ROLE OF BIOSURFACTANT IN BIOREMEDIATION PRODUCED BY SOIL FUNGI

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Abstract: An alternative and eco-friendly method of remediation technology of environments contaminated with the pollutants is the use of biosurfactants and biosurfactant-producing microorganisms. The diversity of biosurfactants makes them an attractive group of compounds for potential use in a wide variety of industrial and biotechnological applications. Many biosurfactants have been produced, although few are produced due to the high costs of production and purification processes. For the potential application of these compounds in the oil and pharmaceutical industries the optimization of modified GN- medium for the production of a biosurfactant by soil fungi was studied. In this paper we used modified GN- medium (Glucose nutrient medium) to produce biosurfactants by soil fungi. The biosurfactant was characterized as crude biosurfactant showing great potential to be used in bioremediation processes, especially in the petroleum industry.

Keywords*:* Bio-surfactant, GN-medium, microorganisms, soil samples, surface active properties.

Introduction: Increasing public awareness of environmental pollution influences the search and development of technologies that help in clean up of organic and inorganic contaminants such as hydrocarbons and metals.

Synthetic surfactants used to increase contaminated solubility are often toxic, representing an additional source of contamination (Bognolo, 1998). Microbially produced surface active compounds have similar properties but are less toxic, biodegradable and can be produced *in situ*, at the contaminated sites (Cha, 2000).

Surfactant molecules are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil / water and air / water interfaces (Desai and Banat, 1997).

Surface active compounds produced by microorganisms are of two types, those that reduce surface tension at the air water interface (Biosurfactants) and those that reduce the interfacial tension between immiscible liquids, or at the solid-liquid interface (Bioemulsifiers). Biosurfactants usually exhibit emulsifying capacity but Bioemulsifiers do not necessarily reduce surface tension (Karanth *et al*., 1999). Thus, emulsifiers are a subclass of surfactants that stabilize dispersions of one liquid in another, e. g. oil-in-water emulsions.

Surfactant molecules act by reducing interfacial energy (interfacial tension) and surface tension through the formation of an ordered molecular film at the interface (Tan H.M. 2000).

Biosurfactants can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area at the aqueous –hydrocarbon interface. This increases the rate of hydrocarbon dissolution and their utilization by microorganisms (Gerson, 1993).

Much interest and attention have been directed towards biosurfactants in recent years, due to their broad-range, functional properties and diverse synthetic capabilities of microbes.

Biosurfactants are likely to gain wide acceptance since they are readily biodegradable and have lower toxicity as compared to their chemically synthesized counterpart. Effect of environmental factors on growth and biosurfactant production by microorganisms were carried out (Kim, 1993, L. Rodrigues *et al*., 2006, Abu-Ruwaida *et al.,* 1991, Banat I. M., 1993).

The objective of the study was to determine the biosurfactant production & its potential use in bioremediation processes, especially in the petroleum industry.

Bioremediation relies on improved detoxification and degradation of toxic pollutants either through intracellular accumulation or via enzymatic transformation to less toxic or nontoxic compounds. Many microorganisms naturally possess the ability to degrade, transform, or chelate various toxic chemicals, but these natural transformations are relatively slow and require information on feasibility, verification, and capability of the process. The principal mediators of the bioremediation are microbes and their products as they transform or mineralize pollutants, thereby decreasing their masses and toxicities in contrast to most other components of environment. Bioremediation uses natural as well as recombinant microorganisms to break down toxic and hazardous substances by aerobic and anaerobic means.

The success of bioremediation is governed by three important factors: availability of microbes, accessibility of contaminants and a conducive environment. The efficiency of the bioremediation is dependent upon the microbial ability to degrade these complex mixtures and their rate-limiting kinetics. The intensity of biodegradation is influenced by several factors, such as nutrients, oxygen, pH value, composition, concentration, and bioavailability

of the contaminants, chemical and physical characteristics, and the pollution history of the contaminated environment.

A typical bioremediation process will consist of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, addition of emulsifiers and surface-active agents in two processes, and biostimulation and bioaugmentation to enhance the biodegradation rate by increasing the capability of biosurfactants and biosurfactant-producing bacterial strains to enhance organic contaminants' availability and biodegradation rates was reported by many authors. The effective microbiological method in bioremediation of hydrocarbon polluted sites is the use of biosurfactant producing bacteria without necessarily characterizing the chemical structure of the surface active compounds. The cell free culture broth containing the biosurfactants can be applied directly or by diluting it appropriately to the contaminated site. The other benefit of this approach is that the biosurfactants are very stable and effective in the culture medium that was used for their synthesis.

Materials And Methods:

Microorganisms: Hydrocarbon-utilizing fungi were isolate from different soil samples and were identified as *Aspergillus niger, Aspergillus flavus, Rhizopus sp., Penicillium* sp. To confirm their ability to grow on hydrocarbons, a few filaments from fungal cultures were transferred in to 100ml Erlenmeyer flasks containing 20 ml liquid medium supplemented with 2%hexadecane or n-paraffins and kerosene, respectively and cultivated at 28°C and 130 rpm (Borjana *et al*., 2001). Fungal cultures were maintained on potato dextrose agar slants at 4°C.

Medium: Modified GN medium- (glucose-10gm, KNo_3-2.5gm, KH_2PO_4-1gm, $MgSO_4$-0.5gm in 1000ml distilled water)

Method of inoculation: A loopful of spore suspension was standardize to contain 20-30 spores per field (10X10) was inoculated in 50ml fermentation medium.

Incubation: In 250ml Erlenmeyer flask 50ml sterilized fermentation medium was inoculated with fungal culture and incubated in static and shake condition for 7 days.

Assessment of Growth: Growth was assessed in terms of dry weight at the end of incubation period. (Gravimetric method)

Biosurfactant production: Biosurfactant production was determined by measuring surface tension by capillary rise method as described in Physical Chemistry Laboratory Manual, 2003.

Screening and identification of biosurfactant-producer: Initially, the screening of biosurfactant production by the isolated strain was carried out by oil-displacement test according to the method described elsewhere (Ebrahimi *et al*., 2012). Further, emulsification activity of the cell-free culture supernatant agains paraffin oil was carried out as mentioned by Paraszkiewicz *et al*., 2002.

To confirm the biosurfactant production from the above mentioned tests, surface tension of the culture samples were measured by DuNouy Tensiometer.

Extraction of bio-surfactant: Biosurfactants released by *Aspergillus niger* at stationary phase mixed with 4ml of water, 5ml of chloroform and 10ml of methanol. After shaking for 5 minutes, the mixture was filtered through Whatman filter paper no.1 with slight suction. The residue on the filter paper was homogenised with 5ml of chloroform and filtered once more. The residue on the filter paper was dissolved in water and freeze-dried (Blish and Dyer, 1959).

For extraction of lipid, two filters were combined, 5ml of water was added and the mixture was shaken thoroughly before separation of layer allowed. The upper methanol-water layer was removed by aspiration, retoevaporated until an aqueous solution was left and subsequently freeze-dried. The lower chloroform layer containing lipid fraction was retoevaporated resuspended in chloroform-methanol-water (4:3:1, vol/vol) and again retoevaporated until a cloudy aqueous emulsion was formed. This emulsion was freeze-dried and stored at -20°C.

Different concentrations of the biosurfactant in the range of 10-80 mg/l were prepared from the stock solution of biosurfactant (0.1 g/l) in water. CMC was determined by measuring the surface tension of the dilutions at room temperature. The CMC was defined by plotting the surface tension as a function of biosurfactant concentration. These experiments were conducted in three independent experiments: The surface tension reaches its minimum value at a concentration of biosurfactant called critical micelle concentration (CMC) above which the molecules are associated, forming super-molecular structures.

Thin – layer chromatography (TLC):

TLC of freeze dried biosurfactant was carried out. On exposure to iodine vapors yellow and brown spots were observed in daylight indicates the presence of glycolipids and phospholipids.

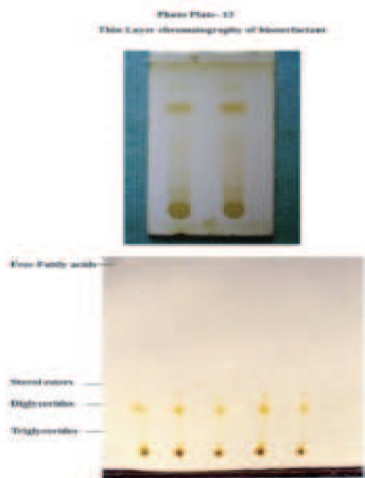

Fig-1 Thin – layer chromatography

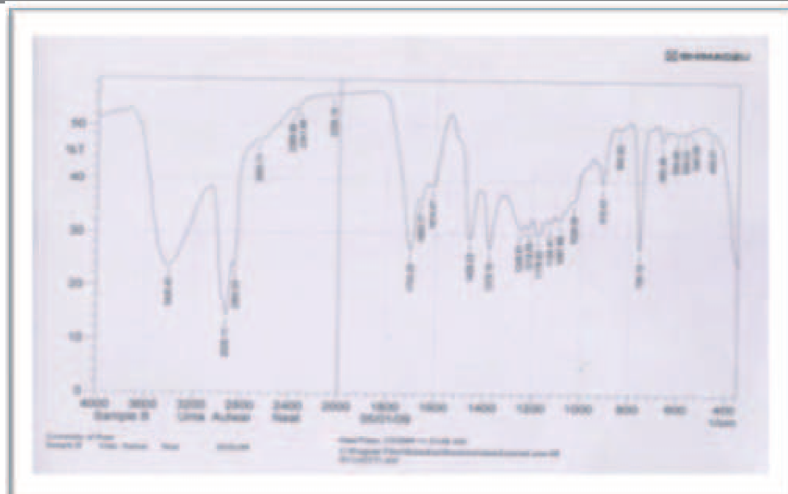

Fig-2 Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy (FT-IR): Fourier transform infrared spectroscopy (FT-IR) is an analytical technique used to identify organic (and in some cases inorganic) materials. This technique measures the absorption of various infrared light wavelengths by the material of interest. These infrared absorption bands identify specific molecular components and structures.

High performance liquid chromatography (HPLC): C-18 reversed-phase high performance liquid chromatography (HPLC) of biosurfactant sample was carried out on a Perkin Elmer series 410 HPLC system.

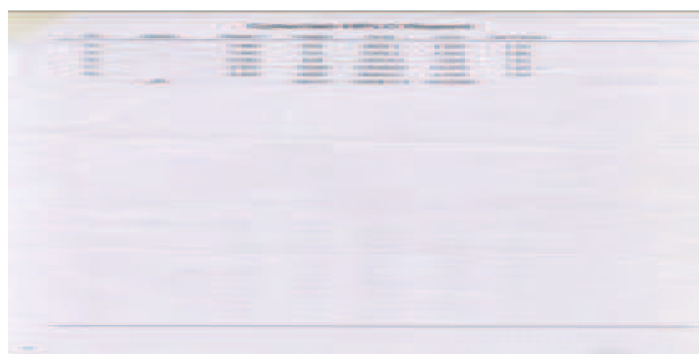

Fig-3 High performance liquid chromatography

Oil-degradation activity of bio-surfactant: Natural oil and fats mostly consists of higher fatty acids and esters of glycerol. The basic hydrolysis of esters is known as saponification. The saponification value indicates about oil degradation. Oils viz., coconut, sunflower, castor bean, palm, soyabean and

cottonseed oil were used. In 100ml oil sample 10ml crude biosurfactant solution was added and it was kept for 12 hours.

Analytical methods: Protein concentration in the isolated biosurfactant was determined by the Lowry method (Lowry et *al*., 1951) using Bovine serum albumin as a standard. Carbohydrates were determined by the phenol-sulphuric method, using D-glucose as a standard (Hanson and Phillips, 1981). The lipid composition of the crude biosurfactant was determined according to Manocha et *al*., (1980).

Result And Discussion: Growth and optimization studies were carried out. On the basis of the growth and emulsification activity of all these isolates were measured in static condition and in suspension culture by using modified GN-medium. Amongst these isolates *Aspergillus niger* was found suitable for more biosurfactant production. Growth measured was 1.9g/L and emulsion activity measured was 21.61. *Aspergillus niger* was selected for further studies. Optimum growth and maximum activity was measured in GN-medium.

Aspergillus niger when grown on modified GN-medium with pH 6.5, temperature 28°C, on orbital shaker at 120 rpm, on seven days of incubation produced 1.9g/L of growth (dry weight basis) and 21.61 emulsification activity. 1995 reported optimal yields of bioemulsifier are usually obtained when carbohydrate and vegetable oil are used as substrate.

Liposan an extracellular emulsifier synthesized by *Candida lipolytica* was composed of 93% carbohydrate and 7% protein (Cirigliano and Carman 1984). Other polymeric emulsifiers containing proteins carbohydrate and lipids were produced by *Candida lipolytica* when grown in babassu oil (Sarubbo et al. 2006). Preliminary analysis of the bioemulisifier produced by *Aspergillus niger* in modified GN-medium indicated that it was a hetropolymer, which consisted of 19 % protein, 25 % carbohydrate, and 5.5 % lipid.

The chemical nature of biosurfactant produced was detected by HPLC, FTIR, IR, NMR and GC/MS.

Joachim Vater et *al*., (2002) performed reverse phase HPLC to separate the isoforms of the lipopeptide biosurfactant complexes produced by *Bacillus subtilis* C-1. They found the lipopeptide products in 23 fractions.

The biosurfactant produced by *Aspergillus niger* was found heteropolymer made up of protein, carbohydrate and lipid. TLC revealed the presence of glycolipids. FT-IR, HPLC, NMR and GC/MS analysis indicated presence of Pentadecanecarboxylic acid and D-xyletol penta-acetate in biosurfactant. Further studies are required for detailed elucidation of structure and chemical nature of biosurfactant.

Oil degradation activity of biosurfactant was determined in terms of saponification value. Biosurfactant have capability of hydrolyzing the esters. Increase in saponification value is an indication of degradation of oil. Oil degradation activity of biosurfactant was checked against six different types of oils. Saponification activity was found most against soyabean and cottonseed oil. It indicates oil degradation by biosurfactant.

Masaaki Morikawa et *al*., (1993) noted the similar report for biosurfactant produced by *Arthrobacter* sp. Strain MIS38.

A promising method that can improve bioremediation effectiveness of hydrocarbon contaminated environments is the use of biosurfactants. They can enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells. By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons. In consequence, biosurfactants enhance biodegradation and removal of hydrocarbons. Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification.

Emulsification is a process that forms a liquid, known as an emulsion, containing very small droplets of fat or oil suspended in a fluid, usually water. The high molecular weight biosurfactants are efficient emulsifying agents. They are often applied as an additive to stimulate bioremediation and removal of oil substances from environments.

The effective microbiological method in bioremediation of hydrocarbon polluted sites is the use of biosurfactant producing microorganisms without necessarily characterizing the chemical structure of the surface active compounds. The cell free culture broth containing the biosurfactants can be applied directly or by diluting it appropriately to the contaminated site. The other benefit of this approach is that the biosurfactants are very stable and effective in the culture medium that was used for their synthesis.

Bioremediation usually consists of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, if necessary, supplying air and often adding bacteria. The addition of emulsifiers is advantageous when bacterial growth is slow (e.g. at cold temperatures or in the presence of high concentrations of pollutants) or when the pollutants consist of compounds that are difficult to degrade, Bioemulsifiers can be applied as an additive to

stimulate the bioremediation process; however, with advanced genetic technologies it is expected that the increase in bioemulsifier concentration during bioremediation would be achieved by the addition of microorganisms that overproduce bioemulsifiers.

Biosurfactants are produced by a variety of oil-degrading microorganisms. These biosurfactants can be of low molecular weight, acting by decreasing the oil–water interfacial tension, or high molecular weight and act as biodispersants by preventing coalescence of oil drops in water. The high molecular weight bioemulsifiers are hetero-polysaccharides and the active components are lipids or proteins. The activity of bacterial biosurfactants in bioremediation stems from their ability to increase the surface area of hydrophobic water-insoluble substrates and to increase the solubility and bioavailability of hydrocarbons. They can be added to bioremediation processes as purified materials or in the form of bioemulsifier over producing micro-organisms. In either case, they can stimulate the growth of oil-degrading bacteria and improve their ability to utilize hydrocarbons.

References:

1. Bognolo, G.Biosurfactants as emulsifyng agents for hydrocarbons.Colloids andSurfaces A: Physicochemical and Engineering Aspects*,* v.152, p.41-52, 1999.
2. Cha, D.K., 2000. The effect of biosurfactants on the fate and transport of nonpolar organic contaminants in porous media. Environ. Eng., 20: 1-17.
3. Desai, J.D., Banat, I.M., 1997. Microbial Production of surfactants and their commertial potential. Microbiol. Mol. Biol. Rev. 61 (1), 47-64.
4. Karanth, N.G., Deo, P.G., and Veenanadig, N.K. 1999. Microbial production of biosurfactants and their importance. Curr. Sci. 77: 116-123.
5. Tan H. M. (2000) Biosurfactants and their roles in bioremediation. MC4203: Environmental Biotechnology. P. 1-12.
6. Gerson D.F. and Zajic J.E. (1979) Microbial biosurfactants. Proc. Biochem. 14: 20-29.
7. Kang, SW; Kim, YB; Shin, JD; Kim, EK. Enhanced biodegradation of hydrocarbons in soil by microbial biosurfactant, sophorolipid. *Appl. Biochem. Biotechnol* **2010**, *160*, 780–790, doi:10.1007/s12010-009-8580-5.
8. Rodrigues, L.R.; Teixeira, J.A.; Van der Mei, H.C.; Oliveira, R. (2006).Physiochemical and functional characterization of a biosurfactant produced by *Lactococcus lactis* 53. Colloid.Surface. B., **49(1)**, 79 - 86.
9. Abu-Ruwaida, A. S., I. M. Banat, S. Haditirto, and A. Khamis. 1991. Nutritional requirements and growth characteristics of a biosurfactant producing *Rhodococcus bacterium*. World J. Microbiol. Biotechnol. 7:53– 61.
10. Banat, I. M. 1993. The isolation of a thermophilic biosurfactant producing *Bacillus* sp. Biotechnol. Lett. 15:591–594.
11. Ebrahimi, A., Tashi, N., Lotfalian, S. 2012. Isolation of biosurfactant producing bacteria from oily skin areas of small animals Jundishapour J. Microbiol. 5: 401-404.
12. Paraszkiewicz, K., Kanwal, A., D ugonski, J. 2002. Emulsifier production by steroid transforming filamentous fungus Curvularia lunata. Growth and product characterization. J. Biotechnol 92: 287-294.
13. Lowry, O H., Rosebrough, N J., Farr, A. L and Randall, R. J. 1951. Protein measurment with Folin phenol reagent. J. Bio. Chem. 193(1):265-275.
14. Hanson R. S. and Phillips, J. A. 1981. Chemical composition. In GHERHARDT, P. ed. Manual of Methods for general Bacteriology, American society of Microbiology, Washington, DC, 328-364.
15. Manocha M. S., San-Blas, G and Centeno, S. 1980 Lipid composition of *Paracocodioides brasilienses:* possible correlation with virulence of different strains. J. Gen. Micro. 177(1):147-154
16. Cirigliano, M. C., and G. M. Carman. 1984. Isolation of a bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol. 48:747–750.
17. Surubbo L. A., de Luna J.M., and de Campos-Takaki G.M. (2006) Production and stability studies of the bioemulsifier obtained from a new strain *Candida glabrata* UCP 1002. Environmental Biotechnilogy, vol. 9, no. 4, p. 1-7.
18. M Morikawa, H Daido, T Takao, S Murata, Y Shimonishi and T A new lipopeptide biosurfactant produced by Arthrobacter sp. strain MIS38. *J. Bacteriol.* 1993, 175(20):6459-6466.

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