



Research Article

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Bioremediation of crude oil and hydrocarbons by actinomycetes with the enhanced production of bioactive compounds

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ABSTRACT

Biosurfactants act as stimulants for biodegradation by increasing the surface area of the hydrophobic substrate available for microbial attachment. The study aims the analysis of biosurfactant production by actinomycetes in order to reduce the toxicity of pollutants in soil by bioremediation. Biosurfactant producing actinomycetes were isolated from polluted environment by oil displacement method and identified by physiological, biochemical and morphological characterization. Production of biosurfactants was measured by Adhesion to Hydrocarbons (ATH) Assay as 91.2% and the emulsification index was determined to be 83.3%. Biosurfactant production kinetics was estimated at different intervals of time and biosurfactant production was maximum in the strain numbered TCD06 at 72 hrs of incubation, the extraction was carried out through chloroform methanol method. Characterization of biosurfactants by Fourier Transform Infra Red Spectrum (FTIR) and Gas Chromatography Mass Spectrometry (GCMS) analysis revealed the presence of terpenoids and cyclopentanol. Dense growth of the strains was observed with olive oil substrate for the strains with bioremediating properties with high emulsification index and production of biosurfactants. This study reveals the presence of new and powerful biosurfactants in actinomycetes which can emulsify the toxins and hydrocarbons and use them as their food source. The study is crucial in utilizing Actinomycetes as a potent organism for the production of biosurfactants in remediating heavily polluted soils. Emulsification index and biosurfactant production are comparatively higher than other microbial strains available for bioremediation. Incorporating such consortia of actinomycete strains into highly polluted soils can mitigate the problem of pollution.

Key words: Biosurfactant, Emulsification index, Hydrocarbon adhesion, Bioremediation

INTRODUCTION

Biosurfactants are amphiphilic compounds produced by the micro organisms on their cell surface or to the media in which they grow to reduce the interfacial tension, enhancing the emulsification of hydrocarbons and their biodegradation [1]. Bioremediation of heavy metals and mercury compounds have been reported in many of the microbes including *Pseudomonas aeruginosa*. The identification and characterization of microbial surfactants produced by various microorganisms have been extensively studied [2], [3]. Biosurfactants may have one of the following structures: mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein or lipopeptide, phospholipids, or the microbial cell surface itself [4]. Hence, biosurfactant producing microorganisms may play an important role in accelerated bioremediation of hydrocarbon contaminated site. Biosurfactants are highly advantageous to their chemical counterparts as they are biodegradable, less toxic, biocompatible, and digestible and can be cheaply produced from raw materials available. They are helpful in environmental control, hardy and are very specific in their action [5].

Oil pollution can cause recreational, economic and aesthetic damage to human and aquatic life [6]. So far, chemically synthesized surfactants are commercially available in market, which are ionic surfactants like fatty acids, ester sulfonates or sulfates and quaternary ammonium salts etc. Branched paraffins, phenanthrene and cyclic alkanes are tough to be biodegraded but the activity can be enhanced by the addition of biosurfactants as they widely reduced the degradation time and adaptation time for these compounds. Biosurfactants from microbial broth cultures have been demonstrated to solubilize and remove hydrocarbons from polluted soil [7].

Production of biosurfactants from microbes has been widely reported from *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Acinetobacter*, *Torulopsis*, and *Candida* etc. But scanty literature is available on biosurfactant production by actinomycetes. Actinomycetes which are told to be the potential candidate for bioremediation have surfactant producing ability, due to the production of extracellular biosurfactants. Glycolipids like the trehalose lipids are produced by *Rhodococcus spp.* [8], lipopeptides by *Arthrobacter spp.* and cellular biosurfactants such as mycolic acids [9]. This study is a novel approach on the promising aspects of actinomycetes in Bioremediation. Therefore Biosurfactant from actinomycetes can act as an alternative to chemicals used for oil recovery and oil spill cleaning. In this present context, the production and property of biosurfactants by actinomycetes are discussed and their functional groups were elucidated.

EXPERIMENTAL SECTION

Isolation of biosurfactant producing strains

Isolation of biosurfactant producing actinomycetes was carried out using the sample collected from fishing harbour of Tuticorin district, Tamil Nadu. The soil samples with high concentration of hydrocarbon were collected from contaminated sites and packed in sterile polythene bags. 1g of soil from each sample was dispensed into 100ml of Bushnell Hass broth supplemented with petrol and kept in shaking incubator for 11 days at 25°C. The cultures were plated for enumeration [10] and screened for Biosurfactant production by Oil displacement method, developed by [11] and Blood Haemolysis Test, where biosurfactants can cause lysis of erythrocytes or haemolysis [12]. The potent actinomycetes species were characterized morphologically [13], [14], physiologically and biochemically following the Bergey's Manual of Determinative Bacteriology.

Analysis of Biosurfactant production

Adhesion of actinomycetes to Hydrocarbons (ATH Assay) was used to measure the production of biosurfactants. Based on the results obtained in hemolytic activity, the hydrocarbonoclastic strains were tested for ATH assay [15]. The method is based on the degree of adherence of cells to liquid hydrocarbons. Before adding hydrocarbons the absorbance of cell suspensions at 660nm were determined (A_0). A turbid aqueous suspension of cells is mixed for 2 min with a distinct volume of petrol (2 ml). After mixing, the two phases are allowed to separate. The optical density of aqueous phase (A) was determined at OD - 660 nm. Maximum activities were then calculated by the formula:

$$H = (1 - A/A_0) \times 100$$

Emulsification Capacity

Emulsification activity of the purified biosurfactant was developed by Cooper and Goldenberg. The emulsification index is measured by the addition of petrol (2ml) to same volume of biosurfactant (2ml) in a graduated screw cap test tube. The tube was vortexed for 2min and left to stand for 24 h. The emulsification index (E_{24}) was determined as the percentage of the height of the emulsified layer (cm) divided by the total height of the liquid column (cm) [16].

Biosurfactant Production Kinetics

The kinetic studies were carried out in batch culture (Strains TCD01, TCD02, TCD03, TCD04, TCD05, TCD06) for the production of biosurfactant. These studies were carried out in 500ml flasks with 100 ml of tryptone broth and 1% oil inoculated with specific strains. The kinetics was followed in batch culture for about 120 hrs at optimum conditions. The OD was measured for every 24 hrs at 660 nm.

Extraction of Biosurfactants

The biosurfactant was extracted from the culture after cell removal by filtration. A mixture of chloroform: methanol (2:1 v/v) was added to the culture medium, after being vigorously shaken, this was allowed to stand until phase separation. The extracts were combined and concentrated by vacuum rotary evaporator and then sodium sulphate anhydrous was added to remove the water. The dry weight of biosurfactants was measured after drying the same for 30 min at 100°C.

Characterization of Biosurfactants

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR (Bruker optics- Alpha T) was used to measure the absorption of various infrared radiations by the target material, to produce an IR spectrum that can be used to identify functional groups and molecular structure in the samples obtained after Chloroform: Methanol extraction of biosurfactants. The powdered and ground biosurfactant powder was used as the sample.

Gas Chromatography/ Mass Spectrometry (GCMS analysis)

Gas chromatographic studies were done with the GCMS system (Shimadzu GC-17A 2P5000). Diphenyl-dimethyl silofane was used as the substrate for column. The sample volume was maintained at 0.1 μ l. Helium gas served as the carrier for chromatographic analysis. The different peaks obtained after the analysis was located for reading the compounds present in the sample.

RESULTS

The Sediment samples collected from the polluted sites were inoculated in Potato dextrose broth and Bushnell Hass (BH) broth, which showed turbidity and uniform growth. The conditioned growth in BH broth was enriched with petrol. It evaluates the presence of Hydrocarbon degraders in the collected sample. When the cultures were placed in an antibiotic supplemented PDA plates, there was an abundant population in Site 2 (120×10^{-2} /ml) when compared to Site 1 and 3.

There was an abundant growth in BH broth supplemented with olive oil, which gave an O.D of 1.961 at 14th day. On screening the strains for biosurfactant production, using Oil displacement assay, six among 18 cultures exhibited oil displacement. The cultures also showed β -hemolytic activity in blood agar. The degradation of RBC cell lipid membrane is the cause for hemolysis which is the principal aspect behind emulsification of hydrocarbon. The cultures were named as TCD01, TCD02, TCD03, TCD04, TCD05 and TCD06.

The positive strains of hemolytic assay were tested for ATH assay and maximum activity of 91.2 % was observed. The absorbance was measured before and after mixing the hydrophobic phase. Emulsification capacity was tested with all the hydrocarbons substrates. TCD06 and TCD02 showed best emulsification activity as 83.3 % and 66.6% respectively due to the formation of critical micelle concentration (CMC) which determines the concentration of surfactants above which micelles form as shown in Table 1.

The E₂₄ plot shows the dependence of growth rate, biosurfactant production and emulsification index E₂₄ in 100 ml of tryptone broth medium containing the hydrocarbon (1 % petrol). At about 48 hours, the surfactant concentration began to increase, reaching its maximum at 72 hours.

Finally, Biosurfactant was extracted from the mixture of culture, chloroform and methanol. White sediment (biosurfactant) remained after removal of solvent and moisture by rotary evaporator. The biosurfactant obtained retained its activity on analysis.

Structural Characterization by Fourier Transform Infrared Spectroscopy (FTIR) showed the presence of five different functional groups were shown in Fig.1, which were primary and secondary amines having transmittance at wave number 3316.7 cm⁻¹, Carboxylic acid (2359 cm⁻¹), Amide (1634 cm⁻¹), n-alkenes (1078 cm⁻¹, 1045cm⁻¹). The presence of various functional groups can be observed as in Fig. 1 and Fig. 2. Which explains that sharp peaks were obtained by GCMS analysis indicating the presence of the compounds such as cyclopentanol and terpenoids in the biosurfactants samples was shown in Fig.3.

DISCUSSION

The population in Site 2 was high (120×10^{-2} /ml) when compared to Site 1 and 3. The main reason for the presence of actinomycetes in these sites is lipase outlet from Industries which facilitates assimilation of fatty acids contained in hydrocarbon fractions. The abundant growth in BH broth supplemented with olive oil, proved the presence of hydrocarbon degraders in the collected sediments. The microbes perform specific biochemical reactions which degrade macromolecules into smaller units in a step-wise fashion. It is evident that the minimal media serves as a differential media for the isolation of specific microbes when the degradable substrate is provided as the sole source of carbon [17]. TCD06 and TCD02 showed best emulsification activity due to the formation of critical micelle concentration (CMC) which determines the concentration of surfactants above which micelles form. Presence of biosurfactant compounds has been indicated by FTIR and GCMS data. Compounds such as cyclopentanol, octanone, heximide enhance solubilization of polycyclic aromatic hydrocarbons and terpenes have the capacity to

lower the surface tension for surfactant substitution during bioremediation [18], [19]. Emulsification index and the bioremediation properties exhibited by the isolated strains from this site proves to be better than other reported actinomycete strains which could exhibit a bioremediation index of only 55% [20].

Table: 1. Actinomycete strains with their Emulsification Index (E_{24}) and ATH assay

Sl. No.	Strains	Height of emulsified layer (h_e) (cm)	Total height of liquid column (h_t) (cm)	Emulsification Index (%)	Maximum activity (ATH) $H = (1-A/A_0) \times 100$
1	TCD01	1.5	3	50	75
2	TCD02	2.0	3	66.6	80
3	TCD03	1.8	3	60	30
4	TCD04	0.9	3	30	64
5	TCD05	1.2	3	40	15
6	TCD06	2.2	3	83.3	91.2

Figure 1. FTIR transmittance graph obtained for different biosurfactant samples

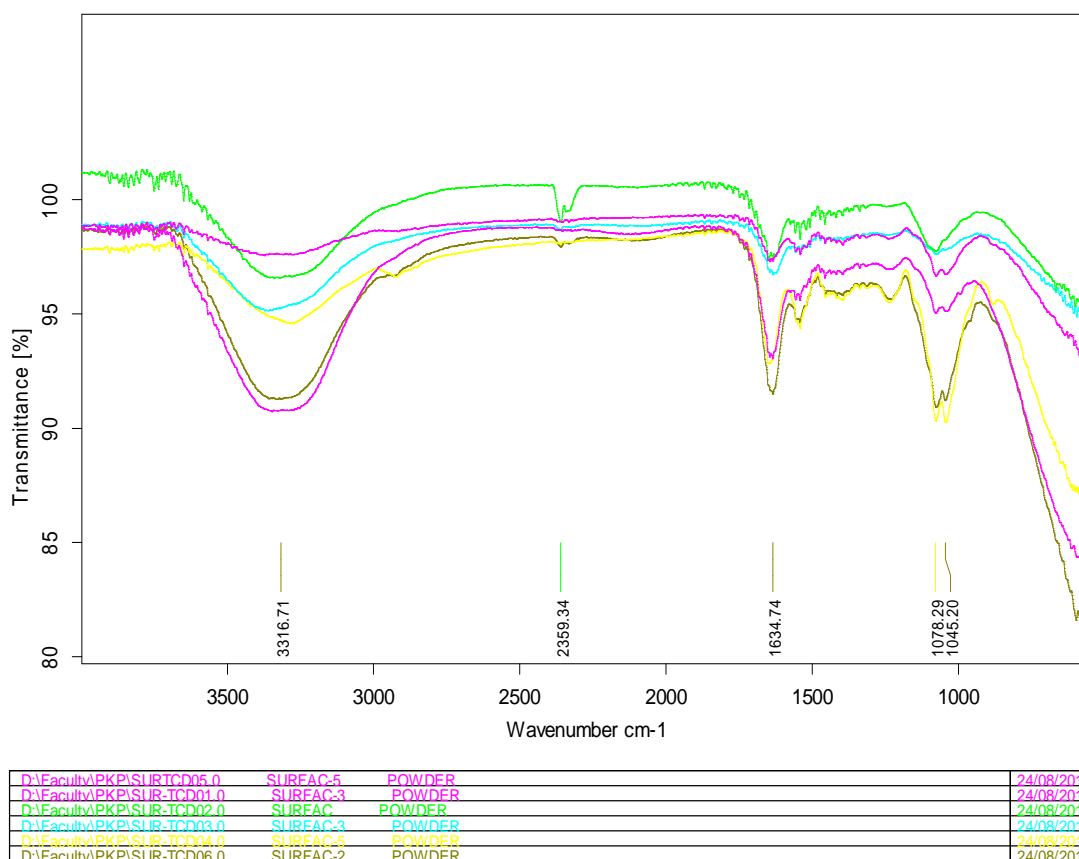


Figure 2. Peaks obtained by GCMS analysis of the biosurfactants

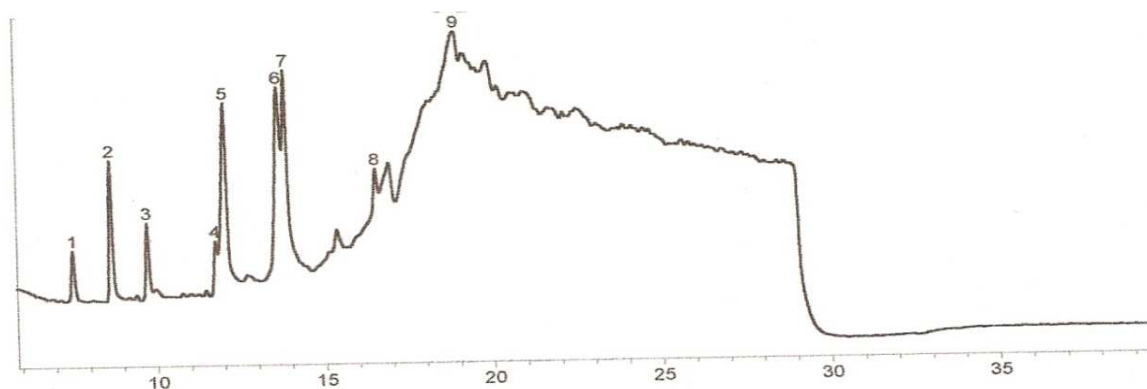
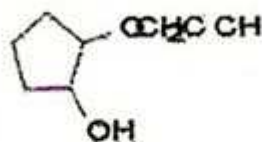


Figure 3. Structural elucidation of compounds from GC and FTIR analysis

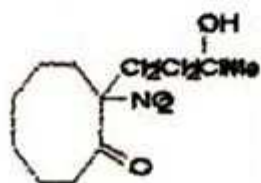
i. Cyclopentanol

(Retn.Time:13.650)



ii. 2-Nitro-2-(3'-hydroxybutyl)- cyclo-octanone

(Retn.Time:13.973)



CONCLUSION

Three different polluted sites of the Tuticorin district were chosen as the study site. 18 strains of actinomycetes were used for the studies among which six strains reported positive for the production of biosurfactants. Biosurfactant production was enhanced and was extracted from the crude source. The biosurfactants were characterized for confirming the functional groups present and the exact structure of the components present in them was elucidated after subjecting them to GCMS analysis which confirmed the presence of various biosurfactants such as cyclopentanol, octanone and terpenoids. The biosurfactants were tested for their bioremediating efficiency. The biosurfactant production and emulsification index was high proving that Actinomycetes are powerful sources of biosurfactants and can tone down oil pollutions in hydrocarbon contaminated soils.

REFERENCES

- [1] M Abouseoud; R Maachi; A Amrane. Communicating Current Research and Educational Topics and Trends in Applied Microbiology, A. Méndez-Vilas, **2007** 340-347.
- [2] R.J Grant; L.M Muckian; N.J.W Clipson,.E.M Doyle. *Letters in Applied Microbiology*, **2007**, 44(3), 293-300.
- [3] S.G Tupe; J.M Rajwade; K.M Paknikar. *Letters in Applied Microbiology*, **2007** 45(1), 36-41
- [4] S Lin. *J Chem Tech Biotech.*, **1996**, 66(2), 109-120.
- [5] N Kosaric; WL Cairns; NCC Gray. *Biosurfactants and Biotechnology*, eds. Marcel Dekker, New York. Surfactant Science Series, **1987**, 1-19
- [6] GA Sorial; AD Venosa; KM Koran; E Holder E; D King. *ASCE Journal of Environmental Engineering.*, **2004**, 130, 1085-1093.
- [7] AS Abu Ruwaida; IM Banat; S Haditirto; A Khamis. *World J. Microbiol. Biotechnol.*, **1991**, 7, 53-61.
- [8] P Rapp. *Appl. Environ. Microbiol.*, **2001**, 67, 3496-3500.
- [9] T Neu; K Poralla. *Appl. Microbiol Biotechnol.*, **1990**, 32(5), 521-525.
- [10] R Balagurunathan. Antagonistic Actinomycetes from Indian shallow sea sediments with reference to unsaturated lactone type of antibiotic from *Streptomyces griseobrunneus*, Ph.D. thesis, Annamalai University, India, **1992**, 33-82.
- [11] M Morikawa; Y Hirata; T Imanaka, T. *Biochim Biophys Acta.*, **2000**, 1488(3),211-218.
- [12] FC Bicca; LC Fleck; MA Zachio. *Rev Microbiol.*, **1999**, 30 (3), 26-31.
- [13] ST Williams; ME Sharpe; JG Holt. *Streptomyces and related genera*. In *Bergey's Manual of Systematic Bacteriology*. Vol. 4. Williams and Wilkins, USA. **1989**, 4, 25-29
- [14] D Singh; VP Agrawal. Diversity of actinomycetes of Lobuche in Mount Everest I Proceedings of International Seminar on Mountains, Kathmandu, **2003**, 357 - 360.

- [15] M Rosenberg; D Gutnick; E Rosenberg. *FEMS Microbiol Lett.*, **1980**, 9(1), 29-33.
- [16] AA Bodour; C Gerrero Barajas; M Maier. *App and Env Microbiol.*, **2004**, 10(6), 1114-20.
- [17] S Asthana; P Rusin; CP Gerba. *Int. J. Environ. Health Res.*, **1997**, 7, 277-287
- [18] KS Zanker; G Blumel. Terpene - induced lowering of surface tension in vitro: a rationale for surfactant substitution. *Res. Exp Med. (Berl)*; **1983**, 182(1), 33-8.
- [19] W Xiaojiang ; LB Mark. *Environ. Sci. Technol.*, **1995**, 29 (9), 2346–2351
- [20] P Chaudhary; R Sharma; SB Singh; L Nain. *Toxicol.*, **2011**, 86(3), 268-71.