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Glycolipid Biosurfactant from *Indigenous Pseudomonas sps*. isolated from Kandigai, Kanchipuram District For the Removal of Cr(III)

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Abstract

Heavy metal contaminants in fresh water is a major cause for most health issues and remediation of these contaminants are quite a tedious process but with the help of biosurfactants produced by bacteria isolated from soil, these contaminants can be removed in an effective manner. Chromium(III) is one of the heavy metal contaminants in fresh water. Experimental samples were prepared in laboratory containing chromium(III) at various concentrations (100ppm, 200 ppm,300,400 ppm,500 ppm) and it is treated with biosurfactant produced by bacteria isolated from soil from Kandigai, Kanchipuram district of Tamil Nadu. Biosurfactant producing bacteria were characterized by phenotypic methods and was identified based on Bergy'smanual and it was identified as *Pseudomonas sps*. Biosurfactant was characterized by oil displacement method,phenol:chloroform qualitative test, protein estimation, carbohydrate estimation, SDS-PAGE and FTIR analysis. Based on these studies, the biosurfactant was characterized as glycolipids. The same biosurfactant was used to remove Cr(III) from aqueous system and 89.3% of 100ppm/lt Cr(III) was removed in 10 minutes at 120rpm by 10mg of biosurfactant. The current research proves that this glycolipid biosurfactant produced by the indigenous bacteria could be effectively used to remove chromium(III) from aquatic environments.

Keywords*: Biosurfactant, bioremediation, FTIR Cr (III) etc.,*

1. Introduction:

Biosurfactants are amphiphilic compounds which are produced on living surfaces, mainly on surfaces of microorganisms or may also secreted extracellularly and it contains both hydrophilic and hydrophobic moieties which reduces the surface and interfacial tension of the surface and interface respectively. Since biosurfactant and bio emulsifiers both exhibit emulsification properties, bio emulsifiers are frequently considered with biosurfactant, even though emulsifiers may not lower surface tension. A biosurfactant can have one of the following structures: glycolipids, mycolic acid, polysaccharide–lipid composite, lipoprotein/ lipopeptide, phospholipid, or the microbial cell surface itself. Significant attention has been given in the past to the synthesis of surface-active molecules from biological source because of their potential use in food-processing (Karanth, 1989), oil industry, and pharmacology. Even though the type and quantity of the microbial surfactants produced depends mainly on the producer organism, factors like nitrogen and carbon, temperature, aeration and trace elements also affect their production by the organism. Hydrophobic pollutants present inside petroleum hydrocarbons, and soil and water environment necessitate solubilisation before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Surfactants can raise the

surface area of hydrophobic resources, such as pesticides in water and soil surroundings, thus increasing their water solubility. Hence, the existence of surfactants might increase microbial degradation of pollutants. The utilization of biosurfactants for the degradation of pesticides in soil and water environment has gained significance recently. The identification and characterization of biosurfactant produced by a variety of microorganisms have been broadly reviewed (Lin, 1996; Desai, 1987; Parkinson, 1985). Therefore, rather than recounting the several types of biosurfactants and their properties, this study specifies the production, characterization, surface tension reduction ability, antimicrobial activity of biosurfactant and its role in the hydrocarbon removal from environment and its efficacy in metal removal.

The tannery industries were released the chromium contaminated effluent in water bodies. Chromium VI is recognized as a human carcinogen when it is inhaled and may also damage the small capillaries in kidneys and intestines. Chromium VI in water has become an issue of growing concern nationwide. In 2010 the environment working group tested and found Chromium VI in 89%.some of the samples are safe maximum limit level only present, but continuous taken that also causes problem. (Larry West,2010). Chromium VI is known it cause various health effects. When it is a compound in leather product, it can cause allergic reaction, such as sin rash. After breathing it in chromium VI can cause nose irritations and nosebleeds. Other health problems that are caused by chromium (VI) are: skin rashes, upset stomachs and ulcers, respiratory problems, weakened immune system, kidney and liver damage, alteration of genetic material, lung cancer.

Release of heavy metals without proper treatment poses a significant threat to public health because of its persistence, bio magnification, and accumulation in food chain. Microbial metal bioremediation is the reversing of the damage caused by industrial effluents and is highly efficient due to its low cost, high efficiency, and eco-friendly nature. Several technologies exist for the remediation of metal-contaminated soils like subsurface barriers, immobilization, pyro-metallurgical, solidification/stabilization, vitrification, extraction, toxicity and/or mobility reduction, electro kinetic treatment, chemical and physical treatments etc. (Evanko et al. 1997). But all these treatments are very expensive and found to affect the texture of the soil. Bioremediation is an efficient tool practiced now a day for the removal of contaminants from the contaminated soil and water. The use of microorganisms such as *Pseudomonas spp*, that secretes biosurfactant that bind to oil containing wastes can be used in removing or detoxifying the pollutants, mainly the contaminants of soil, water, or sediment which may otherwise threaten public health. Bioremediation has been used as a strategy of using microorganisms for complete transformation of organic pesticides to harmless end products such as CO2 and H2O. Similarly, microorganisms can transform inorganic pollutants, not necessarily completely, but to compounds with decreased solubility, mobility, and toxicity. We could able to isolate potent biosurfactant producing bacteria from oil contaminated soil which able to effectively remove chromium(III) from experimental samples prepared in laboratory. Rapid developments and increase in mining and industrial activities have gradually redistributed many of the toxic metals from the earth's crust to the environment. This has substantially raised the chances of human exposure to these heavy metals, which increase in excess of their natural con-centration, through ingestion, inhalation or skin contact. All metals that are mined are normally dissipated into the environment, thereby endangering the components of the eco-system. As metals cannot be degraded further to non-toxic products (Khan et al. 2009), their deleterious effects tend to be permanent unless measures are taken to recover the metals economically from the contaminated site.

Remediation of metal-contaminated environments is particularly challenging given that, unlike organic molecules, metals cannot be biodegraded or mineralized. As such, remediation approaches must focus either on changing the redox state of a metal contaminant to a less toxic form, or on physically removing the metal from the environment. Biological processes can play a central role in the remediation of metal-contaminated water, soil, and sludge as microbes are well known to interact with, and change the properties of, a wide range of toxic and nontoxic metals. For example, metals may be used as electron donors or electron acceptors for energy production within a cell, may be used to shuttle electrons between organisms in syntrophic relationships, or possibly used as cofactors for intracellular and extracellular enzymatic reactions (Croal et al., 2004; Haferburg and Kothe, 2007). Microorganisms have, therefore, evolved mechanisms to oxidize, reduce, transport, bind, and

sequester metals to either avoid toxic effects or to assist with basic cellular processes. These physiological responses to metals can be harnessed for bioremediation (Gadd, 2010; Singh et al., 2007; Van Hamme et al., 2006), and the focus of this chapter is on the use of biosurfactants for mobilizing and removing metals from contaminated environments. Specifically, the mechanisms underlying biosurfactant–metal interactions will be described together with applied examples of the use of biosurfactants for treatment of metal-contaminated industrial effluents and contaminated sites.

2. Review Of Literture:

According to Bhatia and ghosh 1999, rapid increase in transport activities of modern society has triggered deterioration of environmental quality due to excessive use of fossil fuel. About 70-75% of lead, present in petroleum is emitted as incombustible exhaust fumes. Therefore, soil and grasses on road side habitats are enriched with heavy metals.

According to Baldi et al.1998, *Thiobacillus ferrooxidans*, an acidophilic bacterium, shows a high, natural level of resistance to heavy metal. This bacterium can tolerate Cr^{3+} up to 75mM during growth on ferrous sulphate. This was achieved at pH1.4.

Chang et al 1996 reported that *Pseudomonas aeruginosa* PU21 has the ability to remove lead, copper and cadmium. These bacterial cells were able to survive metal concentration as high as 500mg/l for Pb2+, range of metal in concentrations, adsorption time pH and CO ions.

Ismail saadoun *et al* 2002 reported that five bacterial strains were recovered from different soils from different locations contaminated with fuel spills. The ability of these different bacteria to degrade diesel fuel was studied. Phenotypic identification of the bacterial colonies showed that the strains belonged mainly to members of the genus *Pseudomonas* and was represented by the following species: *P. maltophilia, P. putida* and *P. mallei*. Other bacterial genera were also identified namely: *Enterobacter cloacae* and *Acinetobacter lowffi.*

Chromium VI is known it cause various health effects. When it is a compound in leather product, it can cause allergic reaction, such as sin rash. After breathing it in chromium VI can cause nose irritations and nosebleeds. Other health problems that are caused by chromium (VI) are: skin rashes, upset stomachs and ulcers, respiratory problems, weakened immune system, kidney and liver damage, alteration of genetic material, lung cancer (Larry West *et al*., 2010).

Removal of heavy metal from bearing from waste water is usually achieved by physicochemical process before discharging the effluents into natural water-body systems. conventional treatment technologies like precipitation and coagulation become less effective and more expensive when metal concentrations are in the range of 1-100 mg/ml high costs, process complexity and low removal efficiency of membrane processes have limited their use in heavy metal, present development in the field of environment biotechnology include the search for microorganisms as sorbents for heavy metals. Bacteria, fungi, yeast and algae can remove heavy metals form aqueous Solution in substantial quantities. The uptake of heavy metals by biomass can take place by an active mode (bioaccumulation) or by a passive mode (sorption and / or complexation) (shumate and strandberg, 1985). Predomination of the members of the genus Pseudomonas in addition to Bacillus, *Streptomyces* and *Rhodococcus* in the various oil-polluted Kuwaiti desert soil samples subjected to various types of management (Radwan et al. 1995).

Cr (VI) levels of more than 75mg/kg is present in soils at many such sites all over the world. Cr 6 is a known carcinogen and mutagen in human and animals. Cr 6 in soil can be dissolved by sweat on exposed skin and such person could become sensitized to allergic contact dermatitis. It also reduces plant growth (Salunhe et al.1998).

Chromium VI is known it cause various health effects. When it is a compound in leather product, it can cause allergic reaction, such as sin rash. After breathing it in chromium VI can cause nose irritations and nosebleeds. Other health problems that are caused by chromium (VI) are: skin rashes, upset stomachs and ulcers, respiratory problems, weakened immune system, kidney and liver damage, alteration of genetic material, lung cancer (Larry West, 2010).

Heavy metals can be removed by chemical precipitation, chemical oxidation or reduction, electrochemical treatment and evaporation recovery. The use of microorganisms for the removal of metal ions from industrial wastes provides an alternative

means to existing technologies (Ginisty et el, 1998).Biomass of brown algae of the *sargassum* family algal biomass binds approximately 2.3 meq/g of Cr (VI) from water by ion exchange (Krtochvil and volesky 1997).

According to Tobin and Roux 1997 waste industrial mucor meihi biomass was found to be an effective biosorbent for removal of chromium from industrial tanning effluents. It can absorb 1.15 m mole Cr (VI) /g at pH 4.0.

Rhizopus arrhizus biomass absorbs a variety of metal ions at pH 4. The alkali ions are not absorbed, and more strongly than smaller ones. The behaviour can be explained by a complexation mechanism involving sites in the biomass containing carboxylate, phosphate and other functional groups (Tobin et al 1984).

Niu et al 1993, removal of Pb^{+2} by Pencillium, that *Pencillium chrysogenum* has the ability to remove Pb^{+2}, and it removes 116 mg/g of Pb at the pH 4to 5.

According to shu et al 1998, the accumulation of $Pb^{+2}$by *Saccharomyces cerevisiae*, the rate of Pb^{+2} removal by *S.cerevisiae* depends on the biomass weight. In the Pb^{+2} accumulation experiments, the time to reach an equilibrium state was significantly shortened from 96h to 24h as the cell dry weight increased from 0.56g/l to 5.18g/l.

Chromium (VI) resistant yeast from tannery waste, *Candida spp* isolated from tannery waste showed resistance towards 500µg/ml of chromium (Baldi,et,al,1989).

The rate of chromium reduction by *Bacillus spp* increased with increasing cell concentration. Cr (VI) reduction was observed over a wide range of oxidation-reduction potential values (Wang and Xiao, 1995).

Potential of microbial produced to heavy metals was investigated. This study appears to be a complexation of a heavy metal. The rhamnolipid surfactant used in this study complexes Cd^{2+} rapidly and the complex remained stable for at least 27 hours (Tan et al 1994).

Biosorption of the Cr (VI) ion was strongly affected by pH. within a pH range of 4 to 5. At pH 4.5, *Pencillium chrysogenum* biomass exhibited selectively for Cr (VI) over other metal ions such as Cd+2, Ca+2, Zn+2. (Niu et al 1993).

3. Materials and Methods:

3.1 Isolation of Biosurfactant producing bacteria:

3.1.1.Sample collection:

Petroleum contaminated soil samples were collected to isolate biosurfactant producing bacteria.

Water samples were collected from pond in Kandigai to analyse the presence of Chromium.

3.1.2.Isolation of bacteria:

1g of each soil samples were taken and serially diluted up to 10-5 dilution and incubated in mineral salt Agar(MSA) . All the plates are incubated for 48-72 hours at 37^0C.. After incubation morphologically distinct colonies were selected for further studies. The bacterial isolates hich could tolerate Cr(III) and could able to produce biosurfactant was identified by phenotypic methods.

3.2 Production & characterization of Biosurfactant**:**

The isolated strains are used to produce Biosurfactant. Mineral Salt Medium was prepared and sterilized by autoclaving. 2% of glycerol was added as a carbon source. The culture was inoculated and kept in rotary shaker for4-5 days at 37^oC at 100 rpm.

3.2.1 Phenol: Sulphuric acid:

Biosurfactant producing strains selected from above screening methods were inoculated in MSM broth& Incubate at37^oC on rotary shaker for 4-5 days. After incubation, broth was centrifuged at 10000 rpm for 15 mints & supernatant was collected while pellet was discarded. 1ml collected supernatant was mixed with of 5% phenol then 5 ml of con.H2SO4 was added in drop wise manner. Presence of Biosurfactant in supernatant produces orange colour from yellow colour.

3.2.2 Oil Spreading Assay:

The oil spreading assay was developed by morikawa. For this assay 10ul of oil is added to the surface of 30 ml of distilled water in Petri dish to form a thin oil layer. Then 10ul of culture or culture supernatant are gently placed on the centre oil layer if Biosurfactant

is present in the supernatant the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity also called oil displacement.

3.2.3 Blood Agar Hemolysis:

Blood agar hemolysis method is used to screen Biosurfactant producing strain. This method is based on the fact that Biosurfactant are able to haemolyse the red blood cell present in blood. cultures of selected isolates were spot inoculated on blood agar plates.these plates were incubated forn24-48 hours at 37^{o}C.after incubation plates were observe for zone f hemolysis. This zone of hemolysis indicates production of Biosurfactant

3.2.4 Carbohydrate estimation:

Carbohydrate estimation of biosurfactant was done by the following methods. Carbohydrate estimation was done by phenol-sulfuric acid method.

3.2.5. Protein estimation**:**

Protein estimation of biosurfactant was done by the following method. Protein estimation was done by Bradford method.

3.2.6.SDS:

SDS (Sodium Dodecyl Sulphate) Polyacrylamide gel electrophoresis is one of the methods used for characterizing proteins. It is most common, rapid and sensitive method. After various modifications on the basic electophoretic technique, various kinds of electophoretic kits are now available.

3.2.7.FTIR:

Fourier Transform Infrared analysis (FTIR) FTIR spectroscopy was carried out using crude biosurfactant extract obtained from the acid precipitation of the cell free culture supernatant. Fourier Transform Infrared spectrophotometer was used to determine the chemical nature of the biosurfactant by the KBr pellet method.

3.3..Bioremediation of chromium**:**

3.3.1. Estimation of chromium:

Chromium estimation of biosurfactant was done by the following methods. Chromium estimation was done by (DPC) Diphenylcarbazide method.Five ml of standard chromium (100ppm,200ppm,300ppm,400ppm,500ppm) and unknown chromium was taken in flask and 1ml of 1N sulphuric acid was added in each flask. 0.5ml of potassium permanganate was added and warmed up the solution in boiling water for 40 minutes to oxidize chromium. The content was cooled and a few drops of sodium azide solution was added and warmed again for 3 minutes to reduce excess potassium permanganate. Further it was cooled in ice water and 2ml of Diphenylcarbazide reagent was added and made up the solution to 25ml in a standard flask.It was incubated at room temperature for 20 minutes and the absorbance was measured at 540nm.

Treatment of chromium:

Aqueous solution of Standard chromium(III) from 100 to 500 ppm was prepared and were treated with 10mg of biosurfactant. The treated samples were kept at static condition and the efficiency of bioremediation was studied based on the previous spectrophotometric method..

4.Results & Discussion:

Pollution by chromium is of major concern as the metal is used in electroplating, metal finishing leather tanning and chromate preparation. Chromium found in water bodies and in soil is in the form of Cr (III) and Cr (VI). The aim of present work was to bioremediate chromium(III) using biosurfactant produced from bacteria –Pseudomonas spp to find the efficiency of biosurfactant to treat chromium in experimental sample prepared in laboratory as well as from pond water. *Pseudomonas* have been described to destroy a wide range of unmanageable xenobiotics (Mulet et al. 2011) as well as PAHs such as pyrene (Ma et al. 2013). *Pseudomonads* are also described to create a wide range of BS plus glycoplipids (mainly rhamnolipids), lipopeptides and viscosins having varied industrial uses, e.g., in the bioremediation, pharmaceutical, food-processing petroleum, cosmetic and industries.

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4.1.Isolation of Biosurfactant producing bacteria:

Sample Collection:

Figure No. 1(a) Soil sample used

Figure No: 2(b) Water sample collected from pond

4.2 Isolation of bacteria**:**

Soil sample was used to isolate bacteria. Nutrient agar plates were used and isolates of bacteria were selected morphologically.

Table No: 1
Phenotypic Characterization of Bacterial Isolates:

S.No	Name of the test	Bacteria
1.	Gram staining	-VE
2.	Capsule	-
3.	Cultural characteristics	White spread colonies
4.	Indole test	+
5.	Methyl red test	-
6.	VogesProskauer test	-
7.	Citrate test	-
8.	Catalase test	+
9.	Oxidase test	+
10.	Urease test	+
11.	Motility test	+
12	**Name of the unknown bacteria**	*Pseudomonas sps*

Fig.No2 (A to F) Isolation & Identification of Bacteria

4.3 Production of Biosurfactant:

Organism was chosen for further study namely, *Pseudomonas sps*.used to produce Biosurfactant. Mineral Salt Medium was prepared and sterilized in autoclave 15 lbs for 20 mints. The culture were inoculated and kept in rotary shaker for4-5 days at 37^{o}C, rotary shaker speed was 120 rpm.After the incubation the culture were centrifuged at 10000 rpm, 4°C for 30 minutes to remove the bacterial cells. The supernatant are collected and add equal volumes of chloroform: methanol was added in the ratio of 2:1. These mixtures were shaken well to ensure proper mixing and using separating funnel separate the solvent phase and aqueous phase. Solvent phase contain the Biosurfactant and were left overnight for evaporation. White colour precipitate if seen at the interface between the two liquids proved the presence of Biosurfactant. Numerous Pseudomonas sp. have previously been explored as glycolipid-producing bacteria [Zhang., et al., 1992]

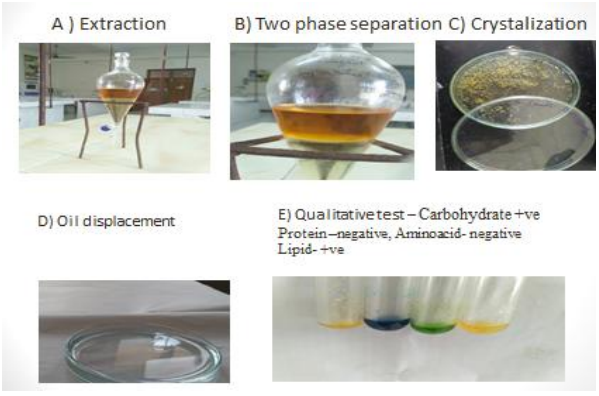

Figure.No:3(A to F) Production & Characterization of Biosurfactant:

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FTIR:

Fourier transform infrared spectroscopy is used to elucidate the chemical structure of unknown samples by identifying type of functional groups. These infrared absorption bands identify specific molecular components and structures. samples are analysed and results were noted.

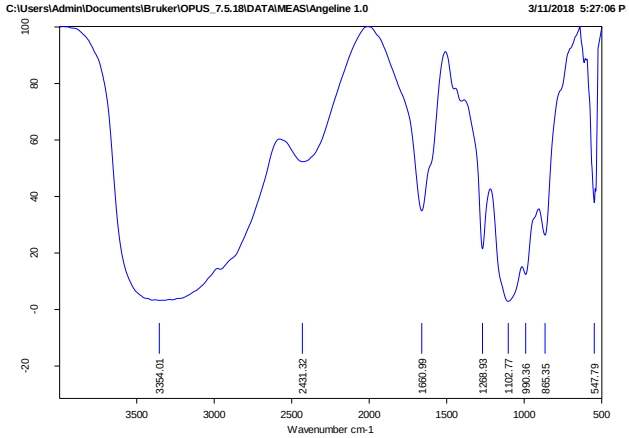

Figure No: 4 FTIR ANALYSIS- Frequency Peaks

Table No.:2, Major Peak Values of FTIR

Peak Frequency range	Intensity	Assignment and remarks	Group or Functional class
33540.1	broad	Carboxylic Acids	O-H stretch
24313.2	Sharp	Alcohols	O-H stretch
16609.9	Sharp	Alkenes	C=C stretch
12689.3	Sharp	aromatic	O=C-O-C
11027.7	Sharp	Alcohols	RR'CH-OH (2o)
990.3	Sharp	Alkenes	RCH=CH2
865.36	Sharp	Alkenes	RR'C=CH2
547.9	Sharp	Alkyl halides	C-Br stretch

Bioremediation of chromium:

Bioremediation of chromium was studied by Diphenycarboxide method. Known Cr(III) was used to construct a standard graph for the treated and untreated experimental samples: Removal of Cr(III) was estimated from the graph.

Figure no: 5, Estimation of Chromium (III) by Diphenylcarbazide method:

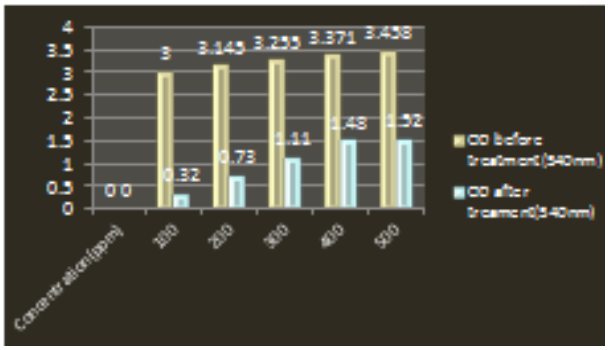

Figure No.6, Graphical representation of OD Values before and after Treatment.

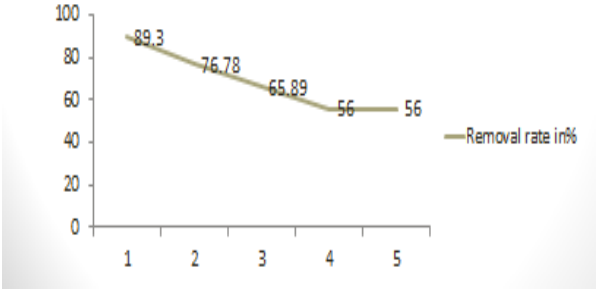

Figure No. 7 , Removal rate in %

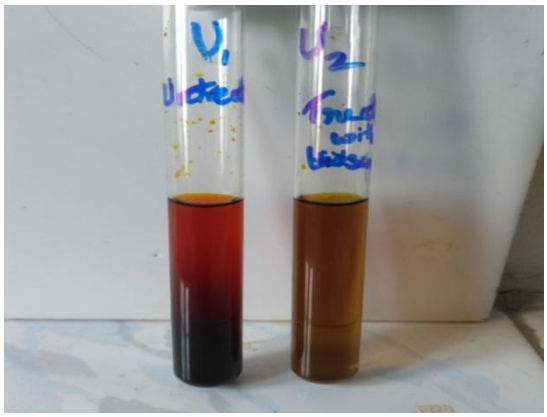

Figure No.8 Cr (III) analysis in Unknown samples

The glycolipid biosurfactant produced by Pseudomonas sps could able to remove 87% of the Cr(III) at the rate of 10mg of biosurfactant within 10 minutes by metal complexation. Release from industry comprises numerous organic and inorganic contaminants. Amongst these contaminants are heavy metals which can be toxic and/or oncogenic and which are damaging to humans and other living species The heavy metals of most alarming industry effluents comprising lead (Pb), zinc (Zn), copper (Cu), arsenic (As), cadmium (Cd), chromium (Cr), nickel (Ni) and mercury (Hg). In established countries, regulation is becoming progressively rigorous for heavy metal restrictions in wastewater. In India, the current maximum contaminant level (ppm–mg/mL) for heavy metals is 0.05, 0.01, 0.25, 0.20, 0.80, 0.006, 0.00003, 0.050 for chromium, cadmium, copper, nickel, zinc, lead, mercury and arsenic, respectively (Gopalakrishnan *et al*. 2015). Various treatment technologies employed for the removal of heavy metals include chemical precipitation, ion exchange, chemical oxidation, reduction, reverse osmosis, ultrafiltration, electrodialysis and adsorption. All these physical and chemical methods have many disadvantages and the current investigation using glycolipid biosurfactant could be an effective alternative eco-friendly strategy to remove all the above said heavy metal pollutants.

5. Conclusion:

Based on the above study it can be concluded that the experimental sample can be effectively treated to remove Cr (III). The Pseudomonas *spp* isolated from soil has produced glycolipids. And the biosurfactant has effectively removed chromium.and the removal was efficient to a greater extent.FTIR was used to confirm the presence of peptides. The previous studies revealed the advantages of using biosurfactant to treat waste water because of its less sludge producing ability and greater efficiency.The present study also supports the same. It is concluded that the biosurfactant produced by Pseudomonas *spp* is a glycolipid with an excellent bioremediating potentials which can also be used for commercial applications. comparing to chemical surfactants biosurfactants are less toxic as well as it is also effective for heavy metal treatment.

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