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Biosurfactant – Isolation, Production, Purification & Significance

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Abstract- The present article is aimed to determine common cultivable surfactant producers from soil and contaminated oil, its purification and significance. Bio-surfactants are surface-active substances synthesized by living cells and as compared to synthetic surfactants, they are more effective, selective, environmental friendly and stable. They have the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable. Interest in microbial surfactants has been steadily increasing due to their diversity, eco-friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection. Bio-surfactants enhance the emulsification of hydrocarbons; have the potential to solubilize it to increase their availability for microbial degradation. These compounds can be used in enhanced oil recovery, environmental protection, herbicides and pesticides formulations, detergents, health care and cosmetics, pulp and paper, coal, textiles, ceramic processing and food industries, uranium ore-processing and mechanical dewatering of peat. Mostly bacteria and yeasts are known to synthesize bio-surfactants. These microorganisms synthesize a wide range of chemicals with surface activity, such as glycolipid (low molecular weight surfactants), phospholipids and others like polyanionic heteropolysaccharides (containing covalently-linked hydrophobic side chains or complexes containing both polysaccharides and proteins). In this study two unique isolates L4 and L15 (screened from soil contaminated with oil) are grown on MSM broth with 2% glucose for the production of bio-surfactant. Bio-surfactant was purified by Ammonium sulphate precipitation or $ZnCl_2$ precipitation method and identified by TLC.

Index Terms- Bio-surfactant , Amphipathic molecules, surface active agents, detergengy, surface tension, bioremediation**.**

I. INTRODUCTION

'Surfactant' are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition favourably at the interface between fluid phases with different grades of polarity and hydrogen bonding such as oil/water or air / water interfaces. These polarities render surfactants capable of reducing surface and interfacial tension and forming micro emulsion where hydrocarbon can be solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, forming and dispersing traits. Almost all surfactants currently in use are chemically derived from petroleum: however, interest in microbial surfactants has been steadily increasing in due to their diversity, environmental friendly and easy production. Biosurfactants are amphiphilic compounds, structurally diverse group of surface active molecules excreted extracellularly, synthesized by large varieties of micro-organisms, which vary in their chemical properties and molecular size. These molecules reduce surface and interfacial tension in both aqueous solutions and hydrocarbon mixture.

The main functions of bio-surfactant in microbial cells are emulsification of water insoluble substrates such as hydrocarbons and facilitate its transport into the cell to stimulate the growth. Similarly adhesion and desorption of cell is essential for the survival of microbes in unfavourable conditions like toxin accumulation, limited nutrients availability.

In this article, have presented the application of bio-surfactant, methods used for its isolation, production and purification.

II. CLASSIFICATION AND MICROBIAL ORIGIN

Bio-surfactants are categorized mainly by their chemical composition and their microbial origin. A major class of bio-surfactants includes:

- **Glycolipids:** most common carbohydrate in combination with long chain aliphatic acid of hydroxyl aliphatic acid. The glycolipid can be categorised as Rhamnolipids(commonly produced by *Pseudomonas aeruginosa*), Trehalolipids (commonly associated with *Actinomycetes, Mycobacterium, Nocardia And Corynebacterium*), Sophorolipids (produced by different strains of *Yeast* and *Torulopis bombicola and T. petrophilum*)
- **Phospholipids, Fatty Acid and Natural Lipids:** several bacteria and yeasts such as *Thiobacillus thiooxidane, Aspergillus spp., Arthobacter , P. aeruginosa etc*. produces large quantities of fatty acid and phospholipids during growth on –n-alkanes.
- **Peptides:** many peptides antibiotics are amphiphilic in nature and exhibit surface active properties. Dipeplide antibiotics (gramicidin), lipopeptide antibiotics (polymyxins) and cyclic lipopeptide producedbyBacillus breve's, B. polymyxa and B. subtilis, respectively were thrown to process remarkable surface active properties.
- **Polymeric biosurfactants:** most recognized polymeric biosurfactant are emulsan, liposan, mannoprotein, polysaccharide-protein complexes, which are mainly

produced by *Acinetobacter calcoacetius, Candida lipolytica, Saccharomyces cerevisiae, Schizonella malanogramma, Ustilago maydis and Pseudomonas Spp*.

- **Particulate bio-surfactant:** surface activity in most hydrocarbon-degrading micro-organisms are attributed to several cell surface constituents, which includes structures such as M protein and lipoteichoic acid in group A *Streptococci*, Protein A in *Staphylococcua aureus*, Layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia spp*., gramicidins in *Bacillus brevis* spores and thin fimbriae in *A. calcoacetius*.

III. AREAS OF APPLICATIONS AND USES

Microbial surfactants are more effective and versatile than many synthetic surfactants owing to their selective action, biodegradable nature and stability at high temperature. There are numerous applications of bio-surfactant as paralleled to their chemically synthesised counterparts: are as follows:

- In food industry: It is used as food additives. Lecithin and its derivatives, fatty acid ester containing glycerol, sorbitol and ethyoxylate etc. are now days used as emulsifier in food industries.
- Health care and cosmetic industries: an appropriate combination of sophorolipids and propylene glycol has remarkable skin compatibility and used commercially as moisturizer. A numerous compounds are prepared by enzymatic alteration of hydrophobic molecules by lipase and whole cell for cosmetic application.
- Medical industries: bio-surfactant is very useful for the treatment of many infectious diseases such as the succinoyltrehalose lipid of *Rhodococcus erythropolis* has been reported to inhibit *Herpes simplex virus and Influenza virus*. 1% emulsion of rhamnolipids is used for the treatment of leaves of Nicotiana infected with *TMV* and to control *Potato virus-x* disease.
- As environment pollution control: it can be efficiently used in handling industrial emulsion, control of oil spill, biodegradation and detoxification of industrial effluents and bioremediation of contaminated soil.
- Economic production: Depending on the application, biosurfactants can also be produced from industrial wastes and by-products (example for use of petroleum related technology).
- Other potential area where it is useful is pulp & paper, coal, textile and uranium ore processing industries.

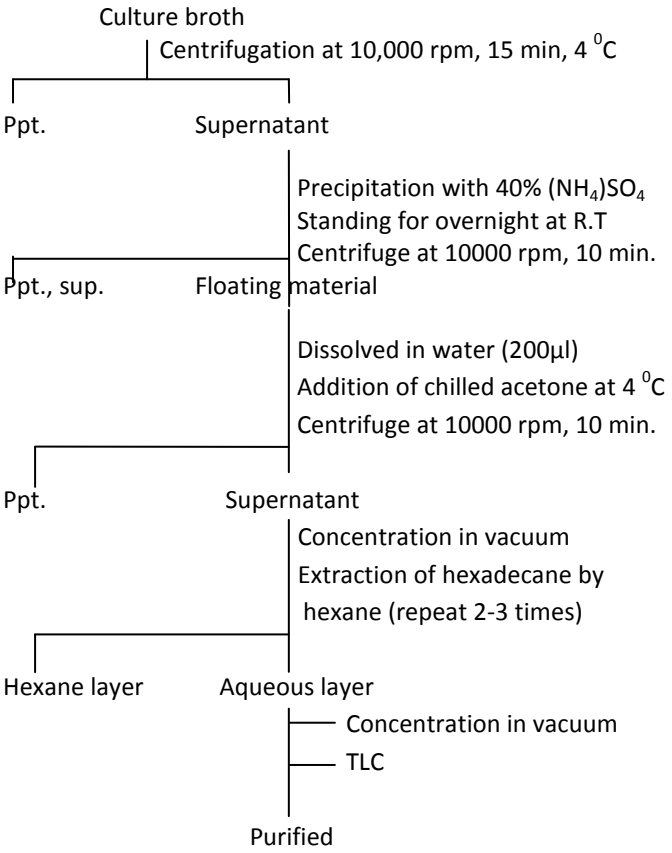
IV. MATERIAL AND METHOD

Isolation and screening: micro-organisms are the most versatile with regards to their ability to live on all kinds of substrates. Soil samples for this study were collected from numerous petrol pumps and waste oil dump areas and inoculated in Mineral Salt Medium(solution A: 2.5g of$NaNO_3$, 0.4 g of $MgSO_4.7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05g of $CaCl_2.2H_2O$, 10 ml of conc. Phosphoric acid(85%) pH of solution was adjusted at 7.2 with KOH pellets. Solution B (per litre): 0.5 g of $FeSO_4.7H_2O$, 1.5 g of $ZnSO_4$. $7H_2O$, 1.5 g of $MnSO_4.2H_2O$, 0.3g of K_3BO_3, 0.15g of $CuSO_4.5H_2O$, 0.1 g of $NaMnO_4.2H_2O$. One ml of Solution B was added to 1000 ml of Solution A). 0.5 g sample of each soil was placed in 250 ml of flask containing tap water (50 ml) and 5 ml oil (petrol, diesel and eatable oil). Mix properly and incubate at room temperature on shaker at 160 rpm for 20 days. On every 4th Day loop full sample were inoculated on nutrient agar, incubated at 37^0C for 24 hrs. Micro-organisms are isolated on the basis of colony characteristics. Isolates were inoculated into 10 ml of MSM medium containing 2% glucose as energy and carbon source and incubated at shaker at 160 rpm for 7-9 days at room temperature.

Screening of isolates: approaches that measure directly the surface activity of bio-surfactants are interfacial tension measurement, axisymmetric drop shape analysis profile (ADSA-P), glass slide test, drop collapse method, blood cell lysis and oil spreading techniques. *Blood Agar lysis*- it has been used to quantify surfactin and rhamnolipids, as preliminary method to screen bio-surfactant activity. The increase in the diameter of lysis on blood agar as the conc. of surfactant increased. *Oil spreading technique*- this technique measures the diameter of clear zone produced when a drop of a bio-surfactant containing solution is sited on oil water surface (20 µl of crude oil on 50 ml distilled water into 25 cm petri plate). The diameter of clear zone on oil surface was measured. *Drop collapse method*- it depends on principle that a drop of liquid containing a bio-surfactant will collapse completely over oil surface. 2µl of mineral oil was added to micro-titre plate and incubated at room temperature and then 5µl of culture was added to the surface of oil, bio-surfactant producing cultures gave flat drops with scoring system ranging from partial to complete spreading on the oil surface after inspecting it for one minute.

Production of bio-surfactant: inoculums of working isolates are poured in the MSM medium with pH 7.2 and incubated at 25 ^{0}C on shaker at 160 rpm. Biomass was determined in triplicate by centrifuging of 10 ml samples (culture liquid) at 5500 rpm for 25 minutes at room temperature. The cell pallets was washed with distilled water, dried at 105 ^{0}C for at least 24 hours and then weighed. The supernatant was used for estimation of glucose, surface or interfacial tension and bio-surfactant concentration. Biomass was determined directly by taking the optical density of culture liquid at 545ηm at calorimeter. Glucose was assayed by *DNSA* method.

Purification of bio-surfactant: isolates producing bio-surfactant were purified by two procedures- Ammonium sulphate precipitation method and $ZnCl_2$ precipitation method. *By Ammonium sulphate precipitation method*: it consists of four steps, ammonium sulphate fractionation, chilled acetone and hexane treatment, silica gel column chromatography.



By $ZnCl_2$ precipitation method: 10 ml of the culture supernatants were concentrated by $ZnCl_2$ to final concentration of 75 mM. The precipitated material was dissolved in 10 ml Sodium phosphate buffer (pH 6.5), extracted twice with equal volumes of diethyl ether. Pooled organic phase were evaporated to dryness and pallets were dissolved in 100 µl of methanol. Further purification was achieved by preparative TLC.

Analysis and Identification of bio-surfactant: the homogeneity was checked and the identification was made by percolated TLC plate spotted with samples (developing system chloroform/methanol/acetic acid at 85:13:02). The components on the plates were visualized by heating after spraying with 50% sulphuric acid. The quantification of bio-surfactant was analysed by the *Orcinol Asay,* extracellular glycolipid concentration was evaluated in triplicate by measuring the concentration of rhamnose. 10 ml of culture liquid is centrifuged at 4 ^{0}C at 10,000 rpm for 15 min, supernatant was extracted twice with 1 ml diethyl ether. Ether fractions were evaporated to dryness and 0.5 ml of water was added. To 100µl of each sample, 900 µl of solution containing 0.19% Orcinol (in 53% H_2SO_4) was added, after heating for 30 min at 80 ^{0}C the samples were cooled at room temperature. The absorbance is measured at 421 ŋm at UV Spectrophotometer. Standard graph were prepared and expressed as mg ml^{-1}.

V. RESULTS AND DISCUSSION

The soil samples were optimized and screened for bio-surfactant producers, 20 isolates (L1 to L20) are initially screened on nutrient agar on the basis of colony characteristics and morphology, which were further grown in MSM broth containing 2% glucose, for week and then tested qualitatively for bio-surfactant production preliminary on blood agar lysing test and secondarily with oil spreading technique. On blood agar plate out of 20 isolates 06(L3, L4, L10,L12,L15, and L16) isolates gave positive result by showing lysis and out of that 06, L4, L12 and L15 given positive result on oil spreading technique, after 120 hours, 48 hours, 48 hours respectively, that means they were producing bio-surfactant. For bio-surfactant production out of 20 isolates 2 were selected on the basis of their efficiency to produce bio-surfactant. The oil spreading technique has shown that the area of displacement by a surfactant containing solution is directly proportional to the concentration of bio-surfactant.

The isolated strains L15 and L4 identified as *Pseudomonas and Bacillus spp*. on the basis of Gram character, cell morphology, biochemical reactions and DNA hybridization studies. Analysis of peptidoclycan, composition of cell wall and its components also has taxonomic importance.

When pure inoculum of L15 (*Pseudomonas spp.)* and L4 (*Bacillus spp*.) were cultured in MSM broth supplemented with 2% glucose , shown bio-surfactant production within 2-4 days and 7-11 days of incubation respectively(fig: 5.1).

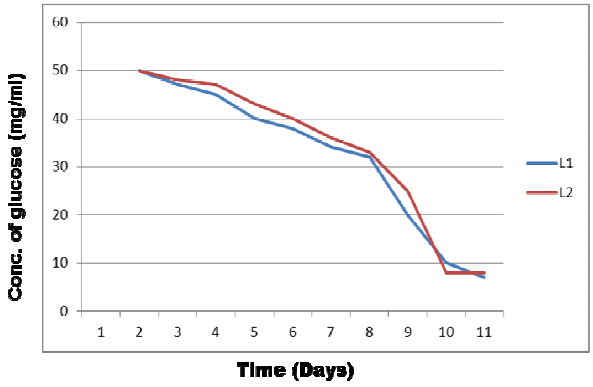


Fig:5.1: shows the profile of biosurfactant production obtained when the strains (L4 and L15) was cultivated in MSM medium with 2% glucose. Since biosurfactant is secondary metabolites maimal Glycolipids production was reached in the stationary growth phase.

The purification technique consists of two autonomous methods: Ammonium sulphate precipitation method and $ZnCl_2$ precipitation method. Floating material resulting from the treatment of 40% ammonium sulphate were collected by centrifugation and then dissolved in small fraction of water. The chilled acetone was added to it to remove proteins; the remained n-hexadecane was removed by extracting it 3 times with hexane. The resultant sample was applied to TLC plate equilibrated with chloroform/methanol (80:20) with standard (L-rhamnose) and one negative control (*E.coli*). The purified bio-surfactant sample produced by *Pseudomonas spp.*were detected as Rhamnolipid whereas the chemical nature of bio-surfactant produced by *Bacillus spp.* (L4) were also glycolipid.

The concentration of glycolipid is calculated from the standard graph prepared with the help of L-rhamnose by Orcinol assay, the graph was plotted on optical density vs. concentration (mg/ml). According to standard graph, the *Pseudomonas spp.*

(L15) produces 73.5 mg/ml glycolipid (bio-surfactant) whereas *Bacillus spp*. L4produces 7.1 mg/ml glycolipid.

VI. Conclusions

The approach is to appreciate the utility and usefulness of bio-surfactant instead of chemically formed surfactant. They are generally non-toxic and ecofriendly. Bio-surfactants improve the emulsification of hydrocarbons; have the potential to solubilize it to increase their availability for micro-organisms.

The article directly highlights the application of bio-surfactant in treatment of diseases, environment pollution control, Health care and cosmetic industry, oil industries and paper and pulp industries.

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