After inoculation 1st hr	**69.3**	**3.86**	**26**	**-80**

10th hr	66.4	3.78	26.6	-82
20th hr	72.3	3.74	27.6	-85
30th hr	65.6	3.74	27.4	-150
40th hr	65.4	3.78	24.4	-197.2
50th hr	28.5	3.8	23.9	-170.4
60th hr	0.2	3.76	25.3	-165.8
70th hr	0.2	3.78	26.5	-100.4
80th hr	0.2	3.82	24.4	-220.7

From the fermenter conditions it was seen that the initial DO was 75.3 and it gradually reduced till the third day of production after which it drastically reduced to 0.2 on the fourth day. The pH and temperature was maintained around 4 and 26˚C respectively. The initial ORP was -75.It decreased to -197.2 on the third day after which it further decreased to -220.7 on the sixth day. These conditions indicated the presence of very little oxyen in the fermenter during the production of biosurfactant.

4.5 Characterization of the biosurfactant

The chemical composition analysis revealed that the biosurfactant produced by *Alcaligenes aquatilis sp.* is most likely a lipopeptide predominantly lipid with relative percent of 60.25 mg/ml and 26.60 mg/ml protein.

4.5.1 Molecular weight determination of lipopeptide biosurfactant by SDS-PAGE

The molecular mass of the produced lipopeptide was found to be 100.2 KDa (Fig. 4.4). It showed the high purity level of the protein. The reported lipopeptide produced from Streptomyces amritsarensis sp. having a molecular mass of 161 Da, is comparatively lesser than the lipopeptide from *Alcaligenes aquatilis sp*.

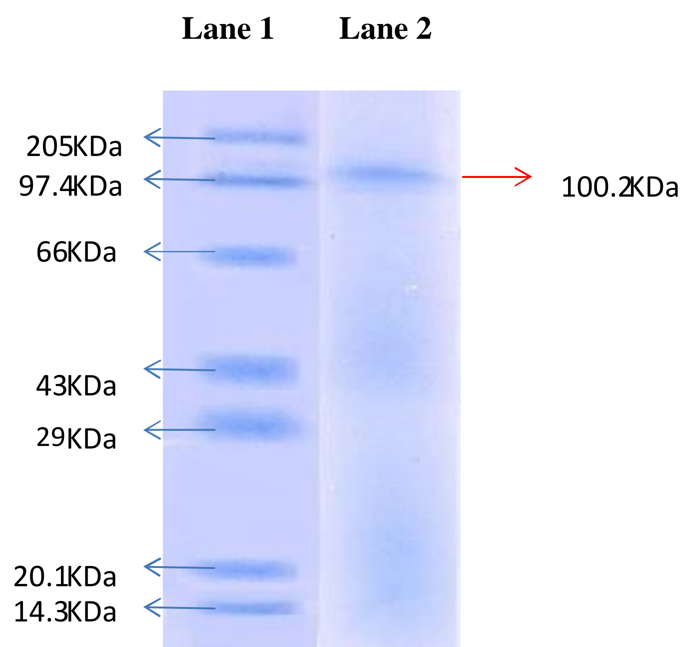


Fig.4.4: SDS PAGE of purified lipopeptide biosurfactant from Alcaligenes aquatilis sp., Lane 1: Molecular mass standards 3.5 KDa-205 KDa. Lane 2: Lipopeptide (10 µg)

4.5.2 Amino acid composition

Table 4.3: Amino acid composition of cationic and anionic biosurfactant

Amino Acids	**Composition (nmoles/ml)**	
	Anionic Biosurfactant	**Cationic Biosurfactant**
Aspartic acid	58.7	361
Glutamic acid	119.6	510
Serine	60.1	225
Histidine	305.9	1195
Glycine	105.1	721
Threonine	12.6	181

Arginine	22.7	23
Alanine	163.3	40
Tyrosine	46.8	23
Methionine	138.1	126
Valine	109.9	130
Phenylalanine	19.4	129
Isoleucine	12.5	76
Leucine	28.3	236
Lysine	71.5	121

The amino acid composition of purified cationic biosurfactant and anionic biosurfactant were determined using HPLC (Table), which shows 64.39 nmoles% and 35.61 nmoles% of polar and non-polar amino acids, respectively. The ratio of polar/non-polar amino acid of cationic biosurfactant is 1.81. And for anionic biosurfactant, the percentages of polar and non polar amino acids were 72.93nmoles% and 27.07 nmoles% respectively. The ratio of polar/non-polar amino acid of anionic biosurfactant is 2.7. Based on the amino acid composition the surfactant is said to be cationic or anionic in nature. In this study, the biosurfactant obtained from *Alcaligenes aquatilis sp.,* was found to be cationic by the presence percentage nmoles of histidine, aspartic acid, glutamic acid, arginine, lysine, tyrosine, phenylalanine were seem to be high and imparts cationic in nature.

4.5.3 UV-Visible spectral analysis

The UV-Visible spectral analysis of biosurfactant and biosurfactant with chromium was studied for the cationic lipopeptide. There was peak observed at 264 nm for biosurfactant. And after it was treated with chromium contaminated soil, there is a reduction in the peak absorbance, and this confirms the binding of chromium with biosurfactant. The Biosurfactant

was subjected to UV spectroscopy studies. The peak obtained at 264nm may indicate the possibility of an aromatic amino acid (Neuhof et al., 2005).

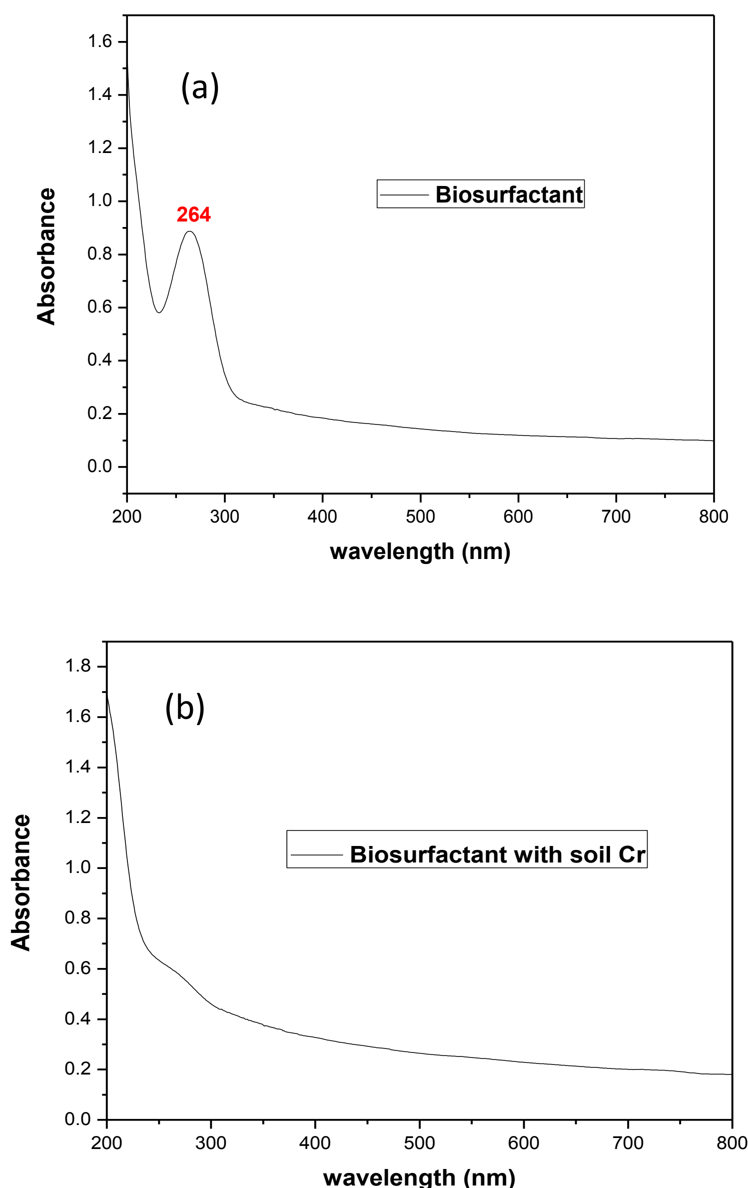
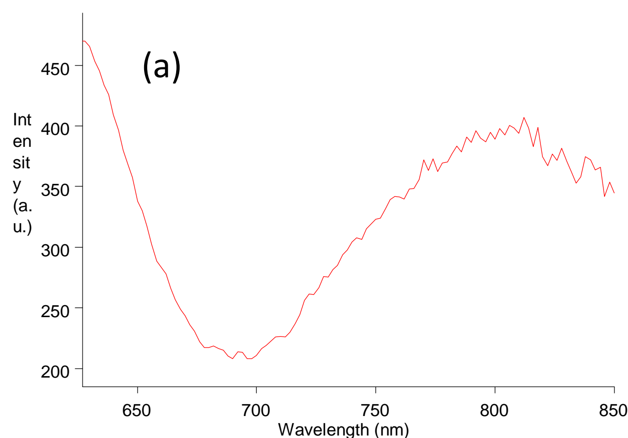


Fig.4.5: a) UV-Visible spectrum of the cationic biosurfactant and b) UV-Visible spectrum of the cationic biosurfactant exposed to Cr contaminated soil

4.5.4. Fluorescence spectral analysis

From the fluorescence spectroscopy, the biosurfactant produced did not possess any fluorescent nature. Whereas, this was allowed to react with contaminated soil and this showed that there was new fluorescent peak appeared. This confirmed the presence of Cr with the biosurfactant.



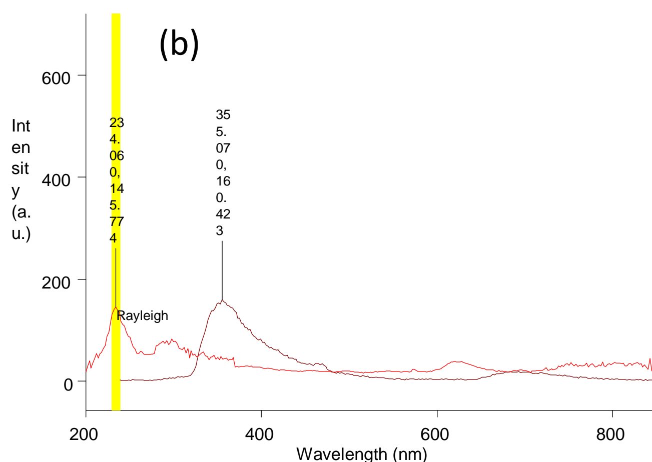
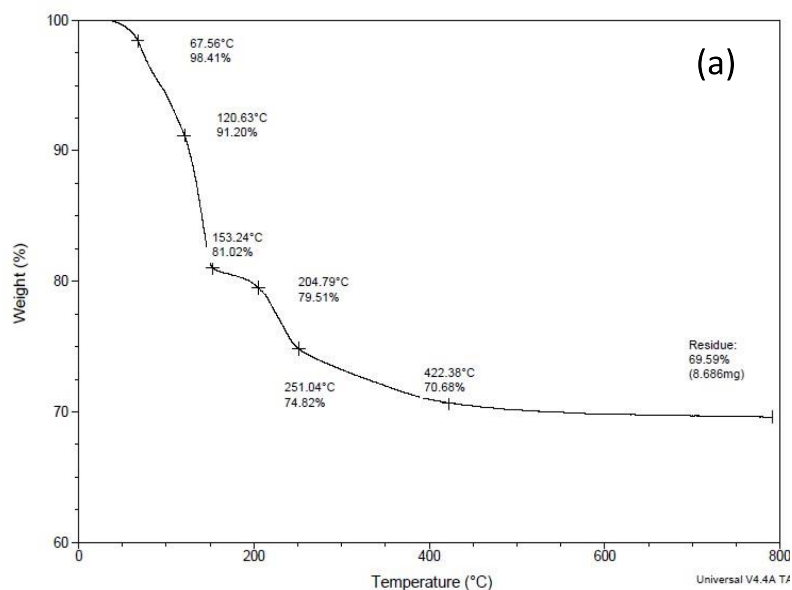


Fig. 4.6: Fluorescence spectroscopy a) Biosurfactant and b) Biosurfactant exposed to Cr contaminated soil

4.5.5 Thermal gravimetric Analysis (TGA) and Differential Scanning Colorimetric analysis (DSC)

The thermogravimetric analysis and Differential Scanning Colorimetry was carried out for the pure biosurfactant. TGA of cationic lipopeptide biosurfactant (Fig.4.7a) showed 1.59% weight loss at the temperature 67.56 °C due to the removal of moisture. Thereafter, 7.21% and 10.18% of weight loss were observed at 120.63 °C and 153.24 °C respectively. At the end of the scan, i.e., at 800 °C, 69.59% was left as residue. The stable components of biosurfactant degraded beyond 251.04°C. The DSC thermogram of cationic lipopeptide biosurfactant showed crystalline temperature (T_c) of 89.87 (onset temperature 75.5) and melting point for T_{m1} and T_{m2} were observed at 125 C (onset temperature 95 C), & 194.50 C (onset temperature 175 C).



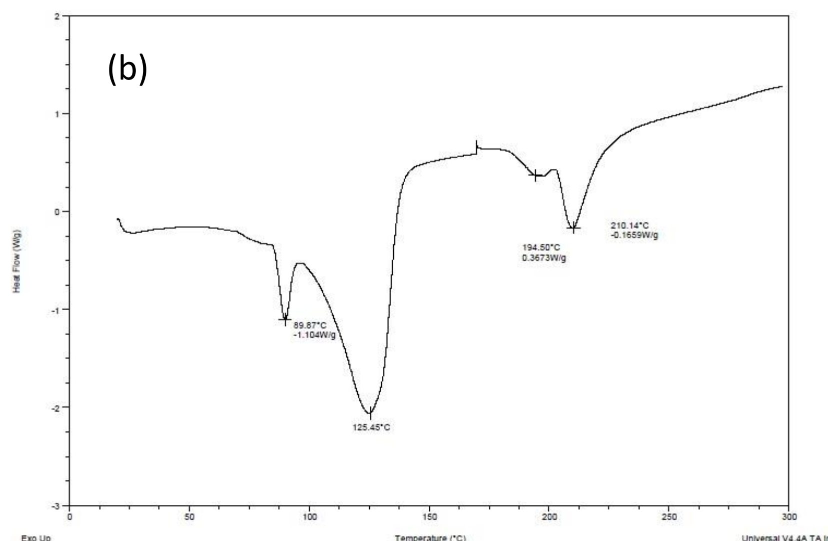


Fig4.7: a) TGA thermogram of cationic biosurfactant obtained from Alcaligenes aquatilis sp. and b) DSC of the pure cationic biosurfactant

4.5.6. Fourier Transform Infrared Spectroscopic studies

The FTIR spectrum of the pure biosurfactant is shown in the above figure. The peak at 3440 cm^{-1} corresponds to the N-H stretching vibrations of the peptide. The peak at 2925 cm^{-1} and 2855 cm−1 corresponds to the asymmetrical and symmetrical stretching of -CH vibration of aliphatic CH2 group.The peak at 1463 cm−1 corresponds to C-H bending.The peak at 1747 cm−1 corresponds to C=O stretching vibration of ester group in lipids.The peak at 1162 cm-1 corresponds to C-O stretching. The peak at 1645 cm-1 and 1540 cm-1 correspond to amide –Istretching ,which confirms the presence of peptide.

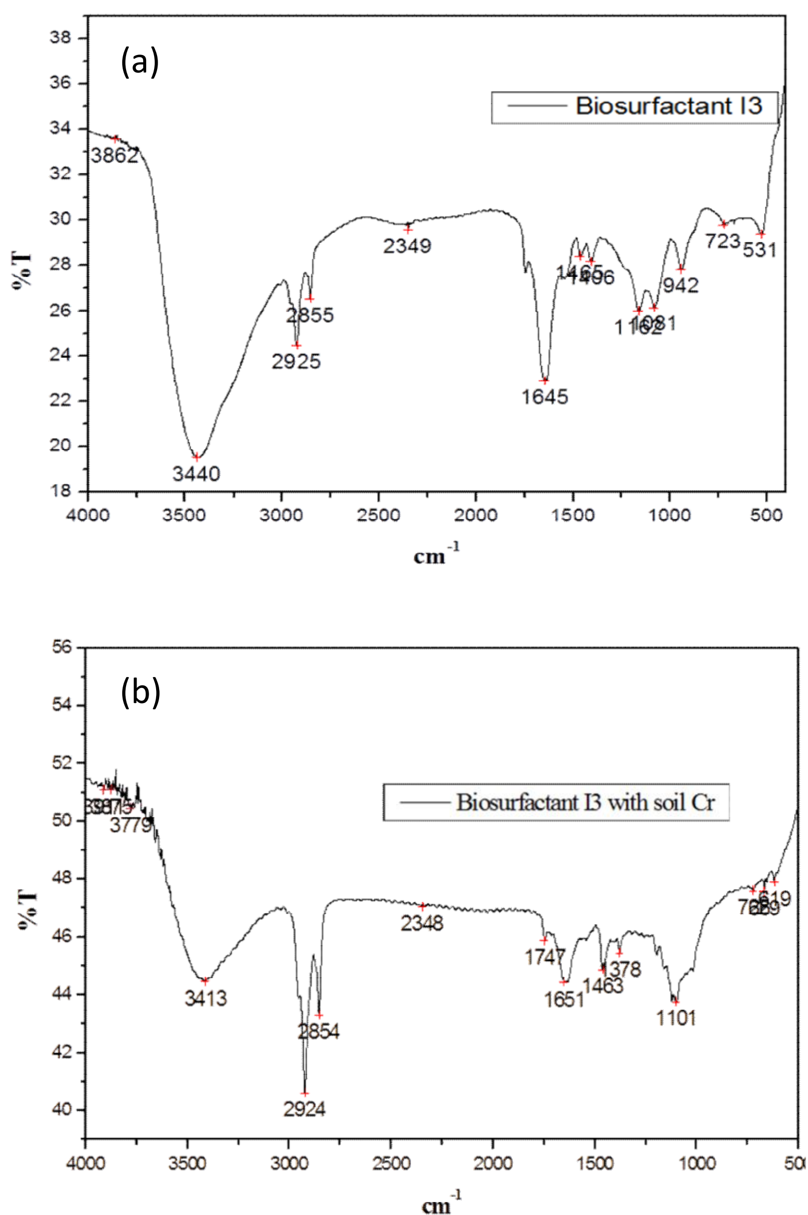


Fig.4.8: a) The FTIR spectrum of lyophilized cationic biosurfactant and b) The FTIR spectrum of the cationic biosurfactant after exposed to Cr contaminated soil

4.6 Removal of Cr (III) from contaminated soil by anionic and cationic biosurfactants: Batch studies

4.6.1 Effect of Time on Cr (III) uptake

The efficiency of biosurfactants both anionic and cationic in chromium removal from contaminated soil was studied. By varying the time of incubation and keeping the biosurfactant concentration (250 mg/50mL) as constant. It showed that the amount of chromium removal was increased with increase in time. It was observed that about 26% of

chromium removal was achieved within two hours by anionic biosurfactant; in case of cationic it is about 34% removal. It was increased from 26% to 60% within 5 hr of incubation time by anionic biosurfactant and from 34% to 59% by cationic. After 4 and 5 hr of incubation time, the Cr in the supernatant was reabsorbed by the soil itself. From this it is concluded that this will be the optimum time for both the biosurfactants and that the biosurfactants strongly binds with the metal.

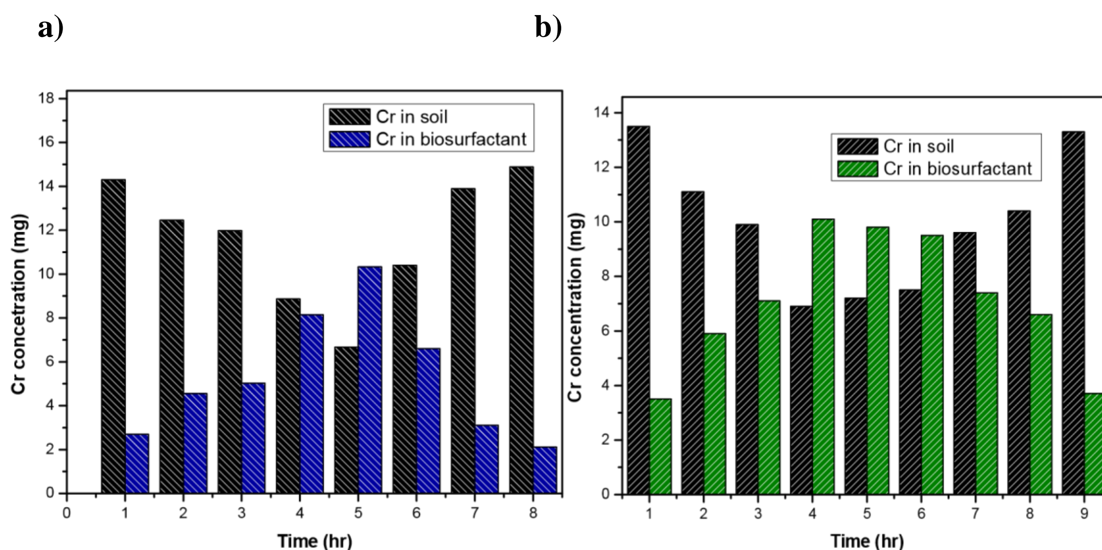


Fig.4.9: Effect of time on chromium removal by a) anionic and b) cationic biosurfactants

4.6.2 Effect of pH on Cr (III) uptake

The role of pH in the removal of chromium by biosurfactants was studied. In this study different buffer systems were used. The amount of biosurfactant used in this was 250 mg/50 mL. At pH 3, the amount of Cr removal from contaminated soil was 50.88% and 33% by anionic and cationic respectively. While increase in pH to neutral it is increased to 55%. In case of cationic, a reduction in the Cr absorption by anionic biosurfactant about 29%. Further increase in pH to alkaline, the amount Cr removal was reduced by the anionic biosurfactant to 22%, whereas in cationic, the uptake of Cr by the biosurfactant was further reduced to 48%. This showed that pH has an effect on both the biosurfactants but cationic is seem to be better than anionic. This showed the stability of cationic over anionic. It is concluded that buffer systems played a main role in the enhancement of chromium removal by the biosurfactant.

a) b)

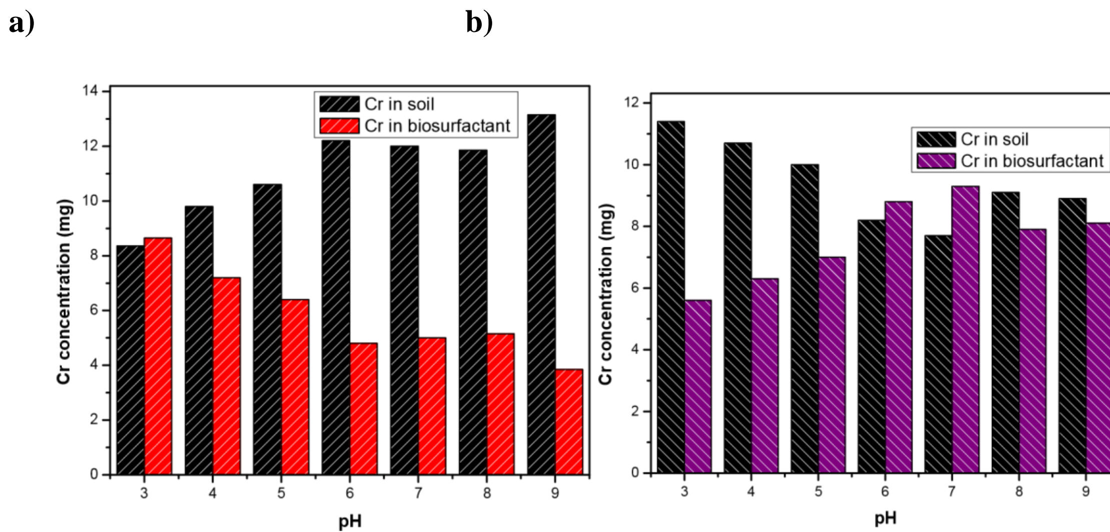


Fig.4.10: Effect of pH on biosurfactants on Cr uptake from contaminated soil by a) anionic and b) cationic biosurfactants

4.6.3 Effect of temperature on Cr (III) uptake

The effect of temperature on biosurfactant in Cr removal was studied. It showed that anionic biosurfactant showed maximum Cr removal at 20-40^{0}C about 62%, after increase in temperature showed a decline in Cr removal. The Cr removal by cationic biosurfactant showed that the activity was increased while increase the temperature up to 40^{0}C and the removal efficiency was 67%. From this it is concluded that cationic biosurfactant produced by Proteus vulgaris showed a better thermal stability.

a) b)

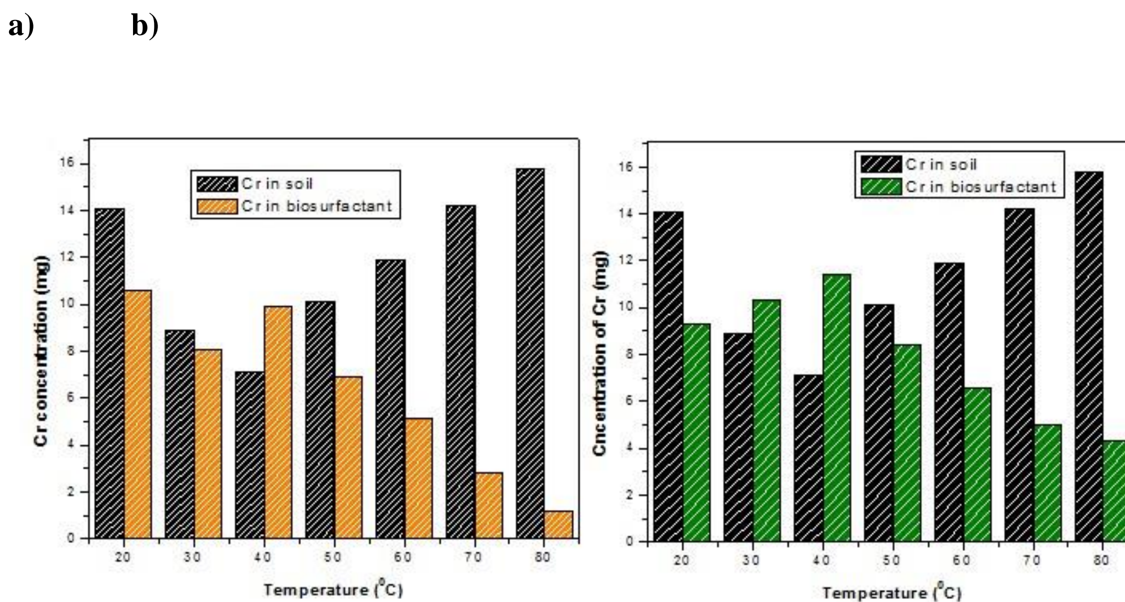


Fig 4.11: Effect of temperature on Cr removal by a) anionic and b) cationic biosurfactants

4.7 Adsorption Isotherms on biosurfactant to the soil

The following are the parameters for ploting Langmuir,FreundlichAdsdorption.

Table 4.4: Parameters required for plotting the Langmuir and Freundlich isotherms for the adsorption studies for biosurfactant

S. No.	Co (mg/l)	Ce (mg/l)	Qe	Ce/Qe	Log Ce	Log Qe
1.	4550	240	431	0.55	2.3	2.63
2.	9100	2211	688.9	3.209	3.34	2.83
3.	13650	4100	955	4.29	3.61	2.98
4.	18200	7560	1064	7.1	3.9	3.02
5.	22750	12540	1021	12.28	4.09	3

4.7.1 Langmuir isotherm

Langmuir Adsorption Isotherm: This describes quantitatively the formation of a monolayer adsorbate on the outer surface of the adsorbent, and after that no further adsorption takes place. Thereby, the Langmuir represents the equilibrium distribution of metal ions between the solid and liquid phases. The model assumes uniform energies of adsorption onto the surface and no transmigration of adsorbate in the plane of the surface. Langmuir adsorption parameters were determined by transforming the Langmuir equation into linear form.Based upon these assumptions, Langmuir represented the following equation:

$$q_e = \frac{K_L C_e}{1 + q_m C_e}$$

where, /Ce = the equilibrium concentration of adsorbate (mg/L), q_e = the amount of metal adsorbed per gram of the adsorbent at equilibrium (mg/g), q_m = maximum monolayer coverage capacity (mg/g), K_L = Langmuir isotherm constant (Lmg).

The values of q_m and $K_L$were computed from the slope and intercept of the Langmuir plot.

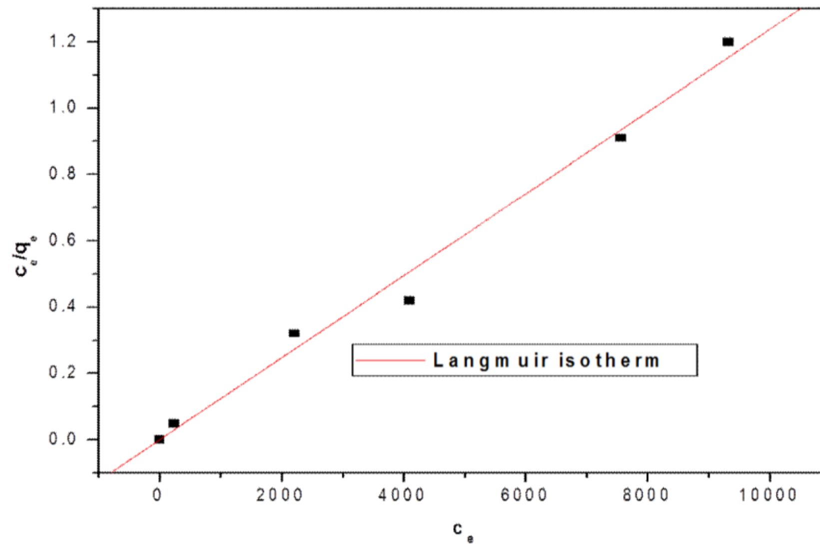


Fig 4.12: Langmuir isotherm of the biosurfactant exposed Cr contaminated soil

Table. 4.5: Variable Values obtained from langmuir isotherm indicates langmuir isotherm is favourable with a high R^2 value

Slope	Intercept	R_L	Q m	K_L	R^2	Qo (mg/g)
1.236	0.051	0.002	24.22	19.6	0.99	18.2

Y = 1.236x+ 0.051R^2 =0.994

K_L is langmuir isotherm constant (L/mg), R_L is separation or equilibrium factor, Qo is maximum monolayer coverage capacity, R_L value less than 1 indicates that langmuir isotherm is favourable.

Hence from the langmuir isotherm it was determined that 18.2 mg of biosurfactant was adsorbed onto 1 gm of soil.

4.7.2 Freundlich Isotherm

This model is commonly used to describe the adsorption characteristics for the heterogeneous surface. These data often fit the empiric al equation proposed by Freundlich.

$$Qe = K_f C_e^{1/n}$$

where, K_f = Freundlich isotherm constant (mg/g), n = adsorption intensity, Ce = the equilibrium concentration of adsorbate (mg/L), Qe = the amount of metal adsorbed per gram of the adsorbent at equilibrium (mg/g).

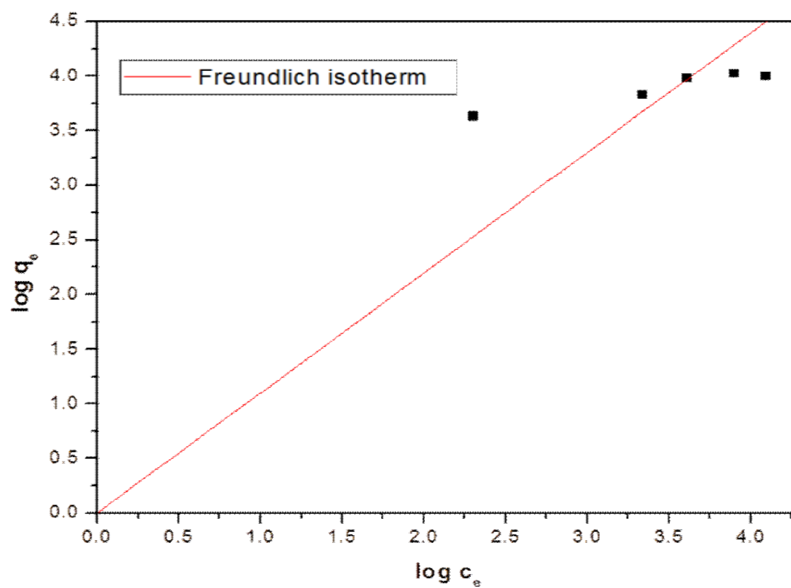


Fig 4.13: Freundlich isotherm for the biosurfactant exposed to Cr contaminated soil

Table 4.6: Values obtained from Freundlich isotherm

Slope	Intercept	n	K_f	R2
0.819	0.423	1.221	0.7	0.971

Y= 0.819x + 0.423 R^2 = 0.971

From the data in table, that value of 1/n = 0.819 while n=1.2 indicating that the sorption of biosurfactant is favourable and the R^2value is 0.97. In this investigation, the equilibrium sorption was carried out at 37 C and pH between 7.0. Other physico-chemical parameters were determined and two adsorption isotherm models were studied. From the Fig. the

sorption data fitted into Langmuir, Freundlich isotherms out of which Langmuir Adsorption model was found to be have the highest regression value and hence the best fit.

4.8 In situ Remediation of chromium contaminated soil

Soil samples from 1-20 sections of the plot were taken out and estimated for chromium concentration using standard protocol. The soil samples were collected after seven days. The following were the results obtained.

Table 4.7: The concentration of Chromium at 20 different points in the soil sample

Sample spots	Concentration of chromium (mg/g)
Point 1	6.025
Point 2	9.42
Point 3	8.55
Point 4	15
Point 5	11.675
Point 6	9.125
Point 7	5
Point 8	4
Point 9	9.12
Point 10	12.5
Point 11	12.75
Point 12	16
Point 13	17
Point 14	17.23

Point 15	12.52
Point 16	11.6
Point 17	10.56
Point 18	12.2
Point 19	13.1
Point 20	11.93

From the Table it is observed that the amount of Cr varied from place to place which shows the mobility of Cr (III) towards the ICB. The anionic biosurfactant played a main role of reducing the surface and interfacial tension of soil. This made the Cr to be released from the soil matrix and free of bound. The values corresponding to each spot is given in the table. And the ash content of ICB after exposure to soil gave the amount of sorbed Cr into the ICB. From this it is concluded that it showed that the ICB having a good affinity towards the metals especially with Cr (III). This was further conformed through seed germination index.

Hence there was a considerable decrease in chromium content in the soil. Also, the average Cr (III) concentration in ash content was found to be 40.71 mg/g. This is supported by FTIR, SEM-EDX and XRD results.

4.9 Soil Characteristics

Table.4.8: Soil characteristics of the contaminated soil before and after treatment

S No.	Characteristics	Untreated soil	Treated soil
1.	pH	7.82	7.21
2.	Bulk density	1.55 g/cm3	1.54 g/cm3
3.	Porosity	0.571	0.59

4.	Moisture content	1.1 % dry mass basis 1.709 % volumebasis	1.41% dry mass basis 2.171% volume basis
5.	Lime content	0.4 mg/g	0.2 mg/g
6.	Organic carbon	0.13 %	0.23 %
7.	TKN content	1.1 mg/g	2.0958 mg/g

From the soil characterization it was seen that the treated soil was more porous as compared to the untreated soil. The moisture content was slightly more in the treated soil. Organic carbon and the TKN content was more for the treated soil than the untreated soil. This could be due to the introduction of the biosurfactant into the soil after which the nitrogen and carbon content may have increased. This confirms the binding of biosurfactant with the soil.

4.10 Characteristics of ICB before and after exposed to Cr contaminated soil

4.10.1 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX) analysis of ICB before and after exposed to Cr contaminated soil

The surface morphology of Immobilized cationic biosurfactant before and after exposure to soil Cr during the soil remediation was observed with the help of SEM-EDAX. Fig.4.14 clearly reveals the surface texture and pores in the ICB before treatment. It shows that the porosity of the Functionalized nanoporous activated carbon with cationic biosurfactant with flower like arrangement. The cationic biosurfactant arranged as white layers on the FNAC. It shows a strong binding and uniform manner. According to Tarley and Arruda (2004), the morphology of the adsorbent can facilitate adsorption of metals in different parts of the material. Therefore, based on morphology we can conclude that this material presents a morphological profile with the capability to retain metal ions.

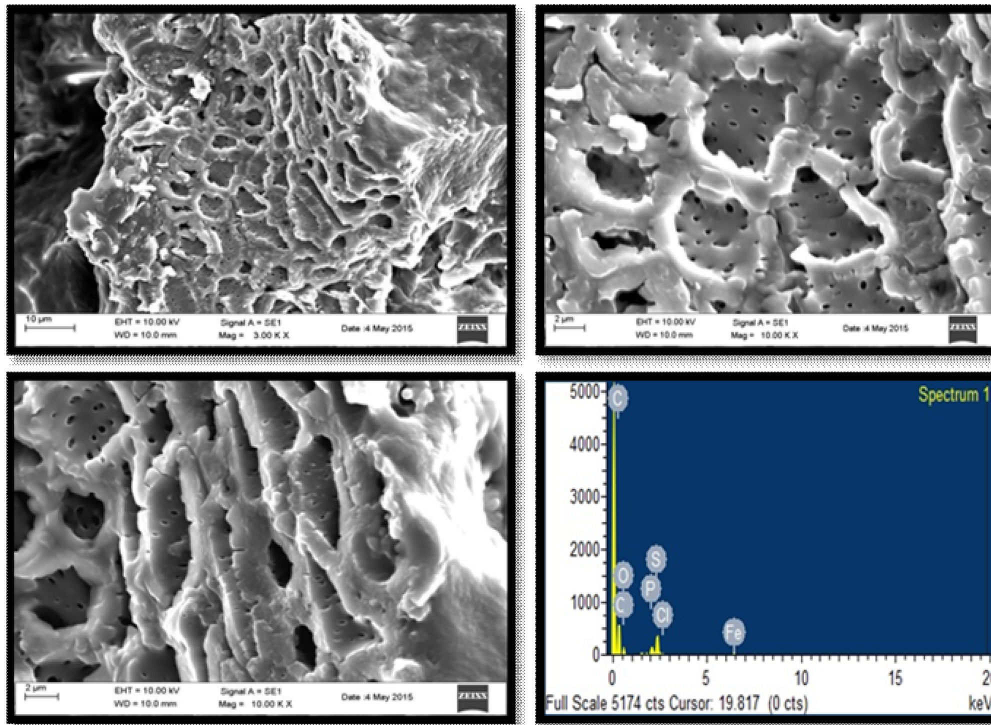


Fig.4.14: SEM-EDX images of Immobilized biosurfactant reveals the honey comb like structure of the FNAC and the biosurfactant attached to it

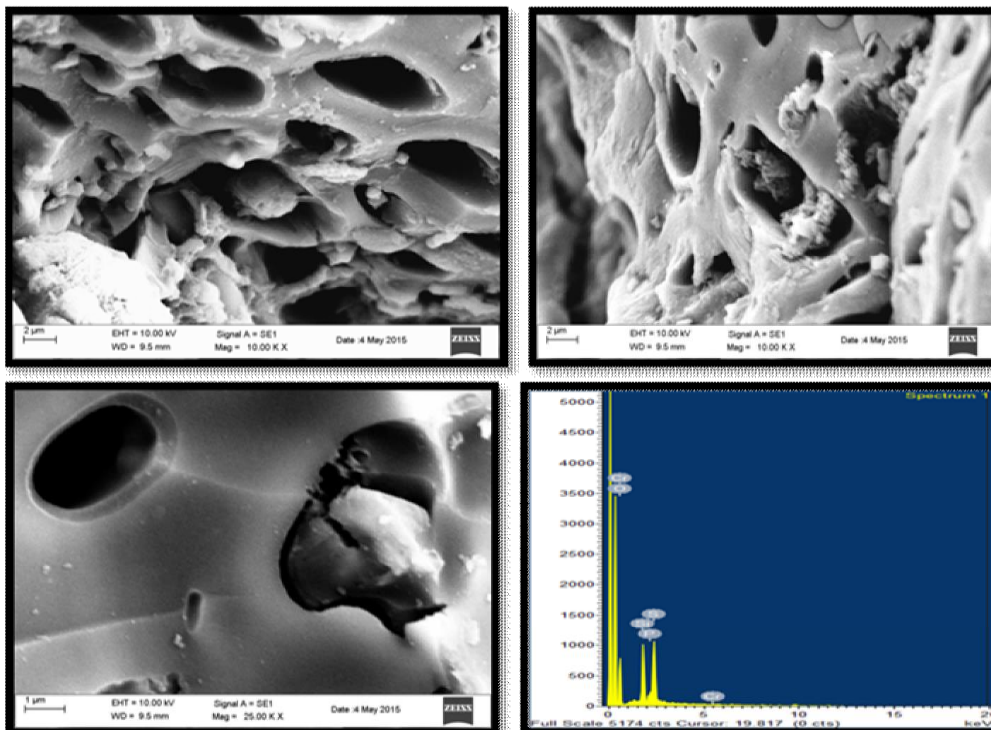
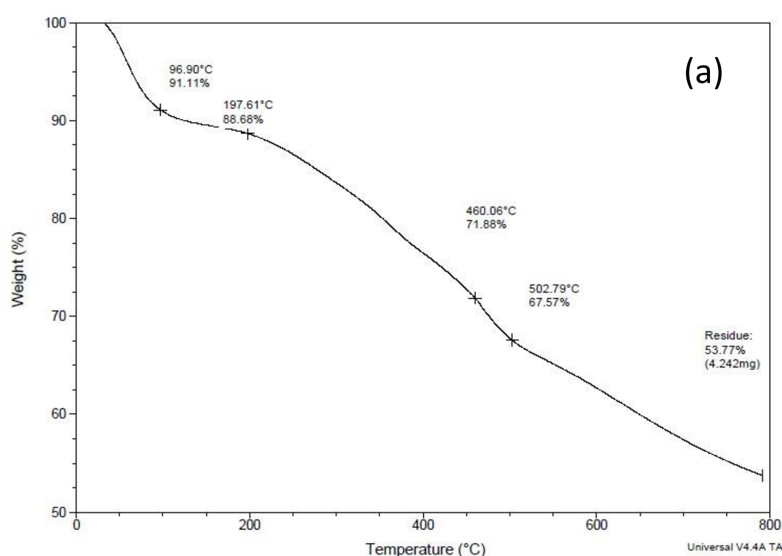


Fig. 4.15: SEM-EDX images of Immobilized biosurfactant after exposure to soil Cr(III) reveals the presence of Cr attached to the ICB. EDX image shows direct evidence of Chromium being attached to the ICB

Fig.4.15 shows the morphology changes with respect to shape and size of the materials after absorption of chromium (III) ions. It can be clearly observed that the surface of materials shape has been changed into a new shiny bulky particle and whitish patches structure after the adsorption of chromium (III) ions.

EDX measurements of the samples were performed to determine the elemental composition of the ICB before and after exposure to contaminated soil. Fig. 4.15 shows the EDX spectra for ICB where as Fig 4.15b shows the EDX spectra of ICB loaded with Cr III. The sample ICB showed distinct peaks for C, S, Fe, Cl. In fig. 4.15 EDX analysis provided direct evidence for the Cr(III) sorption onto the ICB. Cr, S, P & O are the major constituents in the ICB. The phosphorus and sulphur metals peaks originate from the contaminated soil. A reduced peak for O (Fig. 4.15b), recorded in the refined ICB after exposure to soil, may indicate some chemical transformations or variations in the biosurfactant. In addition, the carboxylic groups (Aziz et al., 2009) may confer ion exchange properties to the treated ICB in the metal elimination process. It seems that a metal could easily penetrate into the inside of the ICB through pores or channels and further be adsorbed at interior sites. A more porous biomass structure would definitely favour the diffusion of metal species to the centre or into the bulk of the used biomass. Changes in the structure and morphology of the biomass surface, as discussed here, may be caused by binding of metals onto active centres of ICB.

4.10.2. Thermal gravimetric Analysis (TGA) and Differential scanning calorimetric (DSC) analysis



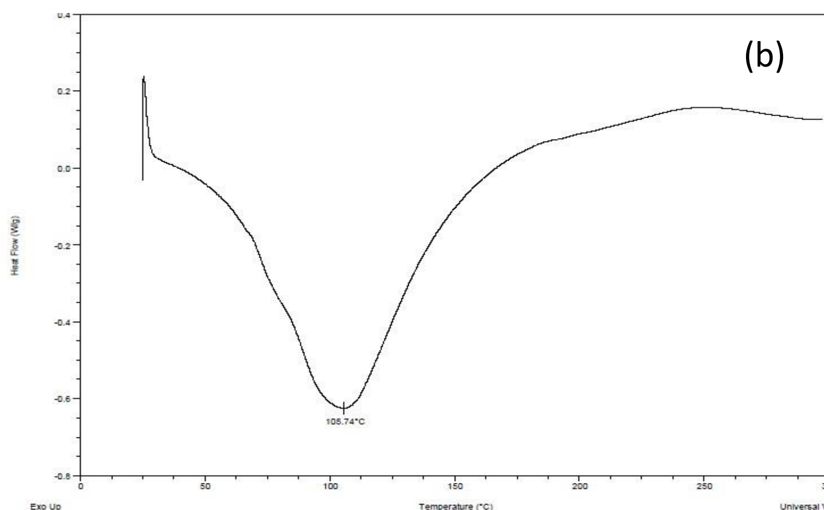


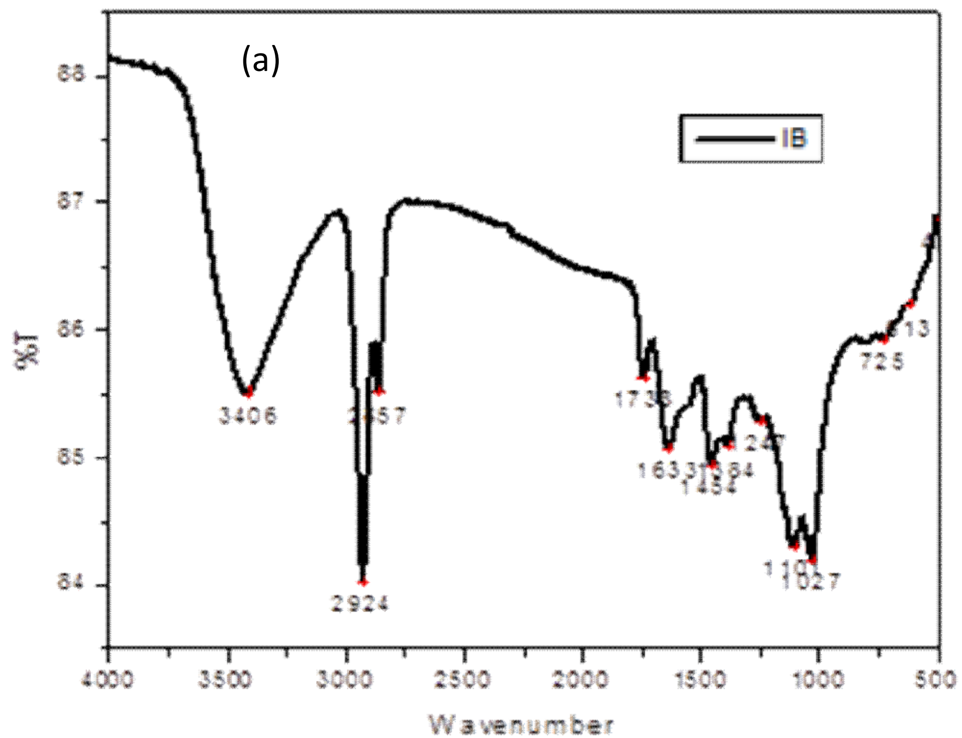
Fig. 4.16: a) TGA for Immobilized biosurfactant and b) DSC for Immobilized biosurfactant

The TGA of ICB (Fig.4.16 a) shows 8.89 % weight loss at 70.31 °C due to the removal of moisture. Thereafter, 2.43 % weight loss occurred at 197.61 °C. A major weight loss occurred from 197.61 to 502.79 °C due to the decomposition of the major components of the immobilized cationic lipopeptide biosurfactant. A drastic decrease in weight by 21.11 % was observed. The final residue remaining was 4.242 mg from the initial weight of 7.89 mg. The DSC of ICB showed the major weight. The DSC thermogram (Fig.4.16 b) of showed an endothermic reaction took place at 105.74 C.

4.10.3 Fourier Transform Infrared spectroscopic studies

The peak at 2924 cm^{-1} indicates the presence of asymmetical and symmetrical stretching of CH vibrations of aliphatic CH2 grp. The peak at 1736 cm^{-1} shows the presence of C=O ester grp. The peak at 1633 cm^{-1} shows the presence of amide I stretching which confirms the presence of peptide bonds. The peak at 1101 cm^{-1}shows the presence of C=O stretching.

The peak at 3440cm^{-1}indicates the presence of N-H stretching vibrations of the peptide. The peak at 2926cm-1 and 2855cm-1 indicates the presence of symmetrical and asymmetrical stretching of CH vibrations of aliphatic CH2 group. The peak at 1393cm-1 indicates CH bending. The peak at 1645cm-1 indicates the presence of amide stretching which confirms the presence of peptide. The peak at 603cm-1 may be due to the presence of chromium.



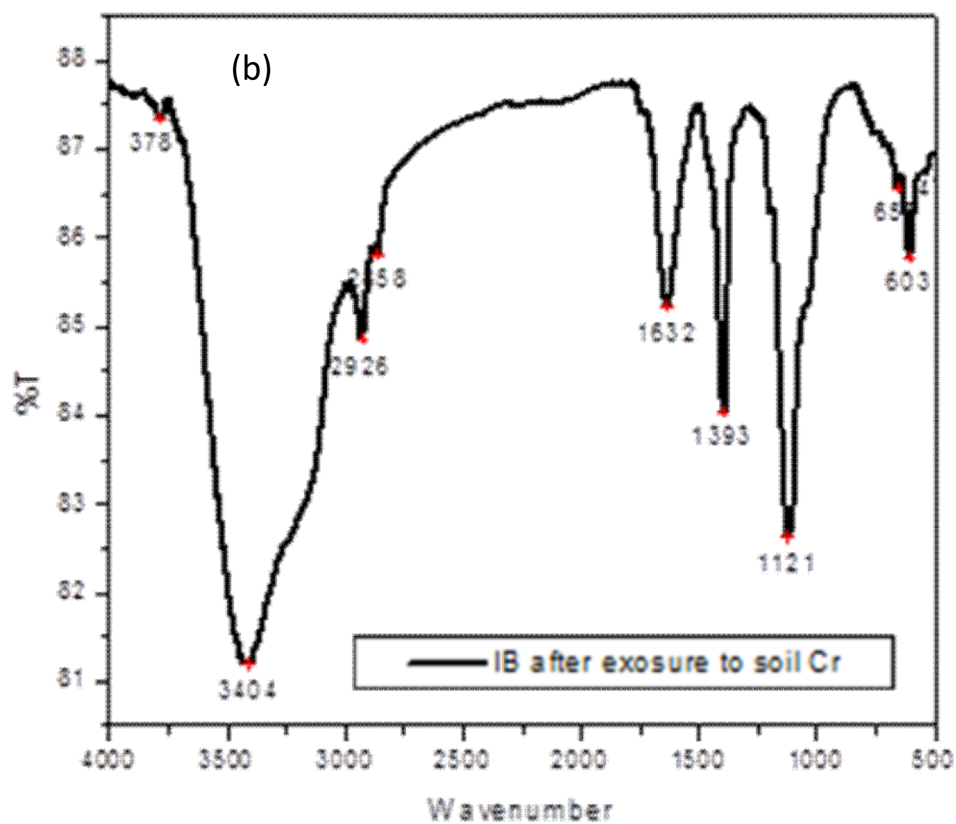


Fig. 4.17: a) FTIR of ICB and b) FTIR of ICB exposed to chromium

4.10.4XRD analysis for the immobilized cationic biosurfactant before and after exposure to soil Cr(III)

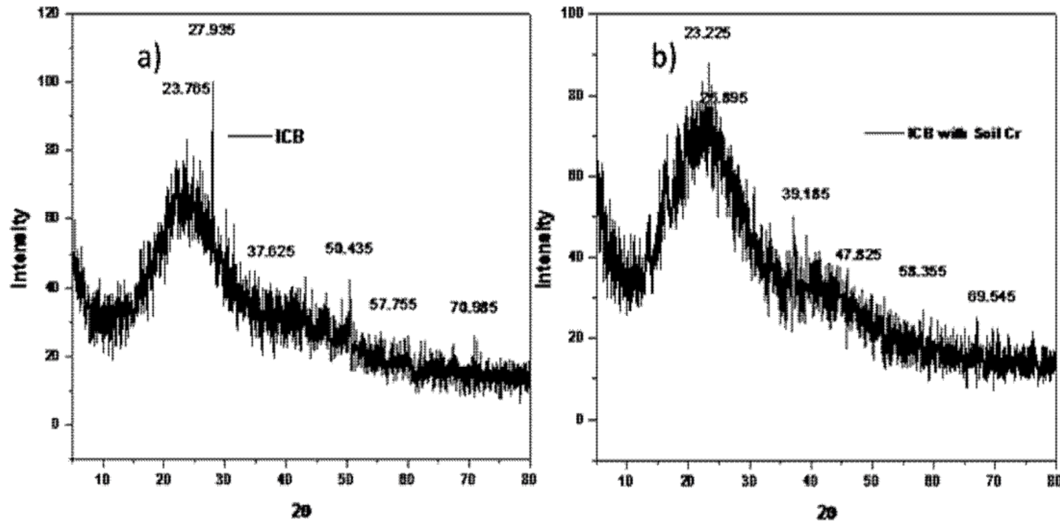


Fig. 4.18: XRD patterns of a) Immobilized biosurfactant and b) Immobilized biosurfactant after exposure to soil Cr(III)

XRD patterns of Immobilized cationic biosurfactant onto the functionalized nanoporous activated carbon (ICB) and Immobilized cationic biosurfactant after exposure to contaminated soil containing Cr(III) were shown in the fig.4.18. Sharp intensity XRD peaks have been observed at typical scanning angles of $2\theta = 23.7^{\circ}$, 27.9°, 50.4°, 57.7° and 70.9° for ICB before Cr(III) adsorption from soil. The sharp peaks 23.2°, 39.1° & 69.5° present in the fig.4.18 (b) indicated the crystalline nature of the material. In addition, several other low intensity peaks corresponding to other crystalline phases of carbons have also been observed.

After adsorption of Cr(III) ions, the porous structures of the carbon adsorbents decreased. These caused low intensity XRD peaks. Hence crystalline phases should have been reduced. So it is concluded that, some of the chromium ions from contaminated soils were incorporated within the porous matrices of Immobilized cationic biosurfactant.

4.11 Seed Germination studies

4.11.1 Zea mays (Maize)

A significant increase in root length (12.69 to 21.02) was seen in the plants grown from seeds in the treated soil when compared to the untreated soil. Phytotoxicity of root and shoot: The phytotoxicity of the root from the seed decreased from 40.67% in the untreated soil to 1.729% for the seed in the treated soil.Tolerance index of the seed increased from 59.33% in the untreated soil to 98.27% in the treated soil.

a.) Untreated soil **b.)Treated soil**

Fig. 4.19: Seed germination index of Zea mays (Maize) in Cr(III) contaminated soil and treated soil

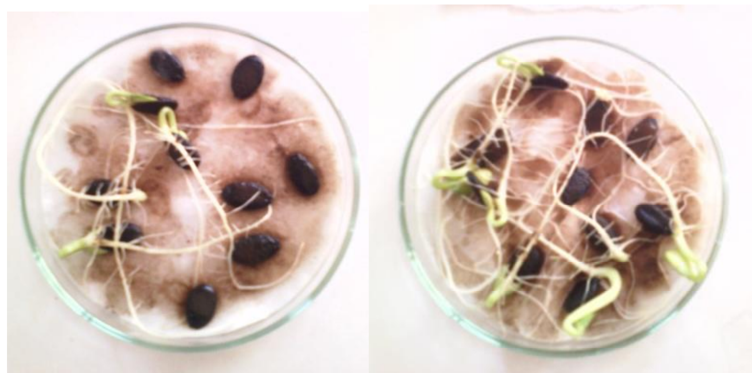
Table 4.9: Comparison of seed germination index of maize between the untreated and treated soil

UNTREATED SOIL	TREATED SOIL
• % Germination = 80% • Mean root length = 12.69 • Mean shoot length =5.29 • Mean seedling length =27.95 • Mean root length in control =21.39 • Mean shoot length in control =7.27	• % Germination =100% • Mean root length = 21.02 • Mean shoot length =6.9 • Mean seedling length = 27.95 • Mean root length in control =21.39 • Mean shoot length in control =7.27

• VI = (12.69+ 5.29)/ 80%) = 22.475 • SVI = 27.95× 80% = 22.36% • TI = (12.69/21.39) × 100 =59.33% • Phytotoxicity of root ((21.39-12.69)/21.39) ×100 = 40.67% • Phytotoxicity of shoot ((7.27-5.29)/7.27) × 100 = 27.24%	• VI = (21.02+6.9)/100% = 27.92 • SVI = 27.95 × 100% = 27.95 • TI = (21.02/21.39) × 100 = 98.27% • Phytotoxicity of root ((21.39-21.02)/21.39) × 100 = 1.729% • Phytotoxicity of shoot ((7.27- 6.9)/7.27) × 100 = 5.089%

4.11.2 Luffa(Ridge gourd)

There was an increase seen in the root length of the plant from the seed growing in the untreated soil to the plant from the seed growing in the treated soil from 15 to 18.275.Similarly the shoot length also increased from 6.05cm to 8.04cm. Phytotoxicity of the root and shoot: The phytotoxicity of the shoot reduced to a large extent from 41.54% to 19.81%. The phytotoxicity of the root reduced from 38.02% to 24.48%.Tolerance index increased from 61.98% in the untreated soil to 75.54% in the treated soil.



a.) Untreated soil **b.) Treated soil**

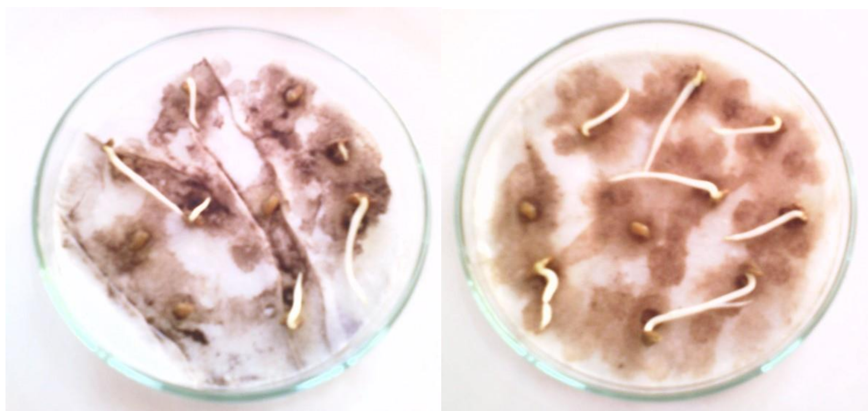
Fig. 4.20: Seed germination index of Luffa (Ridge gourd) in Cr(III) contaminated soil and treated soil

Table 4.10: Comparison of seed germination index of Ridge gourd between the untreated and treated soil

UNTREATED SOIL	TREATED SOIL
• % Germination =40% • Mean root length =15 cm • Mean shoot length =6.05 cm • Mean seedling length=21.05 cm • Mean root length in control soil =24.2 cm • VI =(15+6.05)/40% =52.625 • SVI= (40/100) ×21.05 =8.42 • TI = (15/24.2) ×100 =61.98% • Phototoxicity of shoot ((10.4-6.08)/10.4) ×100 = 41.54% • Phototoxicity of root ((24.2-15)/24.2) ×100 =38.02%	• % Germination = 80% • Mean root length =18.275 cm • Mean shoot length =8.34 cm • Mean seedling length =19.61 cm • Mean root length in control soil =24.2cm • VI=(18.275+8.34)/80% =33.269 • SVI= 80% ×19.61 =15.688 • TI=(18.28/24.2)×100 =75.54% • Phytotoxicity of shoot ((10.4-8.34)/10.4) × 100 = 19.81% • Phytotoxicity of root ((24.2-18.275)/24.2) × 100 =24.48%

4.11.3Trigonella foenum-graecum (Fenugreek)

There was an increase seen in the root length of the plant from the seed growing in the untreated soil to the plant from the seed growing in the treated soil from 0.52cm to 0.99cm.Similarly the shoot length also increased from 0.98cm to 2.25cm. The phytotoxicity of the shoot reduced to a large extent from 40.24% to 22.9%. The phytotoxicity of the root reduced from 0% to 1%. Tolerance index increased from 61.98% in the untreated soil to 75.54% in the treated soil.



a.) Untreated soil **b.) Treated soil**

Fig. 4.21: Seed germination index of Trigonella foenum-graecum (Fenugreek) in Cr(III) contaminated soil and treated soil

Table 4.11: Comparison of seed germination index of fenugreek between the untreated and treated soil

UNTREATED SOIL	TREATED SOIL
• % Germination =60% • Mean seedling length =1.5 cm • Mean root length= 0.52 cm • Mean shoot length= 0.98 cm • Mean root length in control =0.99 • VI= ((0.52+0.98)/ 60%) =2.5 • SVI = (60% ×1.5) = 0.9 • TI= (0.52/0.99) ×100=52.53 % • Phytotoxicity of shoot ((1.64-0.98)/1.64) ×100 =40.24% • Phytotoxicity of root	• Germination % =80% • Mean shoot length =1.263 • Mean root length =0.99 • Mean seedling length=2.25 • Mean root length in control = 0.99 • VI=((0.99+1.26)/80%) =2.82 • SVI = 80% × 2.25 = 1.8 • TI=(0.99/0.99)×100= 100% • Phytotoxicity of shoot ((1.64-1.263)/1.64) ×100 =22.99% • Phytotoxicity of root ((0.99-0.99)/0.99) ×100 =0%

((0.99-0.98)/0.99) ×100 =1%	

Hence, from the seed germination studies carried out it was concluded that the seed which is the most chromium sensitive was ridge gourd. 4 out of 10 seeds germinated in the untreated chromium contaminated soil where as 8/10 germinated in the treated soil.This could possibly be due to the interference of chromium with the plant metabolism and growth which inhibited the germination of the seed.

To determine whether the Cr(III) in the soil has translocated and effected the germination of the seed, the seed of the plant Luffa (Ridge Gourd) which did not germinate in the untreated soil and the seed which germinated in the treated soil was made to ash at 600˚C for 4hrs and was estimated for Chromium concentration.It was seen that the Cr(III) of the seed which did not germinate in the untreated soil was 14.7 mg/g of soil. The Cr(III) conc of the seed which germinated in the treated soil was 1.2 mg/g. This proved that the Cr(III) in the soil has translocated and effected the germination of the seed of Luffa (Ridge Gourd).

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